

4-Biphenylaldehyde and 9-anthraldehyde: two fluorescent substrates for determining P450 enzyme activities in rat and human

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Received 25 March 2002

1. 4-Biphenylaldehyde (4-BA) and 9-anthraldehyde (9-AA) were examined as substrates for cytochrome P450 (CYPs) enzymes in rat and human. Both aldehydes were oxidized by CYPs to fluorescent carboxylic acids, which can be assayed with a high sensitivity by an easy fluorimetric method.

2. With liver microsomes from control and induced rats, the oxidation of both 9-AA and 4-BA followed simple Michaelis–Menten kinetics. Only microsomes from rats pretreated with phenobarbital (a strong inducer of P4502B1/2) could increase (about threefold) the oxidation rates ($V_{\max}$) of both aldehydes above the control values, which were 6.7 ± 1.1 and 3.3 ± 0.6 nmol min⁻¹ mg⁻¹ protein for 4-BA and 9-AA, respectively. On the other hand, the K_m 's, which were similar for both aldehydes (about 25 μ M), did not change significantly with any inducer. The use of purified rat CYP1A1, 2E1, 2B1 and 2C11 in a reconstituted system showed that only 2B1 and 2C11 could oxidize both substrates with a high turnover.

3. In human liver microsomes, the oxidation rates of these aldehydes (1.6 ± 0.2 and 0.42 ± 0.1 nmol min⁻¹ mg⁻¹ protein for 4-BA and 9-AA, respectively) were lower than those of rat but with similar K_m 's (20–26 μ M).

4. The oxidation of these aldehydes was also determined with cDNA-expressed CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4 and with a characterized bank of 14 human liver microsomes. In a reconstituted system, only CYP2B6, 2A6, 3A4 and with a lower turnover 2C9 oxidized both substrates.

5. Among the CYP marker activities of the 14 human samples, good correlations were only observed between CYP3A-dependent 6 β -testosterone hydroxylase and the oxidation of 4-BA ($r = 0.74$) or 9-AA ($r = 0.80$) and between the oxidation of 4-BA versus 9-AA ($r = 0.74$). Weak correlations were also found between the 2B6-linked *S*-mephenytoin *N*-demethylase and the oxidation of 4-BA ($r = 0.58$) or 9-AA ($r = 0.65$).

6. Inhibition experiments revealed that the oxidation of these aldehydes was inhibited by ketoconazole, 8-methoxypsoralene and sulphophenazole, selective inhibitors for P4503A6, 2A6 and 2C9, respectively.

7. In summary, based on the use of cDNA-expressed CYPs, correlation analysis and chemical inhibition, the metabolism in human liver microsomes of these aldehydes appears primarily catalysed by CYP3A, although CYP2A6, 2B6 and 2C9 may play a role. 9-AA and particularly 4-BA, owing to the high rate of its metabolism, may be two novel useful fluorescent probe substrates for assaying CYP activities in various species.

Introduction

Aldehydes are commonly considered to be converted to acids in biological systems by the action of aldehyde dehydrogenase (Lindahl and Peterson 1991). More recently, some examples have demonstrated that hepatic microsomes from

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various animals have the ability to oxidize xenobiotic aldehydes to carboxylic acids by the cytochrome P450 system (Watanabe *et al.* 1990, 1992). It has been reported that the oxidation of aromatic aldehydes such as tolualdehydes and tetrahydrocannabinol to the corresponding acids is primarily catalysed in rat and mouse by hepatic CYPs belonging to the 2C subfamily (Watanabe *et al.* 1993, 1995). Aliphatic aldehydes such as acetaldehyde and 2,2,2-trifluoroacetaldehyde are oxidized mainly by CYP2E1 (Kaminsky *et al.* 1992, Kunitoh *et al.* 1997). On the other hand, retinals have been reported to undergo oxidative conversion to the corresponding retinoic acids primarily by the CYP1A2 and CYP3A4 forms, although with different affinities (Zhang *et al.* 2000). It has also been reported that 9-anthraldehyde (9-AA) is a good substrate for CYPs and may be assayed by an easy fluorimetric method (Watanabe *et al.* 1991), although the CYPs involved in this reaction are not known. Many studies have been devoted to identifying probe substrates for specific CYPs. Fluorimetric assays, for example, the *O*-dealkylation of coumarins (Mayer *et al.* 1990) and resorufins (Burke and Mayer 1983), are particularly useful because of their sensitivity and ability to select fluorophores in a wavelength range that obviates artefacts deriving from endogenous compounds.

In the current study, we have investigated the metabolic specificity of 9-AA and of another novel aromatic aldehyde, 4-biphenylaldehyde (4-BA), against microsomes from control and induced (male and female) rats, and some purified rat CYP1A1, 2E1, 2B1 and 2C11. In addition, we have studied the metabolic activity and specificity of these two aldehydes in man by using a well-characterized bank of human liver microsomes, a series of cDNA-expressed human P450 enzymes and selective CYP inhibitors.

Materials and methods

Chemicals

9-Anthraldehyde, 9-anthracene carboxylic acid (9-ACA), 4-biphenylaldehyde, 4-biphenyl carboxylic acid (4-BCA) and cytochrome b₅ were obtained from Sigma Chemical Co. (St Louis, MO, USA). CYP2B6 was purchased from Gentest Corp. (Woburn, MA, USA). All other chemicals and solvents used were obtained from common commercial sources.

Animals and treatments

Male and female Sprague–Dawley rats (Charles River, Como, Italy), weighing 200–250 g, were used. They were fed with food pellets (Altromin[®], Rieper, Bolzano, Italy) and water *ad libitum*. Inducers administered intraperitoneally (i.p.) in water were: sodium phenobarbital (PB) and pyrazole (PYR) at 80 and 200 mg kg⁻¹, respectively, for 3 days. β -Naphthoflavone (β NF) at 40 mg kg⁻¹, dexamethasone (DEX) at 50 mg kg⁻¹ and clofibrate (CLO) at 250 mg kg⁻¹ were given in corn oil for 3 days. Animals were killed by CO₂ asphyxia; the livers were removed and microsomes prepared as described by Longo *et al.* (1986). The washed microsomal pellets were resuspended in 100 mM phosphate buffer, 1 mM EDTA, pH 7.4, and stored at -80°C. Protein content was determined according to Lowry *et al.* using bovine albumin as standard.

Human liver microsomes

Three pooled liver microsomes (HL-1, HL-2, HL-3) and 14 individual samples (10 male, four females, aged 2–68 years), negative for HIV and hepatitis, were obtained from Xeno Tech's reaction phenotyping kit (Kansas City, KS, USA). Each human sample was supplied with the CYP content and major CYP marker activities such as 7-ethoxyresorufin *O*-deethylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin, tolbutamide methyl-hydroxylase, testosterone 6 β -hydroxylase, chlorzoxazone 6-hydroxylase, lauric acid 12-hydroxylase, paclitaxel (taxol)-6 α -hydroxylase, dextromethorphan *O*-demethylase, mephenytoin *N*-demethylase and *S*-mephenytoin 4'-hydroxylase.

cDNA-expressed enzymes

Human recombinant CYP1A1, 1A2, 2A6, 2C9, 2D6, 2E1 and 3A4 were expressed from *E. coli* using plasmids containing cDNA of various forms kindly supplied by F. P. Guengerich (Vanderbilt University, Nashville, TN, USA).

4-BA and 9-AA oxidation assay

4-BA and 9-AA oxidation activity was determined by measuring the formation of 4-BCA or 9-ACA, as described by Watanabe *et al.* (1991). In brief, the incubation mixture contained (in 1 ml), hepatic microsomes (0.1–0.5 mg protein ml⁻¹), 0.5 mM NADP⁺, 10 mM glucose 6-phosphate, 10 mM MgCl₂, 1 unit glucose 6-phosphate dehydrogenase, and 4-BA or 9-AA as substrate in 100 mM phosphate buffer, pH 7.4. The reaction was carried out at 37°C generally for 15 min and terminated with 1 ml 0.5 N NaOH and extracted with 4 ml ethyl acetate. Aqueous phase (1 ml) was transferred to another tube and 4-BCA or 9-ACA extracted with 4 ml ethyl acetate after the addition of 1 ml 0.5 N HCl. The organic phase was scanned for fluorescence spectra with a Perkin-Elmer spectrofluorimeter. The formation of 4-BCA was determined at 290 nm excitation and 330 nm emission, whereas 9-ACA was determined at 255 nm (excitation) and 458 nm (emission). Both 4-BCA and 9-ACA carried through the extraction procedure under identical conditions were used as standards and were linear at least up to 50 nmol. The extraction phase is necessary to remove completely the fluorescence of substrate (4-BA or 9-AA).

Reconstituted system

CYP1A1, 2B1, 2C11, 2E1 and NADPH-cytochrome P450 reductase were purified from microsomes of male Sprague–Dawley rats (Amato *et al.* 1996). The reconstituted system contained 100 mM phosphate buffer, pH 7.4, 0.1 μ M P450, 0.3 μ M P450 reductase, 30 μ g dilauroyl phosphatidylcholine (DLPC) and substrate (9-AA or 4-BA) in 0.5 ml of the incubation mixture. DLPC was prepared in water and sonicated immediately before use. After a 30-min pre-incubation at room temperature, the reaction was carried out at 37°C for 30 min after addition of an NADPH-regenerating system and analysed for the formation of 4-BCA or 9-ACA as already described.

Membranes from *E. coli* expressing human recombinant CYPs (50 pmol) were pre-incubated with 50 pmol purified rat P450 reductase, 15 μ g dilauroylphospha-

tidylcholine and 50 pmol cytochrome b₅ (in the case of incubation with CYP3A4) for 60 min at room temperature. Potassium phosphate buffer (100 mM, pH 7.4), MgCl₂ (3 mM) and substrate (4-BA or 9-AA) were added at the final concentrations indicated. The reactions were started by addition of an NADPH-generating system. After 60 min of incubation at 37°C, reactions were terminated with 1 ml 0.5 N NaOH and analysed as described above.

Inhibition studies

This approach involves an evaluation of the effects of chemical inhibitors of selected CYPs on the metabolism of 4-BA or 9-AA by human liver microsomes. In the chemical inhibition experiments, α -naphthoflavone (α -NF), 8-methoxypsoralene (8-MOP), sulphaphenazole (SULF), quinidine (QUIN), 4-methylpyrazole (4-MP), *S*-mephenytoin (MEF) and ketoconazole (KET) or troleandomycin (TAO), were used as inhibitors of CYP1A2, 2A6, 2C9, 2D6, 2E1, 2C19 and 3A4, respectively (Parkinson 1996, Koenigs *et al.* 1997, Sai *et al.* 2000). The inhibitors were dissolved in methanol and added to the incubation mixture before the reaction was initiated. The incubations were carried out with 0.2 mg pooled samples of human liver microsomes in a final volume of 0.5 ml and at a concentration of 4-BA or 9-AA 100 μ M.

Results

Fluorescence characteristics of 4-BCA and 4-BA

The fluorescence spectrum of 4-BCA in ethyl acetate has excitation and emission maxima at 290 and 330 nm, respectively. No fluorescence was detected with 4-BA at a concentration up to 0.01 mM. However, the 4-BA may be completely separated by the extraction method described above.

These properties make 4-BA a suitable substrate for a direct assay of microsomal monooxygenase activities.

Oxidation of 4-BA and 9-AA by rat liver microsomes

As previously reported for 9-AA (Watanabe *et al.* 1991), 4-BA is also oxidized by CYPs in rat liver microsomes to the corresponding carboxylic acid 4-BCA. This oxidation was dependent on NADPH and molecular oxygen and was inhibited by CO, typical for a CYP-dependent reaction; NADH was much less effective as the cofactor and both NADP⁺ and NAD⁺ were ineffective (data not shown).

To establish which CYPs were most active in the oxidation of 4-BA and 9-AA, incubations were carried out using liver microsomes from control rats and rats treated with classical CYP inducers; β NF, PB, PYR, DEX and CLO were used to induce CYP1A1/2, 2B1/2, 2E1, 3A1/2 and 4A1/2, respectively (Ryan and Levin 1990). With all types of microsomes, the oxidation of the two aldehydes followed simple Michaelis–Menten kinetics and were linear up to 30 min and for 0.3–0.5 mg microsomal protein ml⁻¹. From Lineweaver–Burk plots of 4-BA and 9-AA oxidation, the apparent kinetic constants were determined (table 1).

Table 1. Apparent kinetic constants for the oxidation of 4-BA and 9-AA by CYP2B1 and 2C11 and by liver microsomes from control and male pretreated rats.

Microsomes or purified CYP	Major CYP	9-AA		4-BA	
		K_m (μM)	$V_{\max}$ ($\text{nmol min}^{-1} \text{mg}^{-1}$ protein)	K_m (μM)	$V_{\max}$ ($\text{nmol min}^{-1} \text{mg}^{-1}$ protein)
Control male	2C11	25 ± 7	3.3 ± 0.6	26 ± 9	6.7 ± 1.1
Control female	2C12	$8 \pm 3^*$	$0.9 \pm 0.3^*$	30 ± 6	$3.8 \pm 0.8^*$
βNF	1A	24 ± 5	$0.7 \pm 0.2^*$	18 ± 5	$4.3 \pm 0.5^*$
PB	2B	38 ± 8	$11 \pm 2.5^*$	47 ± 15	$14 \pm 2.7^*$
PYR	2E1	21 ± 5	$1.6 \pm 0.5^*$	43 ± 12	5.6 ± 0.6
DEX	3A	19 ± 7	5.4 ± 1.3	55 ± 18	8.5 ± 2.1
CLO	4A	23 ± 6	3.7 ± 0.5	27 ± 7	4.8 ± 0.8
P4502B1			7.2		5.2
P4502C11			9.6		7.4

Values are means $\pm$ SD of three experiments performed with different microsomal preparations. Each preparation contained pooled livers from two to three rats. The incubations were performed at 37°C with a substrate concentration ranging from 1 to 100 μM and a protein concentration of 1 mg ml^{-1} . The incubation of the reconstituted system containing CYP2B1 or 2C11 was carried out as described in the Materials and methods.

$V_{\max}$ and K_m are expressed as $\text{nmol min}^{-1} \text{mg}^{-1}$ protein and μM , respectively; in the case of purified P450, the activity was expressed as $\text{nmol min}^{-1} \text{nmol}^{-1}$ P450.

*Significantly different from male control microsomes, $p < 0.5$, Student's *t*-test.

The formation rates of both 4-BCA and 9-ACA by hepatic microsomes from PB-treated rats were increased about threefold with respect to those of microsomes from control male rats indicating that CYP2B1, highly induced in PB microsomes, may play an important role in these reactions. On the other hand, with microsomes from βNF -, PYR-, DEX- and CLO-treated rats, the kinetic parameters were similar or lower than those of microsomes of male control rats implying that the CYPs induced in these microsomes CYP1A1/2, 2E1, 3A1/2, 4A1/2 are not involved in the oxidation of these aldehydes.

The oxidation rates of 4-BA and 9-AA were also significantly higher in the male than in female microsomes suggesting a catalytic role of the male-specific CYP2C11, the major CYP constitutively expressed in rat liver (Fujita *et al.* 1987). The apparent involvement of CYP2C11 and 2B1 in the oxidation of these aldehydes was also supported by the finding that these enzymes in a reconstituted system could catalyse these oxidations with a consistent turnover rate. On the contrary, CYP1A1 and 2E1 were unable to perform these reactions.

Oxidation of 4-BA and 9-AA by human liver microsomes

Both 4-BA and 9-AA are readily oxidized by human liver microsomes following simple Michaelis–Menten kinetics and were linear with time up to 20 min and with protein up 0.5 mg ml^{-1} (data not shown). The apparent kinetic parameters estimated by Eadie–Hofstee plots in three pooled human liver microsomes are shown in table 2. The mean apparent K_m 's for the two substrates were similar whereas the mean apparent $V_{\max}$ for 4-BA was larger than that for 9-AA.

Table 2. Kinetic parameters for the oxidation of 4-BA and 9-AA in three pooled human liver microsome samples.

Liver microsomes	4-BA		9-AA	
	K_m	V_{max}	K_m	V_{max}
HL-1	15.4	1.8	21	0.3
HL-2	17	1.7	11	0.45
HL-3	26	1.4	35	0.51
Mean ($\pm$ SD)	19.4 ± 5.7	1.6 ± 0.2	22.3 ± 12	0.42 ± 0.1

V_{max} is expressed as $\text{nmol min}^{-1} \text{mg}^{-1}$ protein K_m as μM .

Correlation studies

The rates of 4-BA and 9-AA oxidation were determined in microsomes from 14 individual human livers at a substrate concentration of $200 \mu\text{M}$ (figure 1). All the human liver microsomal samples examined demonstrated the ability to oxidize both aldehydes. The rates of 4-BCA formation exhibited a 2.8-fold range from 1.1 to $3.05 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein, and the rates of the 9-ACA formation showed a 3.2-fold range from 0.25 to $0.81 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein. These rates were then correlated against marker catalytic activities for the major human CYPs, determined in these samples. The marker activities were 7-ethoxyresorufin *O*-deethylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), *S*-mephenytoin *N*-demethylase (CYP2B6), 7-ethoxy-4-trifluoromethylcoumarin deethylase (CYP2B6), paclitaxel 6α -hydroxylase (CYP2C8), tolbutamide methyl-hydroxylase (CYP2C9), *S*-mephenytoin 4'-hydroxylase (CYP2C19), dextromethorphan *O*-demethylase (CYP2D6), chlorzoxazone 6-hydroxylase (CYP2E1), testosterone 6β -hydroxylase (CYP3A4), lauric acid 12 hydroxylase (CYP4A9/11). The correlation coefficients obtained are detailed in table 3. For 4-BA and 9-AA, a high correlation was obtained with CYP3A4 (testosterone 6β -hydroxylase) ($r = 0.76$ and 0.8 , $p < 0.001$) and a weak correlation with the catalytic activities

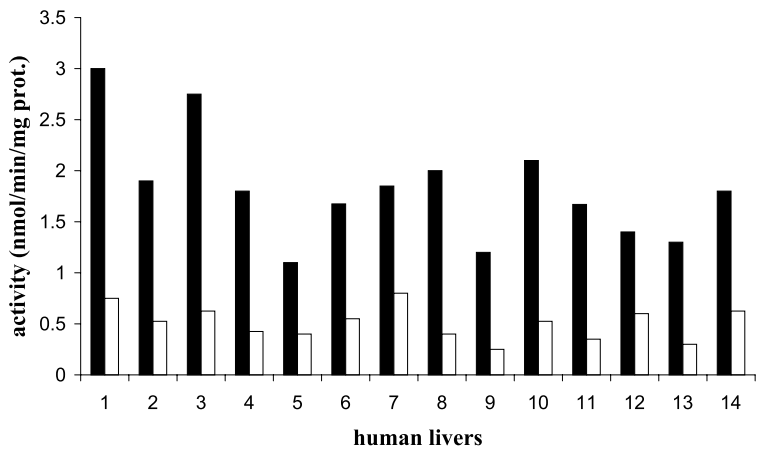


Figure 1. Oxidation rates of 4-BA (■) and 9-AA (□) by 14 human liver microsome samples. Each incubation performed for 20 min at 37°C contained, in a volume of 0.5 ml, 0.2 mg of microsomal protein, NADPH-generating system and $200 \mu\text{M}$ of each substrate; the corresponding acids were analysed as described in Materials and Methods. Data are the average of two determinations.

Table 3. Correlation of the oxidation rates of 4-BA and 9-AA with specific CYP activities in 14 human liver microsomes.

Activities	Correlation coefficient (<i>r</i>)	
	4-BA	9-AA
7-Ethoxyresorufin <i>O</i> -deethylase (1A2)	0.30	0.04
Coumarin 7-hydroxylase (2A6)	0.32	0.63
7-Ethoxy-trifluoromethylcoumarin deethylase (2B6)	0.62	0.60
<i>S</i> -mephenytoin <i>N</i> -demethylase (2B6)	0.58	0.65
Paclitaxel 6 α -hydroxylase (2C8)	0.14	0.46
Tolbutamide methyl-hydroxylase (2C9)	0.38	0.48
<i>S</i> -mephenytoin 4'-hydroxylase (2C19)	0.19	0.34
Dextromethorphan <i>O</i> -demethylase (2D6)	-0.17	-0.08
Chlorzoxazone 6-hydroxylase (2E1)	0.55	0.48
Testosterone 6 β -hydroxylase (3A4)	0.76	0.80
Lauric acid 12-hydroxylase (4A9/11)	-0.30	0.07
4-Biphenyl aldehyde oxidase	—	0.74

Correlation coefficients were calculated from the data of figure 1. Marker activities were supplied by Xenotch. **Bold** numbers mean a good or weak correlation among the various activities.

7-ethoxy-4-trifluoromethyl coumarin *O*-deethylase ($r = 0.62$ and $r = 0.60$, $p < 0.01$) and *S*-mephenytoin *N*-demethylase ($r = 0.58$ and 0.65 , $p < 0.01$), both markers for CYP2B6. In addition, a good correlation was also observed between the oxidation rates of 4-BA versus 9-AA ($r = 0.74$).

Oxidation of 4-BA and 9-AA by cDNA expressed human CYPs

Rates of 9-AA and 4-BA oxidation by the cDNA expressed human CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6, 2E1, 3A4 and 3A4 + b₅ are shown in figure 2.

Conversion of 9-AA and 4-BA to the corresponding acids was only detected with CYP2A6, 2B6, 2C9, and 3A4; with 9-AA as substrate, turnover numbers

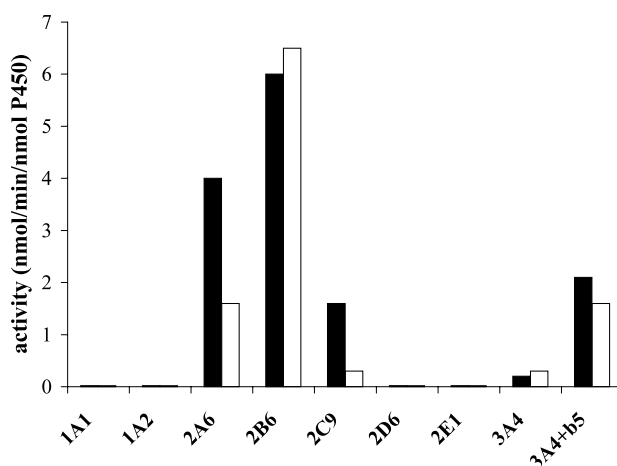


Figure 2. Oxidation rates of 4-BA (■) and 9-AA (□) to the corresponding carboxylic acids by various cDNA expressed human CYPs. Activities were measured with 200 μ M aryl aldehydes under conditions described in Materials and Methods. Data are the average of two determinations.

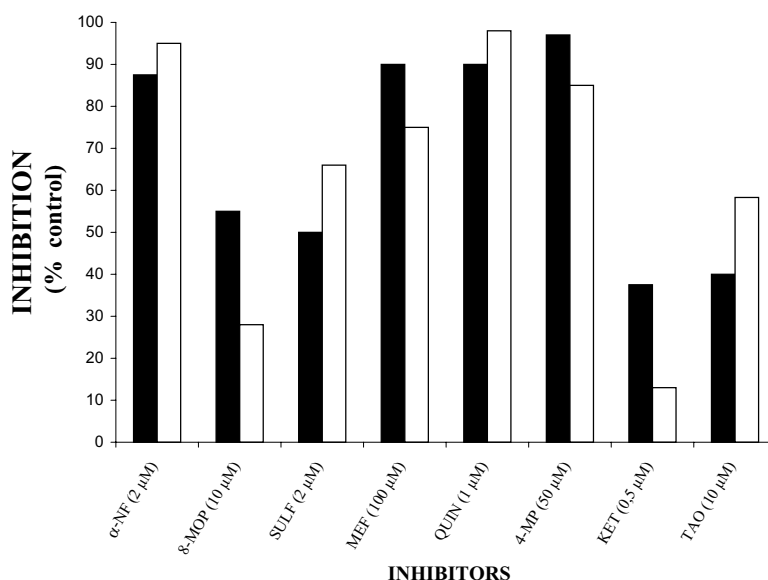


Figure 3. Effect of specific CYP inhibitors on the oxidation rate of 4-BA (■) and 9-AA (□), both at 100 μM. Values are the average of two determinations performed with two pooled human liver microsomes (HL-1 and HL-2) as described in Materials and Methods.

were 1.6, 6.4, 0.3 and 1.6 nmol min⁻¹ nmol⁻¹ CYP, whereas with 4-BA as substrate, turnover numbers were 4, 6, 1.5 and 2.1 nmol min⁻¹ nmol⁻¹ CYP, respectively. With both substrates, CYP2B6 exhibited the highest activity and CYP3A showed an increase in activity in the presence of cytochrome b₅.

Chemical inhibition

Two pooled human liver microsomes (HL1 and HL2) were incubated with 4-BA and 9-AA (at 100 μM) in the presence of selective chemical CYP inhibitors and their conversion rates to the corresponding acids were determined. The inhibitors used were α-NF (2 μM), 8-MOP (10 μM), SULF (2 μM), MEF (100 μM), QUIN (1 μM), 4-MP (50 μM), KET (0.5 μM) and TAO (100 μM), selective for the CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4 (Parkinson 1996, Koenigs *et al.* 1997, Sai *et al.* 2000), respectively.

As shown in figure 3, the oxidations of 4-BA and 9-AA were markedly inhibited by KET, TAO and 8-MOP and, at a lesser extent, by SULF. These data suggest that CYP3A4, 2A6 and 2C9 are likely involved in the oxidation of the two aldehydes.

Discussion

A previous study reported that mouse liver microsomes can catalyse the oxidation of 9-AA to 9-ACA and that this reaction may be assayed by a simple fluorimetric method (Watanabe *et al.* 1991). In the present paper, we describe that this fluorometric assay may also be used to determine the microsomal oxidation of 4-BA to the corresponding carboxylic acid 4-BCA. The assay takes advantage of

the fluorescence of 4-BCA, which is easily separated from the interference of 4-BA by using the same extraction method described for 9-AA metabolism. As found for 9-AA and other aldehydes (Watanabe *et al.* 1991, Kunitoh *et al.* 1997), it was demonstrated that 4-BA, which has not been investigated previously, may also be substrate for the CYP system. The CYP forms responsible for 9-AA metabolism and, of course, those ones for 4-BA are unknown. We obtained evidence that more than one form metabolized both 9-AA and 4-BA in the rat. The results indicated that in the untreated male rats the oxidation of these aldehydes is mediated primarily by the sex-specific CYP2C11, the major CYP constitutively expressed in male rat liver (Fujita *et al.* 1989). The experiments with microsomes from pre-induced rats and purified enzymes provided evidence that CYP2B1 and 2C11 but not CYP1A1/2, 2E1, 3A1/2 and 4A1/2 could oxidize these aldehydes with a consistent turnover.

The 4-BA and 9-AA metabolism to the corresponding acids was also studied in three preparations of pooled liver microsomes. Investigations into the oxidation kinetics of these compounds revealed a single phase in Eadie-Hofstee plots with similar mean K_m 's (19.4 ± 5.7 and $22.3 \pm 12 \mu\text{M}$ for 4-BA and 9-AA, respectively), supporting the hypothesis that a major CYP(s) with similar affinities (K_m) may be responsible for their oxidation. Despite the similarity in K_m , in a bank of 14 human liver microsomes the mean oxidation rate of 9-AA ($0.51 \pm 0.16 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) was about 4-fold lower than that of 4-BA ($1.8 \pm 0.54 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein).

A comparison of the kinetic parameters for the oxidation of 4-BA and 9-AA in rat microsomes versus human microsomes shows similar K_m 's, but a 4–10-fold higher V_{max} for both 4-BA and 9-AA in rat microsomes (tables 1 and 2). This difference may be explained by considering the high constitutive expression of the CYP2C11 (about 40% of total CYP) in rat (Lewis 1998), its catalytic turnover observed in the oxidation of these aldehydes (table 1) and the lack of a similar expression of homologous CYPs in human liver.

To evaluate the contribution of CYPs in the oxidation of 4-BA and 9-AA in human, studies were conducted using cDNA-expressed CYPs, correlation analysis, and selective chemical inhibitors. When the oxidation of 4-BA and 9-AA was examined by cDNA-expressed CYPs, it was found that both were extensively metabolized by CYP2A6, 2B6 and, at lower extent, 2C9 and 3A4, the latter in the presence of b₅. Only trace amounts of aldehyde metabolism were observed with other cDNA-expressed CYPs.

Using a bank of 14 human liver microsomes, good correlations were obtained between the oxidation rate of 4-BA and 9-AA ($r = 0.74$) and between the oxidation rates of both aldehydes ($r = 0.76$ for 4-BA, $r = 0.80$ for 9-AA) with testosterone 6 β -hydroxylase (table 3). This enzymatic activity, which is considered to be catalysed by CYP3A4 (Parkinson 1996, Wrighton *et al.* 1993), is the most abundant form in human liver microsomes, accounting for up to 40% of total expressed CYP (Shimada *et al.* 1994). A weak correlation was also observed with *S*-mephenytoin *N*-demethylase ($r = 0.65$ for 9-AA, $r = 0.58$ for 4-BA) and 7-ethoxy-4-trifluoromethylcoumarin ($r = 0.60$ for 9-AA, $r = 0.62$ for 4-BA), both dependent on CYP2B6 (Ekins *et al.* 1997, Jo *et al.* 1998). Since this enzyme is expressed at low levels (about 1%) in human liver microsomes (Lewis 1998), its contribution to the oxidation of these aldehydes is likely rather small.

A weak correlation was also found between coumarin-7-hydroxylase, a marker for CYP2A6 (Lewis 1998), and 9-AA oxidation ($r = 0.63$). However, no correlation was found between the 4-BA oxidation rate and coumarin 7-hydroxylase ($r = 0.32$); on the basis of the finding obtained with the cDNA-expressed CYP2A6, a correlation with the oxidation of 4-BA would be expected. Possibly, this discrepancy may be due to the low expression (about 4%) of this enzyme in human liver (Shimada *et al.* 1994) and the limited number of human samples used.

Experiments were also performed with chemical inhibitors selective for CYPs. The results indicated that the oxidation of 4-BA and 9-AA was extensively inhibited by TAO and ketoconazole (at low concentration), both considered inhibitors of CYP3A4 (Say *et al.* 2000). An inhibition of metabolism of both aldehydes was also observed with 8-MOP, a mechanism-based inhibitor of CYP2A6 (Koenigs *et al.* 1997) and, with sulfaphenazole although at a lower extent, selective for CYP2C9 (Jo *et al.* 1998). In the light of the data of the correlation analysis, cDNA-expressed CYP catalysis and chemical inhibition presented, it is apparent that, in human liver, CYP3A4 makes a major contribution to the biotransformation of both 4-BA and 9-AA.

However, as the oxidation of these aldehydes was also demonstrated with CYP2B6, 2A6 and 2C9 then, under certain conditions, these forms may play an important role particularly in extrahepatic tissues, where the expression of CYP3A4 is low.

Overall, the results of the present study demonstrate that both 9-AA and 4-BA can be substrates for a numbers of different CYPs in rat and in human liver. Owing to sequence homology of CYPs between species (Smith 1991, Soucek and Gut 1992), we would expect similar enzymes to be responsible for the 4-BA and 9-AA oxidation. Indeed, for the orthologous rat CYP2B1 and human CYP2B6, the substrate specificity towards these aldehydes appears to be maintained. However, between human CYP3A4 and rat CYP3A1/2 a different catalytic behaviour was apparent: whereas CYP3A4 appeared the most important catalyst, the highly expressed CYP3A1/2 in microsomes from DEX-treated rats did not enhance the oxidation rates of these aldehydes above their control values, suggesting a minor contribution, if any, of these rat forms. As it is well known that the members of CYP2A and 2C subfamilies have a very different substrate specificity (Lewis 1998), no comparison can be made between rat CYP2A1/2 and human CYP2A6 and between CYP2C11- and CYP2C9-mediated catalysis.

In conclusion, 4-BA and 9-AA are not CYP-specific substrates whose oxidation is mediated by CYP2C11 and CYP2B1 in rat and by CYP3A4, 2B6, 2A6 and 2C9 in human. Consequently, these fluorescent probes should be used with caution when comparing metabolism across species. Furthermore, our results obtained show that 9-AA and particularly 4-BA undergo oxidation with a high V_{max} and may offer a distinct advantage over other fluorescent substrates in testing CYP activities in a certain species when only a small amount of tissue is available or when extrahepatic tissues have to be examined.

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Role of specific cytochrome P450 enzymes in the N-oxidation of the antiarrhythmic agent mexiletine

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Received 7 February 2002

1. Mexiletine is extensively metabolized in man by C- and N-oxidation and the aim of the present study was to characterize major cytochrome P450 enzyme(s) involved in the formation of *N*-hydroxymexiletine.

2. Incubations with genetically engineered microsomes indicated that the formation rate of *N*-hydroxymexiletine was highest in the presence of microsomes expressing high levels of either CYP1A2 or CYP2E1 and the formation of *N*-hydroxymexiletine by human liver microsomes was inhibited about 40% by antibodies directed against CYP1A1/1A2 or CYP2E1. Additional incubations demonstrated that formation of *N*-hydroxymexiletine was decreased 47 and 51% by furafylline, 40 µM and 120 µM, respectively, and decreased 55 and 67% by α -naphthoflavone, 1 µM and 3 µM, respectively (all $p < 0.05$ versus control).

3. The formation rate of *N*-hydroxymexiletine in human liver microsomes was highly correlated with CYP2B6 (*RS*-mexiletine, $r = 0.7827$; *R*-(-)-enantiomer, $r = 0.7034$; *S*-(+)-enantiomer, $r = 0.7495$), CYP2E1 (*S*-(+)-enantiomer, $r = 0.7057$) and CYP1A2 (*RS*-mexiletine, $r = 0.5334$; *S*-(+)-enantiomer, $r = 0.6035$).

4. In conclusion, we have demonstrated that CYP1A2 is a major human cytochrome P450 enzyme involved in the formation of *N*-hydroxymexiletine. However, other cytochrome P450 enzymes (CYP2E1 and CYP2B6) also appear to play a role in the N-oxidation of this drug.

Introduction

Mexiletine is an orally effective Class Ib antiarrhythmic agent used as a racemic mixture in the treatment of ventricular arrhythmias (Campbell 1987). It has a narrow therapeutic index and in-depth knowledge about its pharmacokinetics properties is essential for effective treatment without toxicity (Campbell *et al.* 1975). Important interindividual variability in dose requirements, efficacy and toxicity has been observed and a number of studies have identified the modulatory roles of disease states, concomitant drug administration, and environmental and genetic factors on the pharmacokinetics of the drug (Pentikäinen *et al.* 1982, 1984, Grech-Bélanger *et al.* 1985, Turgeon *et al.* 1991a).

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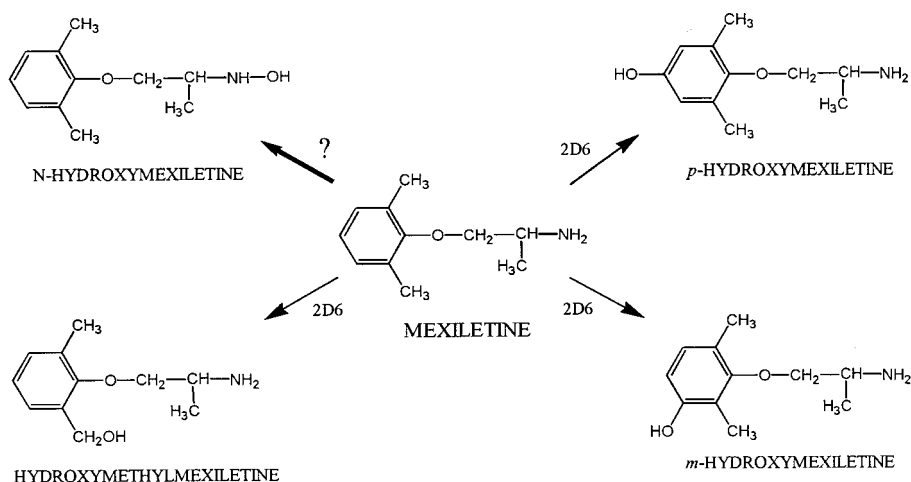


Figure 1. Major metabolic pathways of mexiletine in man.

In man, mexiletine undergoes extensive metabolism by the liver to several pharmacologically inactive metabolites and <10% of an administered oral dose is recovered unchanged in the urine (Prescott *et al.* 1977, Häselbarth *et al.* 1981). The cytochrome P450 enzyme CYP2D6 is a key determinant of mexiletine disposition and is the primary enzyme involved in the formation of three major metabolites, namely hydroxymethylmexiletine, *m*-hydroxymexiletine and *p*-hydroxymexiletine (figure 1) (Turgeon *et al.* 1991a). Partial metabolic clearances of mexiletine to hydroxymethylmexiletine, to *m*-hydroxymexiletine and to *p*-hydroxymexiletine are increased approximately six- to 15-fold in subjects with genetically determined high CYP2D6 activity (EMs) compared with poor metabolizers (PMs). Moreover, quinidine, a selective inhibitor of CYP2D6 (Kobayashi *et al.* 1989), decreases partial metabolic clearances of mexiletine to these metabolites only in EMs (Turgeon *et al.* 1991a).

Previous studies have demonstrated that CYP2D6 is not involved in the formation of *N*-hydroxymexiletine (figure 1) that is present at high levels as a glucuronide conjugate both in plasma and urine (Grech-Bélanger *et al.* 1985, Turgeon *et al.* 1991a, 1992). Partial metabolic clearance of mexiletine to *N*-hydroxymexiletine is similar in CYP2D6 EMs compared with PMs and is unaltered by the co-administration of quinidine in both phenotypes (Turgeon *et al.* 1991a).

Comparison of mexiletine metabolic clearance between EMs and PMs indicates that CYP2D6-dependent metabolic pathways correspond to 19% of mexiletine total metabolic clearance in EMs. In contrast, in PMs, partial metabolic clearance to *N*-hydroxymexiletine represents 28% of mexiletine total metabolic clearance. Independent of CYP2D6 activity, urinary recovery of *N*-hydroxymexiletine is the largest of all identified metabolites and represents 14% of the dose in EMs and 22% in PMs (Turgeon *et al.* 1991a).

Mexiletine is administered as a racemic mixture of equal parts of the *R*-(-)- and *S*-(+)-enantiomers. The drug undergoes stereoselective disposition in man and both enantiomers possess different antiarrhythmic potency with the *R*-enantiomer being slightly more potent (Grech-Bélanger *et al.* 1986, Turgeon

et al. 1991b). We have demonstrated that CYP2D6 polymorphism is not implicated in the stereoselective disposition of mexiletine (Abolfathi *et al.* 1993). Following oral administration of mexiletine, the area under the plasma concentration–time curve (AUC) of both *R*-(–)- and *S*-(+)-mexiletine were greater in plasma of PMs than in those of EMs. However, the *R/S* ratio of mexiletine plasma concentrations was similar in PMs and EMs. Co-administration of quinidine did not alter plasma concentrations of either *R*-(–)- or *S*-(+)-mexiletine in subjects with the PM phenotype. In contrast, mean plasma concentrations of both enantiomers were increased by quinidine in EMs. However, quinidine increased plasma concentrations of *R*-(–)- and *S*-(+)-mexiletine to a similar extent such that the *R/S* ratio of mexiletine plasma concentrations was not modified. Moreover, the *R/S* ratio of the clearance of mexiletine and the ratio of the urinary recovery of both enantiomers were similar in EMs and PMs. These ratios were unaltered by quinidine administration (Abolfathi *et al.* 1993).

On the other hand, formation of *N*-hydroxymexiletine is highly stereoselective. We have reported that the urinary recovery of mexiletine *N*-glucuronide and the partial metabolic clearance of mexiletine to *N*-hydroxymexiletine were more than 10 times greater for the *R*-(–)-enantiomer (Grech-Bélanger *et al.* 1986, Abolfathi *et al.* 1993). These *R/S* ratios were similar in EMs and PMs and were unaltered by quinidine co-administration (Abolfathi *et al.* 1993). Data obtained in the latter study indicate that CYP2D6 is not responsible for the stereoselective disposition of mexiletine in man. On the other hand, enzymes involved in the *N*-oxidation of mexiletine may be responsible for the stereoselective disposition of the drug.

Although formation of *N*-hydroxymexiletine is not mediated by CYP2D6, the cytochrome P450 form(s) involved in the *N*-oxidation of mexiletine is not known yet. Therefore, the goal of the present study was to determine the role of various cytochrome P450 enzymes in the formation of *N*-hydroxymexiletine *in vitro*. Studies were conducted using human liver microsomes and microsomes from human lymphoblastoid or yeast cell lines transfected with cDNA coding for specific P450 isozymes.

Materials and methods

Chemicals and reagents

Mexiletine hydrochloride was a gift from Boeringer Ingelheim (Canada) Ltd (Burlington, ON, Canada). Furaflavine was provided by Dr Kent Kunze (Department of Medicinal Chemistry, School of Pharmacy, University of Washington, USA). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP⁺, α -naphthoflavone and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were obtained from usual commercial sources.

Biological materials

Microsomes from lymphoblastoid cells transfected with cDNAs encoding for specific human cytochrome P450 enzymes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4) and monoclonal antibodies directed against CYP2A6, CYP2B6, CYP2E1 or CYP3A4/3A5 were obtained from Gentest Corp. (Woburn, MA, USA). Polyclonal antibodies directed against CYP1A1/1A2, CYP2C or CYP2D6 were purchased from Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan). Microsomes from yeast expressing high levels of CYP1A2 were provided through the Bioavenir program, Rhône-Poulenc Rorer, Roussel Uclaf, Ministère Français de la Recherche, France. Liver microsomes from 15 human liver samples were obtained from Human Biologics (Phoenix, AZ, USA). Enzymatic activities of these hepatic microsomes for specific cytochrome P450 probe drugs were determined by Human Biologics. Three other human liver samples were obtained from a human liver bank (Dr Philippe Beaune through the Bioavenir program). Livers were

Table 1. Characteristics of human liver samples.^a

Sample	Gender	Age (years)	Ethnic background	Smoke	Alcohol use
HBI 101	M	37	Hispanic	yes	yes
HBI 102	M	43	Hispanic	unknown	unknown
HBI 103	F	45	Caucasian	unknown	unknown
HBI 104	F	72	Caucasian	unknown	unknown
HBI 105	M	64	Caucasian	unknown	unknown
HBI 106	F	58	Caucasian	yes	unknown
HBI 107	F	49	Caucasian	yes	yes
HBI 108	M	50	Caucasian	unknown	unknown
HBI 109	M	60	Caucasian	yes	yes
HBI 110	F	17	Caucasian	unknown	unknown
HBI 111	M	45	Caucasian	unknown	unknown
HBI 112	F	63	Caucasian	unknown	unknown
HBI 113	M	32	Hispanic	yes	yes
HBI 114	M	38	African-American	yes	yes
HBI 115	F	68	Caucasian	unknown	unknown
HL 76	M	Unknown	Unknown	unknown	unknown
HL 537	unknown	Unknown	Unknown	unknown	unknown
HL 1004	M	49	Unknown	unknown	unknown

^a Adapted from Human Biologics data with permission

collected in agreement with French laws and stored at -80°C until preparation of microsomes. Information relative to donors of human liver samples is listed in table 1.

Synthesis of N-hydroxy [1-(2',6'-dimethyl)phenoxy-2-aminopropane]

N-hydroxymexiletine was synthesized according to a method described previously for other alkylamines starting with 4.16 g mexiletine base (Beckett *et al.* 1975). The oxalic acid salt was recrystallized three times from a mixture of ethanol/ether. NMR (d-DMSO) δ (ppm from TMS) 1.22 (d, 3, CHCH_3), 2.23 (s, 6, CH_3), 3.40 (m, 1, CH), 3.77 (m, 2, CH_2), 7.01 (m, 3, H-3, H-4, H-5); GC/MS (diacetyl derivative) m/z 116 (100%), 43 (85.4%), 158 (70.5%), 105 (14.4%), 122 (14.3%), 121 (11.3%), 135 (4.3%). Elementary analysis (expected for dibasic salt): C 59.7%, H 7.5%, N 5.7%, (calculated): C 60.0%, H 7.5%, N 5.8% and O 26.7%. No other major peak ($>1\%$) was detected during GC-MS analysis of the compound.

Incubation procedures

A series of experiments were performed with microsomes from lymphoblastoid cells or yeast expressing high levels of specific cytochrome P450 enzymes. In addition, *in vitro* studies were conducted using human liver microsomes. The latter experiments were mostly performed using human microsomes obtained from Human Biologics. Microsomes from three additional livers were prepared in house to characterize further metabolic enzymes involved in the N-oxidation of mexiletine. Microsomal suspensions from these three human liver samples were prepared according to the method described by Wang *et al.* (1983). Liver samples were obtained from liver/kidney transplant donors. In brief, liver samples were defrosted in NaCl 0.9% at $0-4^{\circ}\text{C}$, dried, weighed and homogenized in Tris-acetate buffer 0.1 M with a polytron Kinematica PT 10-35. The homogenate was centrifuged at 10 000g for 30 min (Sorval centrifuge RC 5B) to eliminate cellular debris, cell nuclei and mitochondria. The supernatant was then centrifuged at 100 000g for 90 min in a Beckman L5-50E ultracentrifuge. The pellet containing the microsomes was resuspended by homogenization in potassium pyrophosphate buffer 0.1 M, pH 7.4, and recentrifuged for 90 min at 100 000g. The new pellet contained the washed microsomes, which were resuspended with the polytron in Tris-acetate buffer 0.01 M, pH 7.4. Of this microsomal suspension, 1 ml was equivalent to 250 mg liver tissue. Fractions of 250 μl of the microsomal suspensions were immediately frozen in liquid nitrogen and stored at -80°C until used for incubation experiments. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

NADPH-generating system

The NADPH-generating solution consisted of glucose 6-phosphate (3.62 mg), NADP^+ (0.6 mg), MgCl_2 25 mM (100 μl) and glucose 6-phosphate dehydrogenase (1.3 units) for each incubation.

Determination of mexiletine N-hydroxylase activity

Each incubation consisted of 100 μ l (20–100 μ l for cDNA-expressed enzymes) microsomal suspension, 200 μ l Tris–KCl buffer 0.25 M, pH 7.4, 100 μ l NADPH-generating solution and 100 μ l substrate (mexiletine solution 5 mM dissolved in MgCl_2 25 mM). The final concentration of the substrate was therefore 1 mM. Incubations containing microsomes, buffer and NADPH-generating system solution were kept on ice and then pre-incubated at 37°C for 5 min. Incubation reactions were initiated by the addition of the substrate to the incubation mixture. Incubations were performed in a shaking water bath at 37°C for 15 min. Substrate was substituted by Tris–KCl buffer 0.25 M, pH 7.4, in control incubations. All incubations were performed in duplicate except for those containing antibodies where only one incubation was performed for each concentration of antibodies.

Determination of mexiletine N-hydroxylase activity in the presence of inhibitors

Antibodies. Incubations using pooled microsomes from human liver samples were performed in the presence of various amounts of monoclonal (0.5, 1, 2, 5 or 10 μ l anti-CYP2A6, anti-CYP2B6, anti-CYP2E1 or anti-CYP3A4/3A5) or polyclonal (3, 6, 12, 24 or 48 μ l anti-CYP1A1/1A2, anti-CYP2C or anti-CYP2D6) antibodies directed against different cytochrome P450 enzymes. Activity is expressed relative to the amount of metabolite formed in a control incubation containing pre-immunoglobulin.

α -Naphthoflavone or furafylline. Mexiletine was incubated with microsomes from human liver samples or yeast in the presence of either α -naphthoflavone (1 or 3 μ M or furafylline (40 or 120 μ M; all final concentrations) to characterize the role of CYP1A2 in the N-oxidation of mexiletine. α -Naphthoflavone solutions were prepared by dissolving 6.8 or 20.4 mg of the drug in 25 ml dimethylsulfoxide/ethanol mixture (4:1). These solutions were diluted 1:10 in the same mixture and 5 μ l of the diluted solutions were added to the incubation mixture. Furafylline solutions were prepared by diluting 1 or 3 mg of the drug in 20 ml Tris–KCl buffer 0.25 M, pH 7.4. Of these solutions, 100 μ l were added instead of 100 μ l Tris–KCl buffer solution usually added to the incubation mixture.

Inhibitors (α -naphthoflavone or furafylline) were pre-incubated for 10 min at room temperature and for 5 min at 37°C in the presence of microsomes, Tris–KCl buffer, pH 7.4, and NADPH-generating solution. Metabolic reactions were initiated by the addition of the substrate (mexiletine).

Determination of $V_{\max}$ and K_m

Incubation procedures were performed using microsomes from human liver or lymphoblastoid cells expressing high levels of CYP1A2. The final concentration of RS-, R(-)- or S-(+)-mexiletine in the incubation mixture varied from 0.03 to 10 mM (0.03, 0.1, 0.3, 1, 3, 6 or 10 mM). Incubations were performed in duplicate (two samples for each concentration). Following determination of $V_{\max}$ and K_m , the intrinsic and predicted hepatic clearances were calculated according the method described by Hoener (1994).

Determination of the amount of N-hydroxymexiletine formed in incubation mixtures

At the end of the incubation period, 100 μ l of an aqueous solution containing the internal standard (rimantadine-HCl 5 μ g ml⁻¹) were added to each incubation mixture. The metabolic reaction was then stopped by the rapid addition of 5 ml distilled diethylether. Extraction with two 5-ml portions of diethylether was performed, the ethereal extracts combined, 50 μ l acetic anhydride added and the organic phase was evaporated at 45°C. The residue was resuspended in 100 μ l diethylether and 1 μ l was analysed by gas chromatography/mass spectrometry (GC/MS).

GC/MS analyses were carried out using a Hewlett Packard 5890 gas chromatograph combined with a Hewlett Packard 5970 mass spectrometer operating at an ionization potential of 70 eV. Samples were injected in a splitless mode (injection port temperature was 250°C) using a DB5 (30 m $\times$ 0.25 mm i.d., Supelco, Toronto, ON, Canada) capillary column. Initial oven temperature was set at 35°C for 2 min followed by an increase of 30°C min⁻¹ to attain a temperature of 150°C. The oven temperature was then increased at a rate of 10°C min⁻¹ until a final temperature of 290°C was attained. This temperature was maintained for 5 min. Helium was used as carrier gas at a rate of 0.9 ml min⁻¹. The temperatures of the transfer line and ionization source were 270 and 250°C, respectively.

Data acquisition was performed using the HP-UX Chemstation software while peak identification and quantification was achieved using the selected ion-monitoring mode. For N-hydroxymexiletine diacetylated derivative, m/z 116 and 158 were selected, while for rimantadine m/z 135 was monitored.

Statistical analysis

Formation rates of N-hydroxymexiletine by microsomes enriched in various P450 enzymes isolated from lymphoblastoid cells were compared using a one-way ANOVA. For inhibition studies, N-hydroxymexiletine formation rates in the presence of inhibitors were compared using a Student's *t*-test. Simple regression analyses were performed between the N-hydroxymexiletine formation rate and

specific activities measured in human liver microsomal preparations using the Pearson correlation coefficient test. The results are presented as means $\pm$ SD and, unless specified, $p < 0.05$ was considered as statistically significant.

Results

In vitro studies with genetically engineered microsomes

Figure 2 shows the formation rate of *N*-hydroxymexiletine using microsomal suspensions from lymphoblastoid cells transfected with cDNA encoding for specific human cytochrome P450 enzymes. Mexiletine *N*-hydroxylase activity was highest with microsomes expressing high levels of CYP1A2 activity (109.4 ± 3.3 pmol nmol⁻¹ of P450 min⁻¹). Formation of *N*-hydroxymexiletine measured with CYP1A2 microsomes was 1.4, 2.6 and 2.7 higher than that measured with microsomes expressing high CYP2E1, CYP2C8 or CYP2A6 activity, respectively (all $p < 0.05$ versus CYP1A2). Moreover, except for CYP1A2, mexiletine *N*-hydroxylase activity was significantly higher in CYP2E1 microsomes compared with microsomes expressing other specific cytochrome P450 enzymes.

Since we had observed in these first series of experiments that CYP1A2 was the major enzyme involved in the *N*-oxidation of mexiletine, we performed experiments with specific inhibitors of this enzyme. The formation rate of *N*-hydroxymexiletine by CYP1A2-enriched microsomes (yeast) was decreased 76 and 81% by furafylline 40 and 120 μ M, respectively, and 88 and 100% by α -naphthoflavone 1 and 3 μ M, respectively.

In vitro studies with human liver microsomes

Figure 3 shows the effects of various antibodies directed against specific cytochrome P450 enzymes on the formation rate of *N*-hydroxymexiletine in

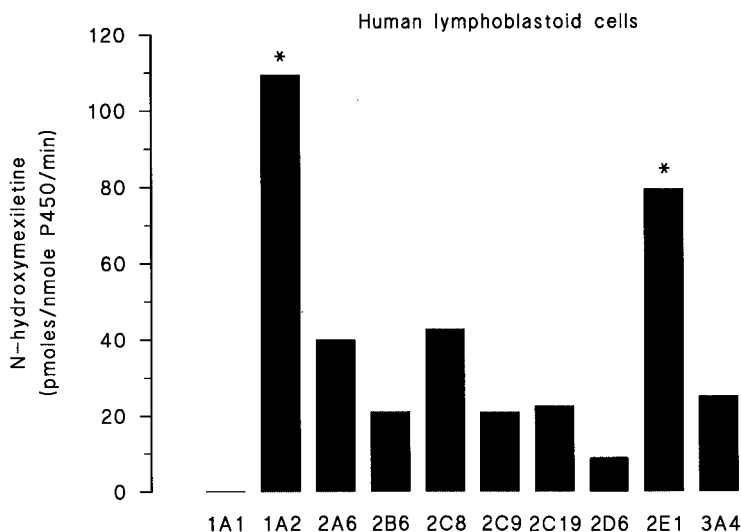


Figure 2. *N*-hydroxymexiletine formation rate measured in microsomes obtained from transfected lymphoblastoid cells expressing high levels of specific human cytochrome P450 isozymes. * $p < 0.05$ versus all other cytochrome P450 enzymes. Data are from two determinations.

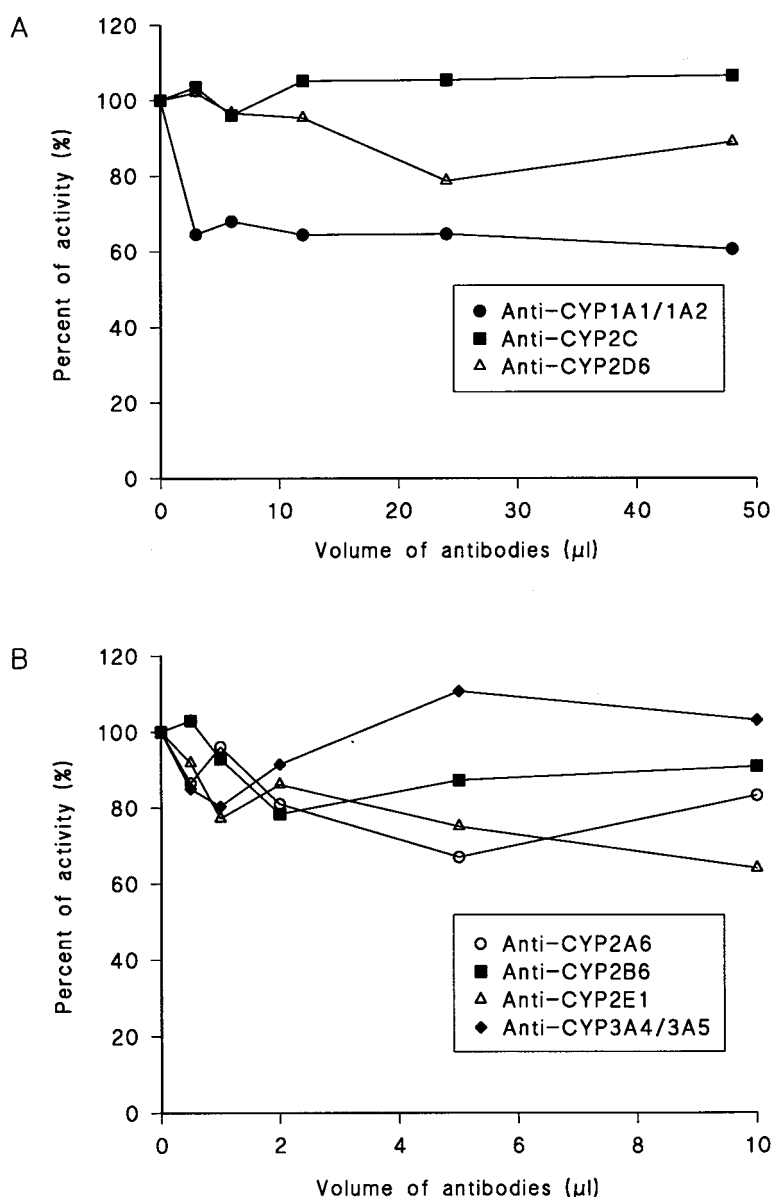


Figure 3. Inhibition of *N*-hydroxymexiletine formation by antibodies directed against various cytochrome P450 enzymes. Incubations were performed with pooled microsomes prepared from 15 human liver samples. The absolute value of control incubations was 95 ± 7 pmol $\text{mg}^{-1} \text{min}^{-1}$.

pooled microsomes from 15 human liver samples (Human Biologics). These experiments demonstrated that an inhibition up to 40% of the formation of *N*-hydroxymexiletine was observed in the presence of antibodies directed against CYP1A1/1A2 or CYP2E1. Qualitative analysis of the results suggests that no major inhibition was noted in the presence of antibodies directed towards CYP2A6, CYP2B6, CYP2C, CYP2D6 and CYP3A4/3A5.

The relationship between the formation rate of *N*-hydroxymexiletine and different cytochrome P450 enzyme activities measured by specific probe drugs was studied in microsomes from 15 human liver samples following incubation with mexiletine and its enantiomers separately (table 2). The best correlations were observed between *S*-mephenytoin *N*-demethylase activity (CYP2B6) and *N*-oxidation of *RS*-mexiletine ($r = 0.7827$; $p = 0.0006$), *R*-(-)-enantiomer ($r = 0.7034$; $p = 0.0034$) and *S*-(+)-enantiomer ($r = 0.7495$; $p = 0.0013$). An excellent correlation was also noted between CYP2E1 activity and *N*-oxidation of *S*-(+)-mexiletine ($r = 0.7057$; $p = 0.0033$). A significant relationship was observed between *N*-hydroxymexiletine formation and caffeine *N*3-demethylase activity (CYP1A2) following incubation with mexiletine racemic mixture ($r = 0.5334$; $p = 0.0406$) and *S*-(+)-mexiletine ($r = 0.6035$; $p = 0.0172$). A good correlation was also noted with CYP2C9 and CYP4A11 activities.

Table 3 shows the effects of different inhibitors on the formation of *N*-hydroxymexiletine in human liver microsomes. Furafylline at concentrations of 40 and 120 μM decreased formation rate of *N*-hydroxymexiletine by 40–61% ($p < 0.05$). Similarly, α -naphthoflavone at concentrations of 1 and 3 μM decreased *N*-hydroxymexiletine formation rate by 37–74 and 44–85%, respectively ($p < 0.05$).

Finally, table 4 shows V_{max} , K_{m} , intrinsic and predicted hepatic clearances of *RS*, *R*-(-)- or *S*-(+)-mexiletine to *N*-hydroxymexiletine in microsomes from human liver or lymphoblastoid cells expressing high levels of CYP1A2. Intrinsic clearances calculated in human liver microsomes following incubation with *RS*-mexiletine varied from 21 to 43 ml min^{-1} . Intrinsic clearances measured with CYP1A2 microsomes were within this latter range. Moreover, calculated intrinsic clearances with CYP1A2 microsomes were 25.0, 23.2 and 21.6 ml min^{-1} following incubation with *RS*, *R*-(-)- and *S*-(+)-mexiletine, respectively.

Discussion

Results obtained in this study with human liver microsomes, as well as with microsomes expressing specific human cytochrome P450 enzymes, suggest that several cytochrome P450s are involved in the formation of *N*-hydroxymexiletine in man. Specifically, CYP1A2, CYP2E1 and CYP2B6 appear to play an important role in the formation of this metabolite *in vitro*.

Evidence has accumulated to support the involvement of CYP1A2 in the metabolism of mexiletine in man. *In vitro* studies performed by Nakajima *et al.* (1998) have demonstrated the involvement of CYP1A2 in the formation of hydroxymethylmexiletine and *p*-hydroxymexiletine. However, these latter authors did not assess the role of CYP1A2 in the formation of *N*-hydroxymexiletine. In addition, two potent inhibitors of CYP1A2 (ciprofloxacin and fluvoxamine) (Brosen *et al.* 1993, Fuhr *et al.* 1993) decrease mexiletine clearance when co-administered with the drug (Labbé 2000, Kusumoto *et al.* 2001). Pharmacokinetic studies in man have also demonstrated that mexiletine reduces the clearance of other CYP1A2 substrates such as caffeine or theophylline by >40% due to metabolic inhibition (Joeres *et al.* 1987, Hurwitz *et al.* 1991, Loi *et al.* 1991, Stoyisch *et al.* 1991). Studies from our group have reported that cigarette smoking (a well-known inducer of CYP1A2) (Sesardic *et al.* 1988) alters the kinetics of mexiletine in humans and increases the formation rate of a *N*-glucuronide

Table 2. Correlation coefficients between *N*-hydroxymexiletine formation rate and cytochrome P450-specific activities in liver microsomes of 15 human samples.

Activity	Substrate concentration	<i>RS</i> -mexiletine		<i>R</i> <i>S</i> (-)-mexiletine		<i>S</i> <i>S</i> (+)-mexiletine	
		<i>r</i>	<i>p</i>	<i>R</i>	<i>p</i>	<i>r</i>	<i>p</i>
Caffeine N3-demethylase (CYP1A2)	1 mM	0.5334	0.0406	0.4803	0.0700	0.6035	0.0172
Coumarin 7-hydroxylase (CYP2A6)	50 μ M	0.1106	0.6947	0.1723	0.5393	0.0396	0.8885
<i>S</i> -mephenytoin <i>N</i> -demethylase (CYP2B6)	400 μ M	0.7827	0.0006	0.7034	0.0034	0.7495	0.0013
Tolbutamide methylhydroxylase (CYP2C9)	1 mM	0.3943	0.1458	0.4014	0.1381	0.5331	0.0407
<i>S</i> -mephenytoin 4'-hydroxylase (CYP2C19)	400 μ M	0.2664	0.3372	0.2388	0.3914	0.2848	0.3036
Dextromethorphan <i>O</i> -demethylase (CYP2D6)	80 μ M	0.2269	0.4160	0.1437	0.6095	0.2059	0.4615
Chlorzoxazone 6-hydroxylase (CYP2E1)	500 μ M	0.5127	0.0507	0.3440	0.2094	0.7057	0.0033
Dextromethorphan <i>N</i> -demethylase (CYP3A4)	250 μ M	0.4679	0.0786	0.4955	0.0603	0.3408	0.2139
Testosterone 6 β -hydroxylase (CYP3A4/5)	80 μ M	0.0767	0.7857	0.0502	0.8589	0.2159	0.4397
Lauric acid 12-hydroxylase (CYP4A11)	100 μ M	0.3688	0.1762	0.2752	0.3208	0.5419	0.0369

Numbers in **bold** indicate statistically significant correlations ($p < 0.05$)

Table 3. Effect of inhibitors on the formation rate of *N*-hydroxymexiletine.

Human liver	Inhibition of <i>N</i> -hydroxymexiletine formation rate (%)			
	α -Naphthoflavone		Furafylline	
	1 μ M	3 μ M	40 μ M	120 μ M
HL76	74	85	61	61
HL 537	37	44	40	45
HL1004	55	71	41	47
Mean $\pm$ SD	55 $\pm$ 19**	67 $\pm$ 21**	47 $\pm$ 12**	51 $\pm$ 9*

* $p < 0.01$, ** $p < 0.05$. Control activities for these incubations were 112 and 120 pmol mg⁻¹ min⁻¹ for microsomes from liver HL 76; 150 and 162 pmol mg⁻¹ min⁻¹ for microsomes from liver HL 537; and 72 and 76 pmol mg⁻¹ min⁻¹ for microsomes from liver HL 1004.

Table 4. V_{max} and K_m determined during *in vitro* metabolism of *RS*, *R*-(-)- or *S*-(+)- mexiletine to *N*-hydroxymexiletine using microsomes from human livers or lymphoblastoid cells expressing high levels of CYP1A2 and the predicted hepatic clearance.

Microsomes	V_{max} (pmol mg ⁻¹ min ⁻¹)	K_m (μ mol ml ⁻¹)	CL _{int} ^a (ml min ⁻¹)	CL _H ^a (ml min ⁻¹)
<i>RS</i> -mexiletine				
HL 76 ^b	168	0.86	42.7	12.7
HL 537 ^b	208	2.22	20.5	6.1
HL 1004 ^b	108	0.75	31.6	9.4
CYP1A2 ^c	39	0.34	25.0	7.5
<i>R</i> -(-)-mexiletine				
CYP1A2 ^c	65	0.61	23.2	6.9
<i>S</i> -(+)-mexiletine				
CYP1A2 ^c	30	0.30	21.6	6.5

CL_{int}, intrinsic clearance; CL_H, predicted hepatic clearance.
^a Calculated using the method of Hoener (1994).
^b Microsomes from human livers.
^c Microsomes from lymphoblastoid cells transfected with cDNA coding for CYP1A2.

metabolite which has been characterized as *N*-hydroxymexiletine glucuronide (Grech-Bélanger *et al.* 1985, Turgeon *et al.* 1992). We have also demonstrated that co-administration of caffeine and mexiletine decreases *N*-hydroxymexiletine formation *in vivo* (Labbé *et al.* 1999).

Results from the current study are in agreement with and further clarify these previous observations. A high-level formation of *N*-hydroxymexiletine was observed in microsomes from lymphoblastoid cells expressing high CYP1A2 activity. In addition, the presence of antibodies directed against CYP1A2 in the incubation mixture with human liver microsomes decreased by 40% the formation rate of *N*-hydroxymexiletine. Moreover, a good relationship was observed between *N*-hydroxymexiletine formation rate and caffeine *N*3-demethylase activity (a CYP1A2 activity) in human liver samples. *N*-oxidation of mexiletine was inhibited by two CYP1A2 inhibitors, α -naphthoflavone (McManus *et al.* 1990, Tassaneeyakul *et al.* 1993) and furafylline (Sesardic *et al.* 1990). Finally, the intrinsic clearance of *RS*-mexiletine calculated in human liver microsomes varied from 21 to 43 ml min⁻¹. Intrinsic clearance measured with microsomes from lymphoblastoid cells encoding for CYP1A2 was within this latter range indicating that this enzyme very likely has a major role in the formation of *N*-hydroxymexiletine.

Results from our current study also suggest that CYP1A2 is not the sole cytochrome P450 involved in the formation of *N*-hydroxymexiletine. Formation of *N*-hydroxymexiletine was partially inhibited by α -naphthoflavone (3 μ M) following incubation with human liver microsomes, whereas a complete inhibition was observed with microsomes from lymphoblastoid cells expressing high CYP1A2 activity. Experiments with microsomes from lymphoblastoid cells expressing high levels of CYP2E1 activity were also associated with a high formation rate of *N*-hydroxymexiletine. Moreover, an inhibition (up to 40%) of *N*-hydroxymexiletine formation was noted in the presence of antibodies directed against CYP2E1. Finally, a high correlation was observed between mexiletine *N*-hydroxylase and *S*-mephenytoin *N*-demethylase (a CYP2B6 activity) activity.

The *N*-oxidation metabolic pathway of mexiletine is highly stereoselective with an *R/S* ratio > 10 observed for the partial metabolic clearance of mexiletine. We have recently proposed that CYP1A2 is implicated in the stereoselective formation of *N*-hydroxymexiletine. In fact, co-administration of caffeine reduced the urinary recovery of *N*-hydroxymexiletine but only for the *R*-(-)-enantiomer (Labbé *et al.* 1999). Consequently, the *R/S* ratio for urinary recovery and the partial metabolic clearance of mexiletine to *N*-hydroxymexiletine are 28% lower following co-administration of caffeine suggesting that CYP1A2 is involved in the stereoselective *N*-oxidation of mexiletine. Results obtained in this study do not suggest that CYP1A2 plays a role in the stereoselective formation of *N*-hydroxymexiletine. In fact, the *R/S* ratio of the intrinsic clearance for *N*-hydroxymexiletine was 1.07. In contrast, significant correlations were found between mexiletine *N*-hydroxylase and CYP2E1, CYP2C9 and CYP4A11 only for the *S*-(+)-enantiomer suggesting that these enzymes may be implicated in the stereoselective disposition of mexiletine.

In conclusion, mexiletine *N*-oxidation is the major metabolic pathway identified in man. Our current *in vitro* study has demonstrated that CYP1A2 is a major cytochrome P450 involved in the formation of *N*-hydroxymexiletine. However, other cytochrome P450 enzymes (CYP2E1 and CYP2B6) also appear to be involved in the formation of this metabolite. Clinical studies are needed to identify the exact role of these enzymes in the metabolism of mexiletine and in the formation of *N*-hydroxymexiletine in man.

Acknowledgements

This study was supported by the Medical Research Council of Canada (MT-11876) and by the Heart and Stroke Foundation of Canada (Québec). L. L. and Z. A. were the recipient of studentships from the Fondation des maladies du coeur du Canada. L. L. was also the recipient of a studentship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR). E. L. was the recipient of a studentship from the Medical Research Council of Canada. J. T. is the recipient of a scholarship from the Joseph C. Edwards Foundation. The authors acknowledge Michel Blouin for technical assistance and Serge Simard MSc for statistical analyses. The access to yeast microsomes was made possible through the Bioavenir programme (Rhône-Poulenc Rorer, Roussel-Uclaf, Ministère Français de la Recherche).

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Metabolic fate of pitavastatin, a new inhibitor of HMG-CoA reductase: human UDP-glucuronosyltransferase enzymes involved in lactonization

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Received 20 May 2002

1. Pitavastatin is a potent competitive inhibitor of HMG-CoA reductase little metabolized in hepatic microsomes. Pitavastatin lactone, which can be converted back to the unchanged form, is the major metabolite of pitavastatin in humans. To clarify the mechanism of the lactonization of pitavastatin and the metabolic properties of the lactone, we performed experiments *in vitro*.

2. On addition of UDP-glucuronic acid, human hepatic microsomes produced pitavastatin lactone and an unknown metabolite (UM-2). UM-2 was converted to its unchanged form by enzymatic hydrolysis and to a lactone form non-enzymatically. Using several human UGT-expressing microsomes, UGT1A3 and UGT2B7 were principally responsible for glucuronidation of pitavastatin leading to lactonization.

3. No marked difference in intrinsic clearance between pitavastatin and its lactone form was detected in human hepatic microsomes.

4. Pitavastatin lactone showed no inhibitory effects on CYP2C9- and CYP3A4-mediated metabolism of model substrates in contrast to other HMG-CoA reductase inhibitors.

5. The mechanism of pitavastatin lactone formation has been clarified, in that glucuronidation by UGT occurs first followed by lactonization via an elimination reaction. It was also found that pitavastatin lactone demonstrates no drug-drug interactions.

Introduction

In general, elevated levels of low-density lipoprotein (LDL)-cholesterol are considered the most important risk factor for coronary artery disease (Brown *et al.* 1991). The most effective method of lowering LDL-cholesterol is the administration of inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the cholesterol synthesis pathway (Havel and Rapaport 1995). Pitavastatin is a highly potent inhibitor of HMG-CoA reductase and causes a significant reduction in serum total cholesterol, LDL-cholesterol and triglyceride levels in animals (Aoki *et al.* 1997, Suzuki *et al.* 1999, 2001). In humans, the persistent effect on serum lipid and safety of pitavastatin have been confirmed in clinical practice (Kajinami *et al.* 2000a), and this drug has an enhancing effect on patients with non-insulin-dependent diabetes mellitus

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(NIDDM) or familial hypercholesterolemia (Kajinami *et al.* 2000b, Yamada *et al.* 2000).

Pitavastatin is minimally metabolized in hepatic microsomes and there are no drug–drug interactions between it and either tolbutamide or taxol *in vitro* (Fujino *et al.* 1999a, 2001). Moreover, no induction of the drug-metabolizing enzymes (aniline hydroxylase, aminopyrine *N*-demethylase, 7-ethoxycoumarin *O*-deethylase and UDP-glucuronic acid transferase) was found in rat after multiple administrations of pitavastatin (Fujino *et al.* 2002). The favourable metabolic characteristic of this drug support efficacy in clinical studies. Pitavastatin is metabolized via biotransformation as follows: lactonization; β -oxidative degradation of the side-chain; hydroxylation of the quinoline ring; and conjugation with β -glucuronic acid and taurine (Kojima *et al.* 1999a). The lactone form of pitavastatin, which can be reversibly converted to the parent drug, is found as a major metabolite in plasma following oral administration in animals and human (Fujino *et al.* 1999b, Kojima *et al.* 1999b). This suggests that pitavastatin and pitavastatin lactone coexist at equilibrium in blood after administration of the drug. To date, only the metabolism of pitavastatin, not of lactone, has been investigated and no studies have been published about the mechanism of lactonization. To clarify this latter mechanism, experiments were performed *in vitro* in the present study. Furthermore, in view of the widespread clinical use of pitavastatin and the importance of drug–drug interactions, the inhibitory effect of pitavastatin lactone on cytochrome P450 enzymes was also investigated.

Materials and methods

Chemicals and reagents

Pitavastatin, monocalcium bis[(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]3,5-dihydroxy-6-heptenoate] and its lactone form were synthesized by Nissan Chemical Industries (Chiba, Japan). Fluvastatin and atorvastatin lactone were synthesized in our laboratory. The chemical purities of all synthesized compounds were > 98.3%. 4-Hydroxytolbutamide and 6 β -hydroxytestosterone were purchased from Ultrafine Chemicals (Manchester, UK). Ketoprofen and *p*-nitrophenol as chemical inhibitors of UGT were purchased from Wako Pure Chemicals (Osaka, Japan). The NADPH-regenerating system (β -NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase) and UDP-glucuronic acid were obtained commercially. All other chemicals and reagents used were commercially available and of guaranteed purity.

Radioisotope-labelled chemicals

(Fluorobenzene-¹⁴C) pitavastatin was synthesized by Amersham Co. (Little Chalfort, UK). The specific radioactivity of the labelled compound was 2.2 MBq mg⁻¹, and the radiochemical purity was > 99.2%. [Ring-¹⁴C] tolbutamide (Amersham, 2.22 GBq mmol⁻¹) and [4-¹⁴C] testosterone (Amersham, 2.11 GBq mmol⁻¹) were purchased commercially and the radiochemical purities were > 98.4%.

Microsomes

Human hepatic microsomes (H23, H30, H42, H66, H70, H93, H112, H161, HK27 and HK31) and recombinant microsomes derived from baculovirus-expressing human UGT (control, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7 and UGT2B15) and human cytochrome P450s (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) were purchased from Gentest Co. (Woburn, MA, USA). Human pooled renal microsomes (HRM-005), intestinal microsomes (HMPM-INT) and pooled monkey hepatic microsomes were purchased from the International Institute for Advancement of Medicine (Exton, PA, USA) and In Vitro Technologies (Baltimore, MD, USA), respectively.

In vitro metabolism of pitavastatin

In Phase 1 reaction experiments, the incubation mixture was prepared as follows: a 0.5 ml reaction mixture containing 1 mg protein ml⁻¹ microsome, 1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 U ml⁻¹ glucose 6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 2.5 μ M ¹⁴C-pitavastatin in 100 mM sodium phosphate buffer (pH 7.4) was incubated for 2 h. In Phase 2 reaction experiments, a 0.5 ml reaction mixture containing 1 mg protein ml⁻¹ microsomes, 1 mM uridine diphosphate glucuronic acid (UDPGA), 10 mM magnesium chloride and 2.5 μ M ¹⁴C-pitavastatin in 50 mM Tris (pH 7.5) was incubated for 2 h. After incubation at 37°C, the reaction was quenched by adding an equal volume of acetonitrile. The reaction-terminated samples were centrifuged at 6000g and the supernatant analysed.

Isolation of ¹⁴C-pitavastatin lactone

¹⁴C-pitavastatin lactone was isolated according to our previous method (Yamada *et al.* 2001). After incubation of ¹⁴C-pitavastatin (50 μ M) with monkey microsomes in the Phase 2 reaction experiment for 2 h, pitavastatin lactone was fractionated from the HPLC elutes while monitoring UV absorption (254 nm) at a retention time of about 20 min. The fractionated pitavastatin lactone solution was purified by a solid-phase extraction method as follows: the solution was diluted with 6 vols water and absorbed on a solid-phase extraction column, Bond Elut C₁₈ (300 mg, Varian Sample Preparation Products, Habor, CA, USA). After being washed with 2 ml water, the residual pitavastatin lactone was eluted with 3 ml ethanol. The radiochemical purity of the isolated ¹⁴C-pitavastatin lactone was >99%. All operations were carried out in the dark to avoid decomposition.

Isolation of unknown metabolites (UM-2)

In the Phase 2 reaction, UM-2 was fractionated from the HPLC elutes while monitoring UV absorption (254 nm) at a retention time of about 7 min. Fractionated UM-2 was evaporated to dryness and subjected to enzymatic hydrolysis with β -glucuronidase/arylsulfatase (5000 Fishman units and 80 units, respectively) at 37°C for 2 h or alkaline treatment with 0.1 N NaOH at room temperature for 10 min.

Inhibition of lactonization by chemical UGT inhibitors

The experiments with known chemical inhibitors of UGT were performed at a substrate concentration of 2.5 μ M and inhibitor concentrations of 150 and 750 μ M for ketoprofen and 200 and 1000 μ M for *p*-nitrophenol using human hepatic microsomes. Pitavastatin was co-incubated with these UGT inhibitors for 2 h. The inhibitory effect was estimated from the amount of pitavastatin lactone formed in the presence and absence of inhibitor.

Possible role for UGT enzymes in pitavastatin metabolism

Using nine recombinant human UGT-expressing microsomes, the human UGT enzymes participating in the lactonization of pitavastatin to pitavastatin lactone were investigated. At pitavastatin concentrations of 2.5, 10 and 50 μ M, the reaction mixtures were incubated for 30 min with UDPGA. In addition, incubation with control microsomes (native insect cells, BTI-TN-5B1-4) was performed in parallel. The incubation conditions were the same as for the Phase 2 reaction.

Kinetic analysis of lactonization

The Michaelis–Menten parameters of the apparent K_m and V_{max} for pitavastatin lactonization were estimated by the analysis of Lineweaver–Burk reciprocal plots. The pitavastatin concentration was 1–80 μ M and incubation was carried out for 30 min in human hepatic and renal microsomes, respectively.

Metabolic stability of pitavastatin lactone

Pitavastatin and its lactone form at a concentration of 2.5 μ M were incubated with human hepatic microsomes and an NADPH-regenerating system and then the metabolic stability was evaluated. The remaining pitavastatin or lactone form were measured at 0, 15, 30, 60 and 120 min after the addition of microsomes. In the determination of *in vitro* clearance (Cl_{int}), the slope of the linear regression from the log concentration of actual data versus incubation time relationships ($-k$) was used in the calculation as follows:

$$Cl_{int} = k \times (\text{ml incubation})/(\text{mg microsome}).$$

In the evaluation of lactone clearance, the unchanged form which was converted from the lactone form non-enzymatically was calculated as the amount of pitavastatin lactone remaining.

Role of CYP enzymes in pitavastatin lactone metabolism

Using six recombinant CYP-expressing microsomes, the human CYP enzymes participating in the metabolism of pitavastatin lactone were investigated. Pitavastatin lactone (2.5 μM) and the reaction mixtures were incubated for 30 min with the NADPH-regenerating systems. The incubation conditions were as for the Phase 1 reaction.

Correlation of pitavastatin lactone metabolism with CYP activities

Using 10 individual human hepatic microsomes, the correlation between the metabolism of pitavastatin lactone and activity of CYP markers was studied for CYP1A2 (phenacetin *O*-deethylase), CYP2A6 (coumarin 7-hydroxylase), CYP2C9 (diclofenac 4'-hydroxylase), CYP2C19 (mephenytoin 4'-hydroxylase), CYP2D6 (bufuralol 1'-hydroxylase), CYP2E1 (chlorzoxazone 6-hydroxylase), CYP3A4 (testosterone 6 β -hydroxylase) and CYP contents. Pitavastatin lactone at a concentration of 2.5 μM was incubated for 2 h with several human hepatic microsomes and total metabolism was estimated from the pitavastatin and lactone remaining.

Effect of pitavastatin lactone on the metabolism of several CYP markers

The inhibitory effect of pitavastatin lactone on CYP-mediated metabolism was examined using ^{14}C -tolbutamide and ^{14}C -testosterone in the concentration range 40–800 μM . In the inhibition experiment for CYP2C9, pitavastatin lactone (1–10 μM) was co-incubated with tolbutamide. In the presence or absence of pitavastatin lactone, the CYP2C9-mediated production of 4-hydroxytolbutamide was evaluated. In the inhibition experiment for CYP3A4, pitavastatin lactone (0.5–5 μM) was co-incubated with testosterone. Fluvastatin and atorvastatin lactone were used as inhibitors of CYP2C9- and CYP3A4-mediated metabolism, respectively. The inhibition experiments for these inhibitors were carried out in the same manner as for pitavastatin. The inhibition constant (K_i) was estimated by Dixon plot analysis.

Analytical methods

Total radioactivity was measured with a liquid scintillation counter (Tri-Carb 1500) for 10 min after addition of a scintillation cocktail (Hionic-Fluor, all Packard Instrument Co., Meriden, CT, USA).

The measurement of pitavastatin and its metabolite was performed according to an HPLC-radioluminography method (Baba *et al.* 1994, Kimata *et al.* 1998) as follows: the HPLC system (L-6000 series, Hitachi, Tokyo, Japan) equipped with a UV detector, a reversed-phase column (Inertsil ODS-3V, 4.6 $\times$ 150 mm, GL Science, Inc., Tokyo, Japan) and auto-injector (AS-4000; Hitachi) was operated with two mobile phases. The separation was achieved with the following program: the initial solvent (A), 0.1 M acetate buffer (pH 3.5)/acetonitrile (55:45 v/v), was delivered for 7.5 min, then the mobile phase was changed with a linear gradient over a period of 25 min to solvent (B), 0.1 M acetate buffer (pH 4.5)/acetonitrile (30:70 v/v), which was delivered for 5 min. The flow rate of the mobile phase was 1 ml min⁻¹. The HPLC elute was fractionated into the wells of polystyrene flat-bottomed microplates (RLG plate Beta48, Toyobo, Inc., Osaka, Japan) at 15-s intervals using a fraction collector (Liquid Handler 222XL, Gilson, Middlenton, WI, USA). The plates were allowed to stand for 12 h at room temperature for evaporation of the solvent.

The measurement of 4-hydroxytolbutamide was performed by TLC (Ludwig *et al.* 1998). The analysis of 6 β -hydroxytestosterone was carried out as follows: the incubates were applied to TLC plates (Silicagel 60F₂₅₄, Merck, Darmstadt, Germany) and developed with dichloromethane–acetone (4:1 v/v) to 16 cm in a horizontal TLC chamber. The amount of unchanged drug and its metabolites were determined using a BAS-2500 (Fuji Photo Film Co., Tokyo, Japan). The radioactive metabolites were positively identified by a comparison of retention times or R_f using authentic unlabelled standard.

Results

Characteristics of pitavastatin in human microsomes

Figure 1 shows typical chromatograms derived from a reaction mixture using human hepatic, renal and intestinal microsomes. In the Phase 1 reaction, ^{14}C -pitavastatin was metabolized only marginally to M-13 with hepatic microsomes but no metabolite was detected with other microsomes. The amount of fractional radioactivity of M-13 in hepatic microsomes was 16.3%. On addition of UDPGA, microsomes produced pitavastatin lactone and an unknown metabolite (UM-2) at retention times of 20 and 7 min in the Phase 2 reaction. The fractional radioactivity

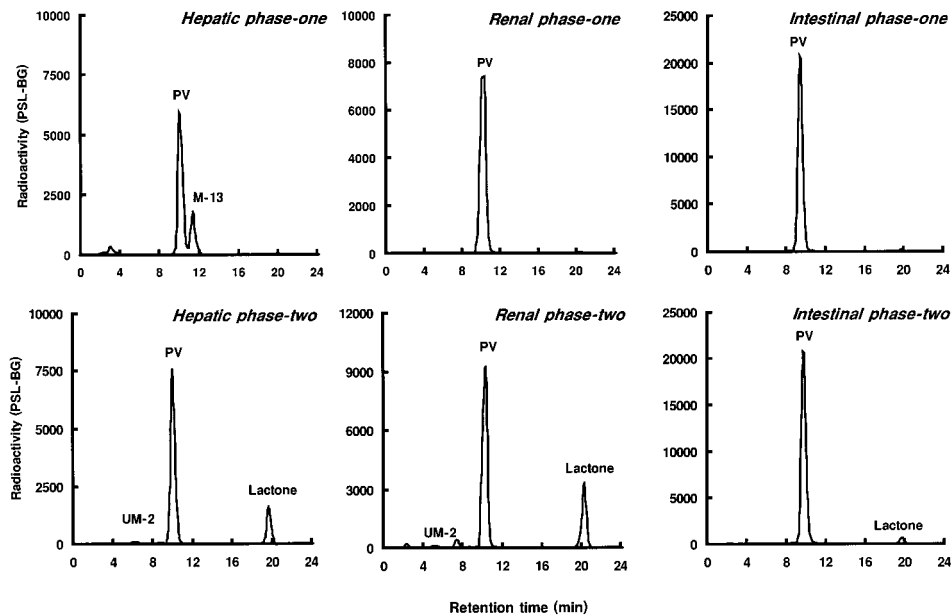


Figure 1. Representative HPLC patterns of radioactivity in human microsomes. PV, pitavastatin; M-13, 8-OH pitavastatin; UM-2, unknown metabolite; lactone, pitavastatin lactone.

Table 1. Composition of pitavastatin and its metabolites after incubation with human tissue microsomes.

Reaction		Percentage of composition		
		Liver	Kidney	Intestine
Phase 1	Pitavastatin	91.5 ± 0.8	99.7 ± 0.2	99.7 ± 0.1
	M-13	6.7 ± 0.7	0.0	0.0
	unknown	1.8 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
Phase 2	Pitavastatin	88.6 ± 1.9	72.8 ± 0.5	96.0*
	Lactone	9.6 ± 1.5	22.1 ± 0.4	2.6*
	unknown	1.8 ± 0.4	10.1 ± 0.4	1.5*

Data are the mean ± SE of three samples.

* *n* = 2

of pitavastatin lactone in hepatic, renal and intestinal microsomes was 9.6, 22.1 and 2.6%, respectively (table 1). These latter peaks disappeared on incubation without UDPGA (data not shown.). In addition, among the UGT chemical inhibitors tested, ketoprofen and *p*-nitrophenol had extensive inhibitory effects on pitavastatin lactone formation (figure 2).

Identification of UM-2 in human microsomes

UM-2 was fractionated from the HPLC elutes by monitoring of UV absorption (254 nm), and the metabolic properties were analysed as follows. Injection of UM-2 led to the formation of pitavastatin lactone. UM-2 was degraded to its unchanged

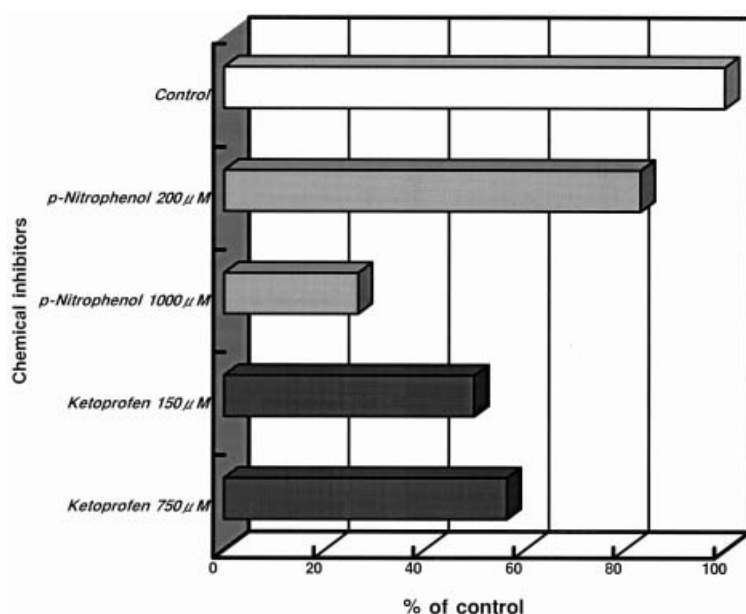


Figure 2. Inhibition of lactonization of pitavastatin in human hepatic microsomes by several UGT inhibitors. The formation of pitavastatin lactone in control samples was 9.6%. Data are the average of duplicate determinations.

or lactone form by enzymatic hydrolysis with β -glucuronidase and also converted completely to the unchanged form by treatment with 0.1 N NaOH (figure 3).

Intrinsic metabolic clearance of pitavastatin lactonization

Lactonization of pitavastatin in human hepatic and renal microsomes with UDPGA followed Michaelis–Menten kinetics as demonstrated by Lineweaver–Burk plots and the curves were all linear (data not shown). The apparent K_m for pitavastatin lactone in hepatic and renal microsomes were 49.4 and 48.8 μ M, respectively, and the V_{max} were 124.6 and 115.6 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein, respectively. The intrinsic clearances (V_{max}/K_m) were 2.5 and 2.4 $\mu\text{l min}^{-1} \text{mg}^{-1}$ protein, respectively (table 2).

Metabolism of pitavastatin by UGT enzymes

Nine microsomal samples expressing human UGTs were used to examine pitavastatin lactonization. The catalytic properties of each human UGT enzyme for the formation of pitavastatin lactone are shown in figure 4. Among the recombinant UGTs examined, UGT1A3 showed the highest activity at all concentrations. UGT2B7 was also capable of catalysing pitavastatin lactonization, although other UGT1A enzymes including UGT1A1, 1A4 and 1A6 were involved as well. In contrast, little metabolite was formed in the presence of the other UGT enzymes up to a concentration of 50 μ M.

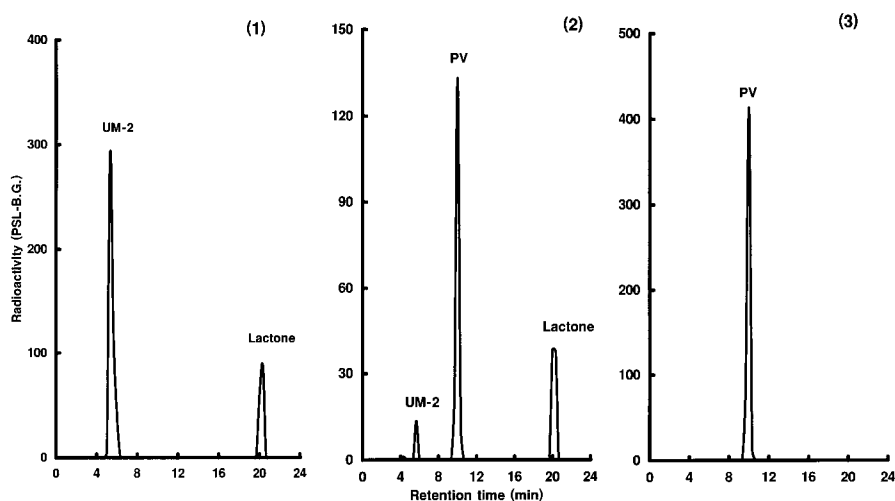


Figure 3. Radiochromatograms of pitavastatin and its metabolites after isolation of the unknown metabolite (UM-2) by several treatments. (1) Direct injection, (2) enzymatic hydrolysis and (3) alkaline treatment. PV, pitavastatin; UM-2, unknown metabolite; lactone, pitavastatin lactone.

Table 2. Kinetic constants of the apparent metabolic reaction of pitavastatin after incubation with microsomes

Tissue	Metabolites	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$)	$V_{\max}/K_m$ ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$)
Hepatic	lactone	49.4	124.6	2.52
Microsomes	M-13*	45.1	77.4	1.79
Renal	lactone	48.8	115.6	2.37
Microsomes	M-13	—	—	—

* Quoted from *Xenobiotic Metabolism and Disposition* (1999), **14**, 415–424.

—, Unable to be calculated

Metabolic stability of pitavastatin lactone

Figure 5 shows the metabolic stability of pitavastatin and its lactone in human hepatic microsomes. The disappearance curves for both compounds were linear up to 120 min. The intrinsic clearance of pitavastatin and its lactone in human hepatic microsomes was 3.4 and $4.6 \mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$, respectively.

Metabolism of pitavastatin lactone by various CYP enzymes

Among the six recombinant CYPs examined (figure 6), CYP3A4 catalysed the metabolism of pitavastatin lactone and the formation of M-3 was predominant. CYP2D6 was also capable of catalysing the formation of M-13. In contrast, metabolite formation was not observed in the presence of the other CYP enzymes at a concentration of $2.5 \mu\text{M}$.

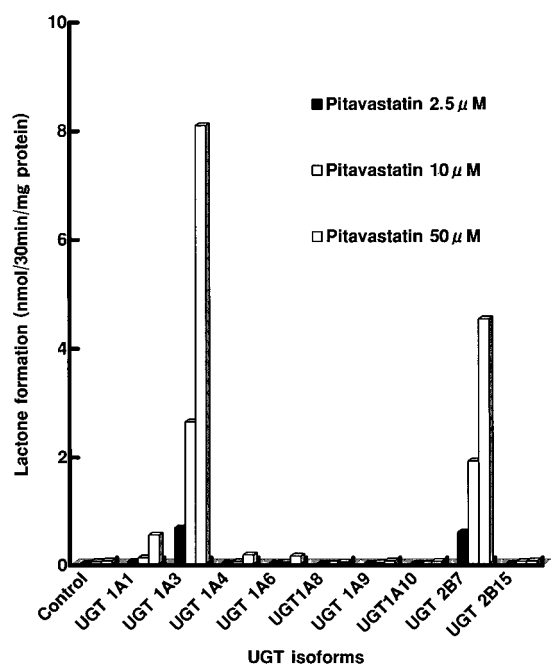


Figure 4. Formation of pitavastatin lactone in microsomes by human UDP-glucuronosyl transferase-expressed enzymes.

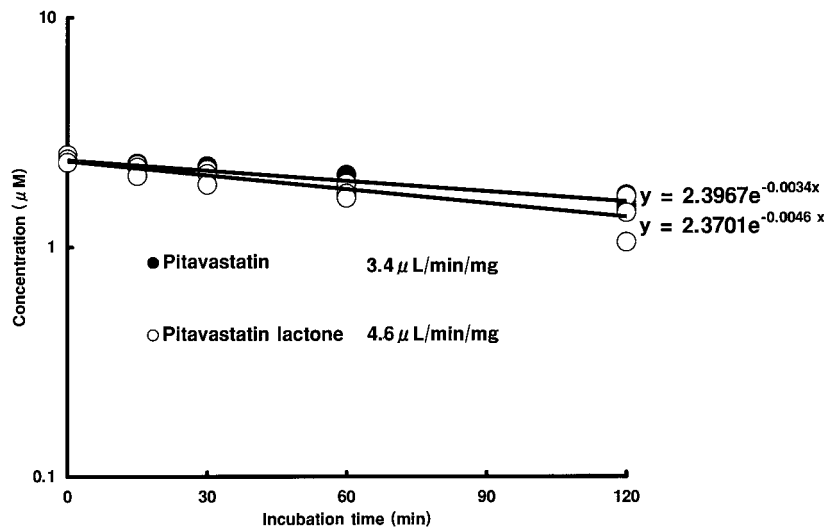


Figure 5. Comparison of intrinsic clearance of pitavastatin and its lactone form after incubation with human hepatic microsomes (1 mg protein ml⁻¹).

Correlation of pitavastatin lactone metabolism with CYP activities

Figure 7 shows the correlation between the metabolism of pitavastatin latone and several CYP marker substrates in ten human hepatic microsome samples. A good correlation was observed for testosterone 6β-hydroxylase (CYP3A4,

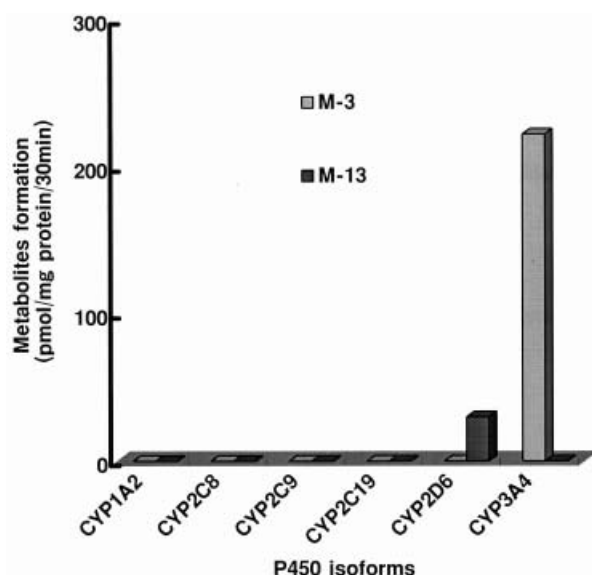


Figure 6. Formation of metabolite in microsomes by CYP-expressed enzymes after incubation of pitavastatin lactone.

$R^2 = 0.911$) and total CYP content ($R^2 = 0.634$), whereas a lower correlation ($R^2 = 0.036$) was observed for lactone metabolism and CYP2C9-mediated diclofenac 4-hydroxylase activity. Only low correlations ($R^2 < 0.06$) were observed for lactone metabolism and markers of enzymic activities catalysed by CYP1A2, CYP2A6, CYP2C19, CYP2D6 or CYP2E1 isoforms.

Effect of pitavastatin on the metabolism of CYP marker substrates

Figure 8 shows the Dixon plots of tolbutamide 4-hydroxylation and testosterone 6 β -hydroxylation, respectively. In the presence of pitavastatin and its lactone, parallel lines without a focal point were obtained, demonstrating that there was no inhibition of CYP2C9- and CYP3A4-mediated metabolism by pitavastatin or pitavastatin lactone. In the presence of fluvastatin or atorvastatin lactone, the metabolic inhibition of tolbutamide hydroxylation and testosterone 6 β -hydroxylation were observed and the K_i were 0.5 and 0.6 μM , respectively.

Discussion

Statins can be classified into the following two groups: those administered as a prodrug, i.e. the lactone form including simvastatin and lovastatin, and those administered in the active form, i.e. the acid form such as pravastatin, fluvastatin, cerivastatin and atorvastatin (Alberts 1990, Lennernas and Fager 1997, Christians *et al.* 1998). The absolute mechanism of lactonization for each statin has not been reported, although the lactone form has been reported to be a metabolite of the acid form (Komai *et al.* 1992, Dain *et al.* 1993, Kearney *et al.* 1993, Boberg *et al.* 1998). The lactone form of pitavastatin was detected although at significantly lower levels than the parent drug in plasma of rabbits, dogs and monkeys (Fujino

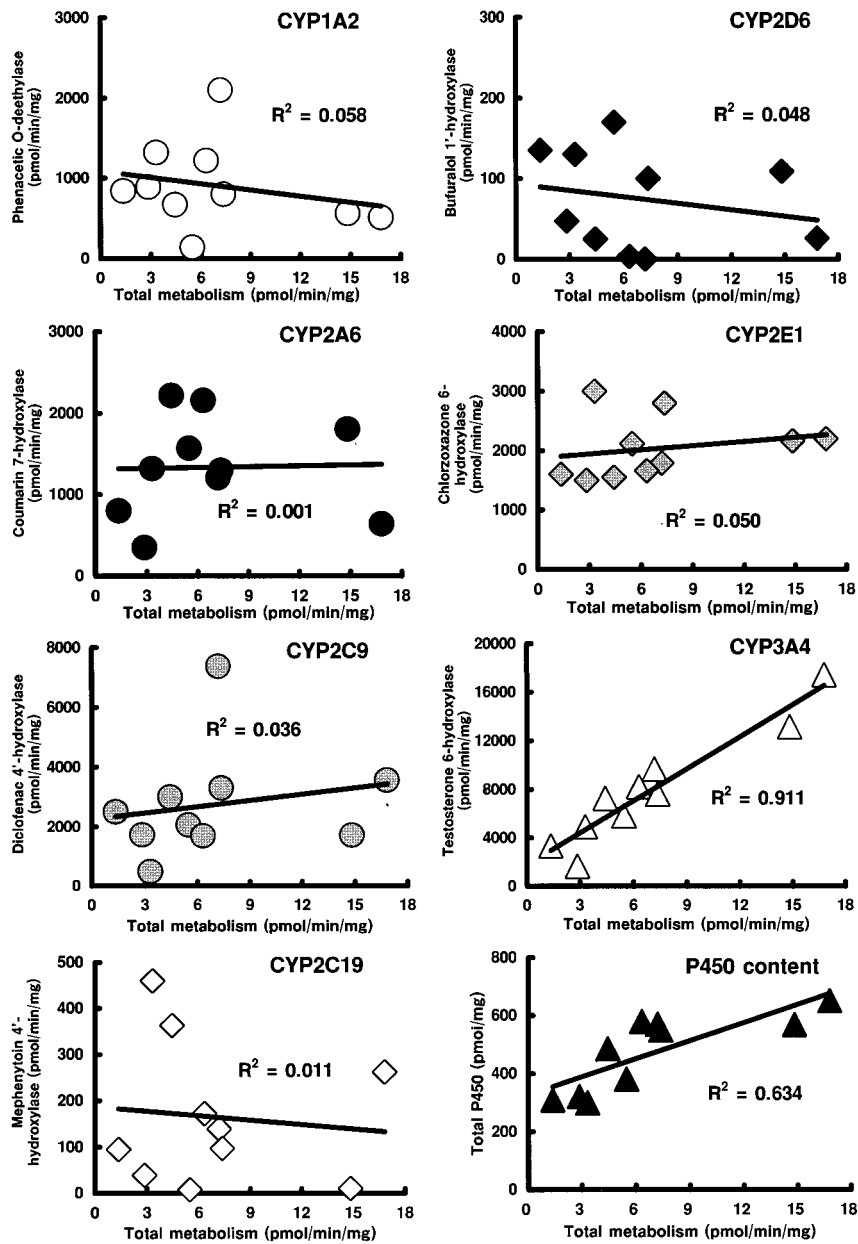


Figure 7. Correlation between total metabolism of lactone and CYP activities in human hepatic microsomes.

et al. 1999b). In humans, the area under the curve for pitavastatin lactone was almost the same as for the parent drug (Kojima *et al.* 1999b), suggesting that lactonization is the major pathway in the metabolism of pitavastatin. To clarify the mechanism of lactonization and the metabolic properties of pitavastatin lactone, experiments were performed *in vitro* in the current study.

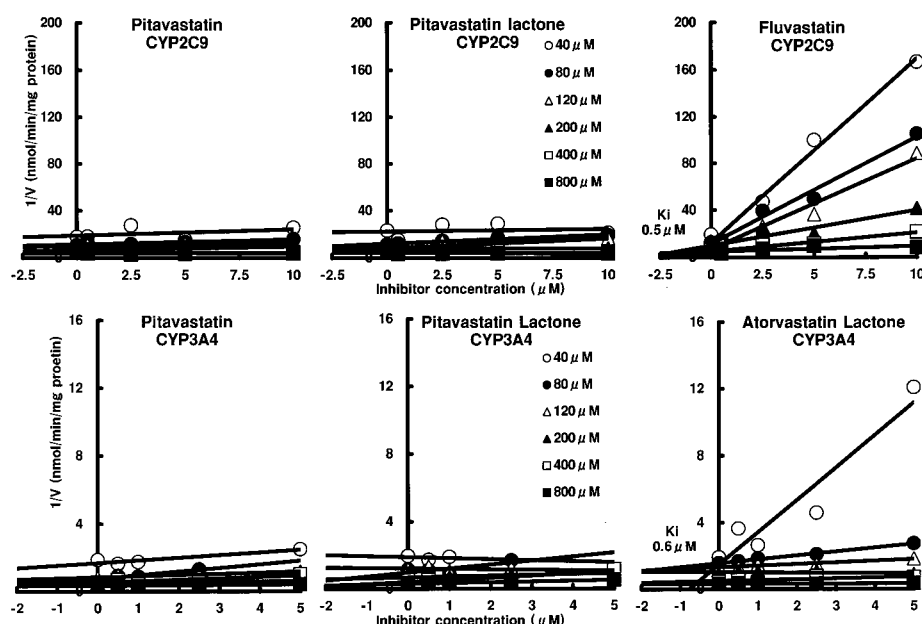


Figure 8. Inhibition of tolbutamide 4-hydroxylation and testosterone 6 β -hydroxylation by HMG-CoA reductase inhibitors in human hepatic microsomes.

Three different *in vitro* approaches using human microsomes, isolated UGT enzymes and UGT chemical inhibitors demonstrated that UGTs play an important role in the lactonization of pitavastatin as follows. The first step in the lactonization of pitavastatin is glucuronidation. UGT involves the formation of UM-2 as an intermediate to the lactone form and catalyses the transfer of glucuronic acid to the carboxyl of the side chain. The glucuronic acid moiety is subsequently converted non-enzymatically to its lactone form. After conversion, some of the resulting pitavastatin lactone is converted to the open acid form. We also found that a portion of pitavastatin lactone is metabolized in human hepatic microsomes. CYP3A4-mediated M-3 formation and CYP2D6-mediated M-13 formation were observed for the metabolism of pitavastatin lactone in the current study. Interestingly, CYP2C9 was the major enzyme responsible for the formation of M-13 from pitavastatin (Fujino *et al.* 1999b), but not in the metabolism of pitavastatin lactone. A good correlation with pitavastatin lactone metabolism was obtained for CYP3A4-mediated testosterone 6 β -hydroxylase activity and for total CYP content. This may be attributable to the fact that the CYP3A4 enzymes are relatively abundant in human hepatic microsomes. On the other hand, no correlation was found for CYP2D6, indicating that the CYP2D6-mediated metabolism of pitavastatin lactone was negligible. We propose the metabolic pathway shown in figure 9, where UGT and multiple P450 enzymes are involved in the metabolism of pitavastatin in humans.

With regard to the metabolism in extrahepatic tissues by UGT enzymes, it has been demonstrated that, as in liver, pitavastatin undergoes Phase 2 metabolism in the kidney and intestine, resulting in the formation of pitavastatin lactone. The results of our *in vitro* investigation revealed that human renal and intestinal microsomes catalyse lactone formation. Kinetic analysis of the lactonization

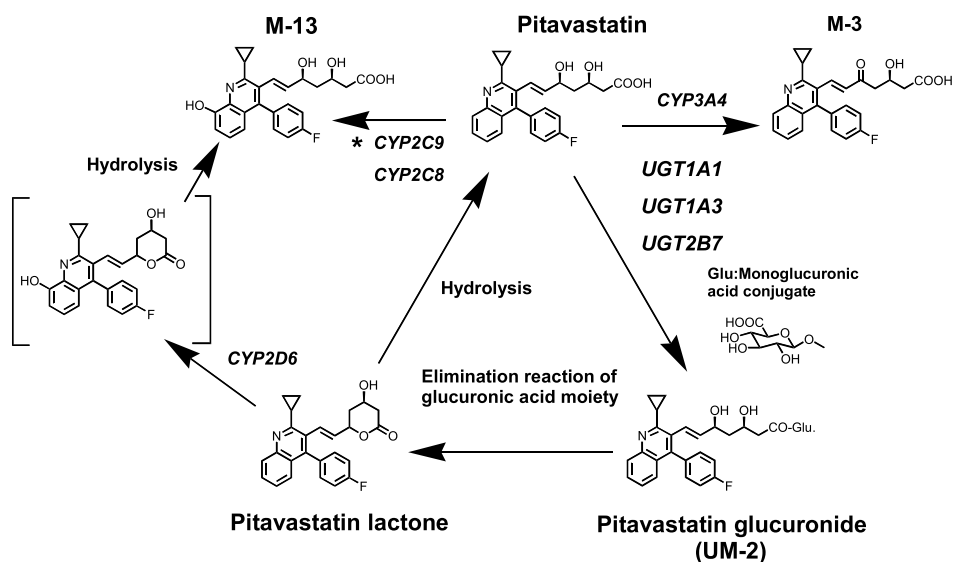


Figure 9. Postulated metabolic pathways of CYP and UGT-mediated pitavastatin metabolism. The unidentified metabolite of pitavastatin is shown in brackets. *Quoted from *Xenobiotic Metabolism and Disposition* (1999), **14**, 415–424.

revealed relatively low $V_{\max}/K_m$ in hepatic and renal microsomes. However, the $V_{\max}/K_m$ of lactonization in both types of microsomes was about twofold higher than that of M-13 formation (Fujino *et al.* 1999a), suggesting that lactonization is the major metabolic pathway in man. This knowledge could be retrospectively applied to provide a pharmacokinetic rationale for our *in vivo* findings (Fujino *et al.* 1999b).

The formation of acyl glucuronides is a major metabolic pathway for many compounds containing a carboxylic acid (Faed 1984). These acyl glucuronides can undergo hydrolysis and are capable of reacting with protein nucleophiles to form covalent adducts (Ding *et al.* 1995). On the other hand, the characteristic migration of the acyl group, which occurs in ester-type glucuronides, was not observed in the metabolism of pitavastatin (Kojima *et al.* 1999a). These findings implied that the elimination of pitavastatin acyl glucuronides to its lactone form was rapid, indicating the covalent adducts might be negligible in pitavastatin metabolism.

To date, at least 70 cDNA-encoding animal and human UGTs have been isolated, composing a superfamily of proteins that have been identified as UGT1 and UGT2 families based upon sequence similarities in humans (Mackenzie *et al.* 1997). The substrate specificity of human UGTs is generally broad, and several genetic polymorphisms have been recently identified in these families (Burchell *et al.* 1995, Tukey and Strassburg 2000). In the present study, mainly UGT1A3 and UGT2B7 catalysed the glucuronidation of pitavastatin, although other UGT enzymes such as UGT1A1, 1A4 and 1A6 are involved. Therefore, we consider that at least five UGT enzymes contribute to the lactonization of pitavastatin in human microsomes, indicating that drug–drug interaction was absent in the competitive inhibition of UGT.

The intrinsic clearance of atorvastatin lactone, which was mediated by CYP3A4-dependent metabolism, was 83-fold greater than that of its acid form (Jacobsen *et al.* 2000). No marked difference was noted in the intrinsic clearance of pitavastatin and its lactone in the current study. Furthermore, we performed an inhibition analysis using several CYP substrates to examine drug–drug interactions. Simvastatin, lovastatin, atorvastatin, cerivastatin and the corresponding lactone forms, all reported to be substrates for CYP3A4, are known to inhibit the metabolism of mexazolam and a strong inhibitory effect on CYP has been reported for the lactone form compared with the acid form of all statins (Ishigami *et al.* 2001). A large difference was noted in the inhibitory effect of the lactone form between pitavastatin and atorvastatin on CYP3A4-mediated metabolism in the present study. CYP2C9 plays an important role in the metabolism of fluvastatin (Fischer *et al.* 1999). CYP2C9-mediated metabolism is inhibited in the presence of fluvastatin but not with pitavastatin or its lactone. Therefore, no inhibitory effect was found on CYP2C9- and CYP3A4-mediated metabolism by either pitavastatin or its lactone form, the main metabolite in humans, indicating that the likelihood of metabolic drug–drug interactions is minimal compared with other HMG-CoA reductase inhibitors.

Acknowledgements

The authors are grateful to Ms Keiko Maekawa for technical assistance.

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Catalytic activities of cytochrome P450 enzymes and UDP-glucuronosyltransferases involved in drug metabolism in rat everted sacs and intestinal microsomes

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Received 19 June 2002

1. The use of everted sacs of the small intestine as an enzyme source for studying the metabolism of xenobiotics by cytochrome P450 (P450, CYP) enzymes and UDP-glucuronosyltransferases has been investigated.

2. Most of the drug oxidation activities for testosterone, bufuralol, ethoxyresorufin and 7-ethoxycoumarin resided in the upper part of the everted sacs and in intestinal microsomes. Testosterone 6 β -hydroxylase activities in the everted sacs were about two times higher than those in the intestinal microsomes. By freezing and thawing treatment, the testosterone 6 β - and 16 α -hydroxylase activities of the everted sacs were considerably decreased, and those of the intestinal microsomes were abolished.

3. Microsomal testosterone 6 β -hydroxylation, bufuralol 1'-hydroxylation, and pentoxyresorufin and ethoxyresorufin *O*-dealkylation were inhibited by ketoconazole, quinine, metyrapone and α -naphthoflavone respectively. Immunoreactive proteins using anti-CYP2B1 and anti-CYP3A2 antibodies were detected in the upper and middle parts of the rat small intestine.

4. Except for morphine 3-glucuronidation, glucuronidation activities in intestinal microsomes or everted sacs were not dependent on the intestinal region. The lower part of the everted sacs exhibited about 10 times higher morphine-3-glucuronidation activities compared with those of the upper part. The glucuronidation activities of 4-nitrophenol in the everted sacs were 10 times higher than those in microsomes.

5. These results demonstrated that the upper part of rat small intestine serves as the major site for intestinal P450-mediated first-pass metabolism. CYP3A enzymes in rat intestinal microsomes may not be stable but probably play an important role in drug oxidations. The high activity of glucuronidation in the rat small intestine should also be considered in terms of drug metabolism.

Introduction

Cytochrome P450 (P450, CYP) and UDP-glucuronosyltransferases (UGTs) catalyse the oxidation and glucuronidation, respectively, of a broad spectrum of endobiotic and xenobiotic substrates (Guengerich 1997). In general, the resulting hydroxylated metabolites and/or glucuronides are more hydrophilic, facilitating renal and biliary excretion. In addition to hepatic metabolism, high rates of gastrointestinal drug oxidation and glucuronidation in rats and humans have been observed (Grams *et al.* 2000). UGT-catalysed glucuronidation and elimination may prevent competing bioactivation of xenobiotics to toxic reactive intermediates

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(Ritter 2000). Drug candidates are not routinely examined for *in vitro* metabolic stability in conjugation or for favorable UGT inhibition profiles in livers and intestines during drug development. This is in part due to the lack of standard microsomal preparations or incubation conditions for the UGTs and the paucity of substrate specificity (Fisher *et al.* 2001). In general, glucuronide formation leads to the termination of pharmacological potency. However, relevant examples exist of glucuronidation being a bioactivation event, which contributes to pharmacodynamic complexities (Mackenzie 1995, Christrup 1997).

It has been reported that human CYP3A4 is the dominant P450 enzyme in the epithelial barrier of the small intestine (Watkins *et al.* 1987, Paine *et al.* 1997). The small intestine may be a major site for CYP3A-mediated metabolism, which would be an important factor in the interindividual variability in disposition after oral administration of CYP3A substrates (Obach *et al.* 2001). The intestinal localization of CYP3A should also be significant in metabolism-based drug-drug interactions, as in the liver. Several species of experimental animals have been used in early toxicology and metabolism studies for drug development. Intestinal microsomes from experimental animals and humans have been prepared by various methods in different laboratories (Zhang *et al.* 1996, Prueksaritanont *et al.* 1997). It is generally accepted that rat intestine may not be suitable because the total P450 contents and CYP3A enzymes in intestine have been shown to be lower, in contrast to rat CYP1A and CYP2B enzymes, than those of other experimental animals (Rich *et al.* 1989, Zhang *et al.* 1997). Recently, we reported that mouse intestinal microsomes could be prepared with glycerol and several protease inhibitors to obtain the Cyp3a activities (Emoto *et al.* 2000b). Everted sacs of the small intestine are also useful for studying drug metabolism as well as transport and absorption (Emoto *et al.* 2000a). It is necessary to clarify the CYP3A activities in rat intestine in the presence of sufficient protease inhibitors in order to use the intestinal microsomes for the investigation of drug metabolism of orally administered medicines. Furthermore, it should also be mentioned that little is known about changes in the stability of intestinal microsomes by freezing and thawing.

In the present study, the enzymatic characteristics of P450s and UGTs were investigated by analysing the drug oxidation and glucuronidation activities in everted sacs and comparing them with those of intestinal microsomes from rats. The stability of rat CYP3A activity in intestinal microsomes and everted sacs after freezing is also reported.

Materials and methods

Chemicals

Uridine 5'-diphosphoglucuronic acid (UGP-GA), alamethicin, 1-naphthol, 4-nitrophenol and their glucuronides were obtained from Sigma-Aldrich (St Louis, MO, USA). Morphine was purchased from Takeda Chemical Industries (Osaka, Japan). Morphine 3- and 6-glucuronide were generous gifts from Professor Kazuta Oguri, Kyushu University (Fukuoka, Japan). Typical drug substrates and their metabolites and reagents used in this study were obtained from sources described previously or were of the highest qualities commercially available (Emoto *et al.* 2000a, b).

Preparation of intestinal and hepatic microsomes and everted sacs

Male Wistar rats, 7 weeks old, were obtained from Japan SLC (Hamamatsu, Japan). Microsomes from rat small intestines were prepared according to the method of Emoto *et al.* (2000b). Briefly, the small intestine (from duodenum to ileum) was divided into three 20-cm segments (upper, middle and lower part), cut longitudinally, then washed in ice-cold washing solution [0.9% NaCl, 1 mM (*p*-amidino-phenyl) methanesulfonyl fluoride hydrochloride (APMSF), 1 mM EDTA] for 1 min by gentle swirling. The mucosal cells were gently scraped off, immersed in 3 vols of ice-cold buffer A [50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl, 20%(v/v) glycerol, 1 mM EDTA, 1 mM APMSF, 1 mg ml⁻¹ trypsin inhibitor, 10 μ M leupeptin, 0.04 U ml⁻¹ aprotinin, 1 μ M bestatin], and homogenized using a motor-driven Teflon-tipped pestle. The homogenate was centrifuged at 9000g at 4°C for 20 min and then the supernatant was centrifuged at 105 000g at 4°C for 60 min. The microsomal pellets were resuspended in ice-cold buffer A.

Everted sacs were prepared according to the method of Emoto *et al.* (2000a). To evert the intestine quickly, a vinyl-covered wire was pushed into the intestinal lumen until it appeared and the eversion was completed by rolling the proximal portion of the intestine. The everted sac was then slipped off the wire and washed in 60-ml ice-cold washing solution for 1 min by gentle swirling. The everted sac was cut into 1-cm segments from the duodenum to the ileum. To study the effects of freezing and thawing of this tissue, everted sacs or intestinal microsomes were frozen in liquid nitrogen, stored at -80°C and then thawed on ice.

Hepatic microsomes were prepared according to the method described by Emoto *et al.* (2000a, b). Protein concentrations were determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Xenobiotic oxidation assays

Testosterone 6 β - and 16 α -hydroxylation, bufuralol 1'- and 6-hydroxylation, and ethoxyresorufin, pentoxyresorufin, and 7-ethoxycoumarin *O*-dealkylation were determined as described previously (Yamazaki *et al.* 1999, Emoto *et al.* 2000a, b, Hanioka *et al.* 2000). The standard incubation mixture (final volume of 0.20 ml for microsomes or 1 ml for everted sacs) contained 0.03 mg protein of intestinal microsomes, 0.01 mg hepatic microsomes or 1-cm segment of the everted sac prepared from rats, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 0.5 U ml⁻¹ glucose 6-phosphate dehydrogenase), and 100 μ M substrates except for ethoxyresorufin and pentoxyresorufin (10 μ M). The reaction mixtures for everted sacs were incubated at 37°C for 30 min, and those for intestinal and hepatic microsomes were incubated for 10 min for all tested xenobiotic oxidations. With the exception of α -naphthoflavone (ANF), which was dissolved in dimethyl sulfoxide, substrates and inhibitors were dissolved in methanol such that the concentration of solvent in the incubation mixture was <1%. The formation of the metabolites was determined by HPLC analysis as described previously (Emoto *et al.* 2000a, b) and the specific enzyme activities of the everted sacs were calculated with the corresponding intestinal microsomal protein content (mean of five rats).

Glucuronidation assays

The glucuronidation activities of 1-naphthol, 4-nitrophenol and morphine were determined according to the methods described previously (Mackenzie and Hanninen 1980, Ghabrial *et al.* 1995, Fisher *et al.* 2000). A typical incubation mixture (200 μ l of total volume) contained 20 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl_2 , 3 mM UDP-GA, 0.02% Triton N-101, 0.2 mg ml^{-1} intestinal or hepatic microsomes, and substrate (0.05 mM 1-naphthol, 0.5 mM 4-nitrophenol or 1 mM morphine). Blank incubations were performed without UDP-GA. After the incubation for 2.5, 5 and 30 min for 1-naphthol, 4-nitrophenol and morphine, respectively, the reaction was terminated by boiling at 100°C for 2 min for 1-naphthol and 4-nitrophenol and by the addition of 200 μ l ice-cold acetonitrile for morphine.

For 1-naphthol and 4-nitrophenol, 2.8 ml 0.2 M sodium glycine buffer (pH 10.4) were added to 200 μ l of the mixtures. After removal of the protein by centrifugation at 12 000g for 5 min, a 50- μ l portion of the sample was subjected to a Shimadzu (Kyoto, Japan) HPLC system equipped with a C18 5- μ m analytical column (Mightysil, 150 $\times$ 4.6 mm i.d., Kanto Chemicals, Tokyo, Japan). The mobile phase was 5% methanol/50 mM potassium phosphate buffer (pH 6.5) at 1.2 ml min^{-1} . The 1-naphthol β -D-glucuronide formed was fluorometrically determined at an excitation wavelength of 293 nm, an emission wavelength of 343 nm with a retention time of 7.6 min (Mackenzie and Hanninen 1980). The 4-nitrophenol β -D-glucuronide formed was monitored at 302 nm with a retention time of 2.9 min. Morphine glucuronides were also analysed by the same HPLC system with 20% acetonitrile/0.8 mM sodium dodecyl sulfate at a flow rate of 1.2 ml min^{-1} . Morphine 3- and 6-glucuronide were fluorometrically determined at an excitation wavelength of 210 nm, an emission wavelength of 350 nm and with retention times of 3.5 and 5.0 min, respectively. The formation of metabolites was quantified by comparing the peak areas in the incubations with a standard curve containing known amounts of metabolite.

When the incubation mixture contained a 1-cm segment of the everted sac, the reaction volume was increased to 1 ml with the same final concentrations of substrates, cofactors and buffers.

Immunoblot analyses

Polyclonal goat anti-rat CYP1A1, CYP2B1, CYP2C6, CYP2E1 and CYP3A2 antisera were obtained from Daiichi Pure Chemicals (Tokyo, Japan). Polyclonal rabbit anti-rat CYP2D1 antiserum was purchased from Funakoshi (Tokyo, Japan). Rabbit anti-human UGT1A and UGT2B7 antisera were obtained from Gentest (Woburn, MA, USA). The anti-rat CYP1A1 shows a minor cross-reactivity with rat CYP2E1. The anti-rat CYP2C6 shows a minor cross-reactivity with rat CYP2C13 in phenobarbital-treated rat liver microsomes. The anti-rat CYP2E1 shows a minor cross-reactivity with rat CYP2C11 and CYP2C13 in acetone-treated rat liver microsomes. The anti-human UGT1A detects human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9 and 1A10. The anti-human UGT2B7 does not detect human UGT1A1, 1A4, 1A6, 1A9, 1A10 or 2B15. Immunoblot analysis was performed according to Emoto *et al.* (2000a). Microsomal proteins were separated on 7.5% polyacrylamide gel electrophoresis and transferred electrophor-

etically to a nitrocellulose membrane. The secondary antibodies used were anti-goat IgG and detection was carried out with diaminobenzidine staining.

Statistical analysis

Statistical analysis was performed by a Student's *t*-test using the InStat computer program (Graphpad Software, San Diego, CA, USA).

Results

Catalytic activities of P450 enzymes in microsomes and everted sacs of rat small intestine

The ethoxyresorufin (CYP1A) and pentoxyresorufin (CYP2B) *O*-dealkylation activities and testosterone 6 β -hydroxylation activity (CYP3A) of intestinal microsomes (figure 1A–C) and everted sacs (figure 1D–F) are shown. The ethoxyresorufin *O*-deethylation activities in microsomes from the upper part of the intestine were significantly higher than those from the other parts (figure 1A), but the microsomal pentoxyresorufin *O*-depentylation activities were not different in the three parts of intestines (figure 1B). These *O*-dealkylation activities were not affected by freezing and thawing of the intestinal microsomes and everted sacs. Testosterone 6 β -hydroxylation activities in microsomes from the upper part of the intestine

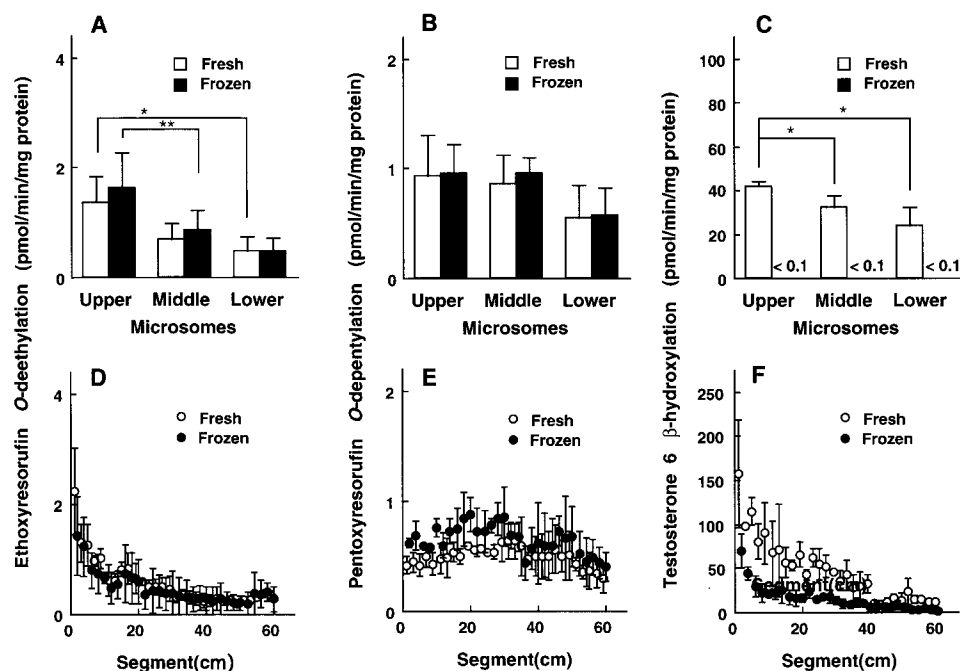


Figure 1. Xenobiotic oxidation activities of ethoxyresorufin (A, D), pentoxyresorufin (B, E) and testosterone (C, F) catalysed by rat intestinal microsomes (A–C) and everted sacs (D–F). Intestinal microsomes or everted sacs which were fresh (open symbols) or frozen and thawed once (closed symbols) were incubated with the substrate and an NADPH-generating system. Data are the mean $\pm$ SD from three rats. Significantly different from the upper part: ***p* < 0.01, **p* < 0.05.

intestine were also significantly higher than those from the other parts (figure 1C). However, no activities were detected in frozen/thawed microsomes. The activities of *O*-dealkylations in everted sacs were the similar to those of the intestinal microsomes (figure 1D, E). In contrast, the testosterone 6 β -hydroxylation activities in fresh (about 160 pmol min⁻¹ mg⁻¹ protein) and frozen and thawed everted sacs (about 60 pmol min⁻¹ mg⁻¹ protein) from the upper part of the intestine were higher than those in microsomes (figure 1F). These activities decreased markedly from the upper to the lower part of the intestine. When compared with the results of microsomes in figure 1C, the low activities of testosterone 6 β -hydroxylation remained even when the everted sacs were frozen and thawed.

The xenobiotic oxidation activities of intestinal and hepatic microsomes from rats are summarized in table 1. Low but significant activities of 7-ethoxycoumarin *O*-deethylation (CYP1A, CYP2B), testosterone 16 α -hydroxylation (CYP2C), and bufuralol 1'-hydroxylation (CYP2D) were also observed in the upper parts of freshly prepared rat intestinal microsomes. These latter activities were significantly decreased or not detected when frozen and thawed intestinal microsomes were used. The high activities were observed for testosterone 6 β -hydroxylation in both intestinal and hepatic microsomes. The testosterone 6 β -hydroxylation activities in everted sacs were significantly higher than those in intestinal microsomes.

Effects of P450 inhibitors on oxidation activities in rat intestinal and hepatic microsomes

The effects of P450 inhibitors on the ethoxyresorufin, 7-ethoxycoumarin, and pentoxyresorufin *O*-dealkylation activities, bufuralol 1'- and 6-hydroxylation activities, and testosterone 6 β - and 16 α -hydroxylation activities in intestinal and hepatic microsomes from rats were investigated (figure 2). Ethoxyresorufin and pentoxyresorufin *O*-dealkylation activities in intestinal and hepatic microsomes were inhibited by ANF and metyrapone, respectively (figure 2A, C) and the 7-ethoxycoumarin *O*-deethylation activities in intestinal microsomes were inhibited by metyrapone but not by ANF. The bufuralol 1'-hydroxylase activities in both intestinal and hepatic microsomes were inhibited by quinine rather than quinidine (figure 2D). In both intestinal and hepatic microsomes, the testosterone 6 β -hydroxylase activities were extensively inhibited by ketoconazole (figure 2E). In contrast, the testosterone 16 α -hydroxylase activities were not affected (figure 2E).

Glucuronidation activities in microsomes and everted sacs of rat small intestine

The glucuronidation activities of 1-naphthol (UGT1A6), 4-nitrophenol (UGT1A1) and morphine (UGT2B1 and UGT2B12) were determined with regard to the position of the intestine and the effects of freezing and thawing (figure 3). These glucuronidation activities in intestinal microsomes or everted sacs were not dependent on the intestinal region (figure 3A–E) except for the morphine 3-glucuronidation of everted sacs, where the lower part of everted sacs showed about eight times higher activity compared with those of the upper part (figure 3F). The glucuronidation activities of 4-nitrophenol in the everted sacs were 10 times higher than those in microsomes (figure 3B, E and table 2). In contrast, 1-naphthol glucuronidation showed almost the same activities in microsomes and everted sacs (figure 3A, D). The effects of freezing and thawing of intestinal

Table 1. Xenobiotic oxidation activities (pmol min⁻¹ mg⁻¹ protein) in intestinal and hepatic microsomes and everted sacs from rats.

Enzyme source		Ethoxyresorufin O-deethylation (CYP1A)	7-Ethoxycoumarin O-deethylation (CYP1A, 2B)	Pentoxylresorufin O-deptylation (CYP2B)	Testosterone 16 α -hydroxylation (CYP2C)	Bufuralol 1'-hydroxylation (CYP2D)	Testosterone 6 β -hydroxylation (CYP3A)
Intestine	Microsomes						
	fresh	1.4 $\pm$ 0.5	10.3 $\pm$ 1.2	0.93 $\pm$ 0.36	5.5 $\pm$ 3.8	18.7 $\pm$ 1.4	42 $\pm$ 2
Everted sacs	frozen	1.6 $\pm$ 0.6	6.9 $\pm$ 0.5*	0.96 $\pm$ 0.26	< 0.1	0.3 $\pm$ 0.1*	< 0.1
	fresh	1.0 $\pm$ 0.1	16.6 $\pm$ 4.5	0.48 $\pm$ 0.08	14.6 $\pm$ 6.8	16.8 $\pm$ 1.7	83 $\pm$ 19 ^a
Liver	Microsomes	0.8 $\pm$ 0.4	6.1 $\pm$ 1.1*	0.70 $\pm$ 0.04	9.1 $\pm$ 2.7	14.3 $\pm$ 2.3	28 $\pm$ 4*
		290 $\pm$ 30	1120 $\pm$ 140	200 $\pm$ 20	320 $\pm$ 80	1210 $\pm$ 30	1530 $\pm$ 180

Everted sacs and microsomes were prepared from the upper parts of the intestine.

Data are the mean $\pm$ SD from three rats.

*Significantly different from fresh group ($p < 0.05$).

^aSignificantly different between microsomes and everted sacs ($p < 0.05$).

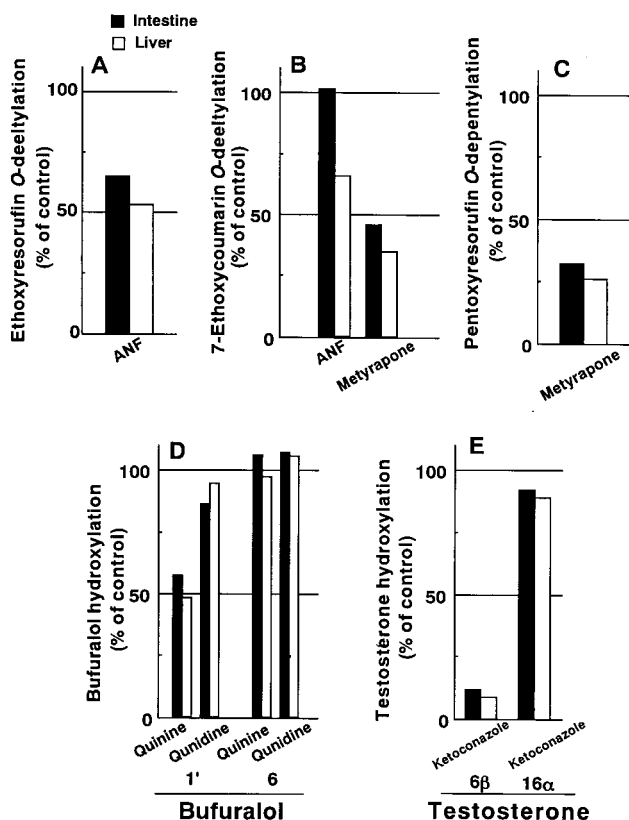


Figure 2. Effects of P450 inhibitors on oxidation activities in rat intestinal (closed) and hepatic (open) microsomes. The concentration of substrate was 0.1 mM. ANF (10 μ M), metyrapone (50 μ M), quinine (100 μ M), quinidine (100 μ M) and ketoconazole (1 μ M) were added as inhibitors. (A) Control activities (without inhibitors) of ethoxyresorufin O-deethylation by intestinal and hepatic microsomes were 1.0 and 180 pmol min⁻¹mg⁻¹ protein, respectively. (B) Control activities of 7-ethoxycoumarin O-deethylation by intestinal and hepatic microsomes were 9.7 and 1100 pmol min⁻¹mg⁻¹ protein, respectively. (C) Control activities of pentoxyresorufin O-depentylation by intestinal and hepatic microsomes were 1.0 and 180 pmol min⁻¹mg⁻¹ protein. (D) Control activities of bufuralol hydroxylation by intestinal and hepatic microsomes were 16 and 1100 pmol 1'-hydroxybufuralol formed min⁻¹mg⁻¹ protein and 1.5 and 10 pmol 6-hydroxybufuralol formed min⁻¹mg⁻¹ protein, respectively. (E) Control activities of testosterone hydroxylation by intestinal microsomes were 36 and 1500 pmol 6 β -hydroxytestosterone formed min⁻¹mg⁻¹ protein and 4.0 and 160 pmol of 16 α -hydroxytestosterone formed min⁻¹mg⁻¹ protein, respectively. Each column represents the average of duplicate determinations.

Table 2. Glucuronidation activities (nmol min⁻¹mg⁻¹ protein) in intestinal microsomes and everted sacs from rats.

	1-Naphthol glucuronidation (UGT1A6)	4-Nitrophenol glucuronidation (UGT1A1)	Morphine 3-glucuronidation (UGT2B1 and UGT2B12)
Intestine			
Microsomes ^a	5.8 $\pm$ 1.7	3.8 $\pm$ 1.6	0.13 $\pm$ 0.02
Everted sacs ^b	4.8 $\pm$ 3.0	39 $\pm$ 12*	0.28 $\pm$ 0.18

^aData are the mean $\pm$ SD of intestinal microsomes obtained from the upper region prepared from five rats.

^bData are the mean $\pm$ SD of the average values of 10 segments from the upper region of the intestine from five rats.

*Significantly different between microsomes and everted sacs group ($p < 0.05$).

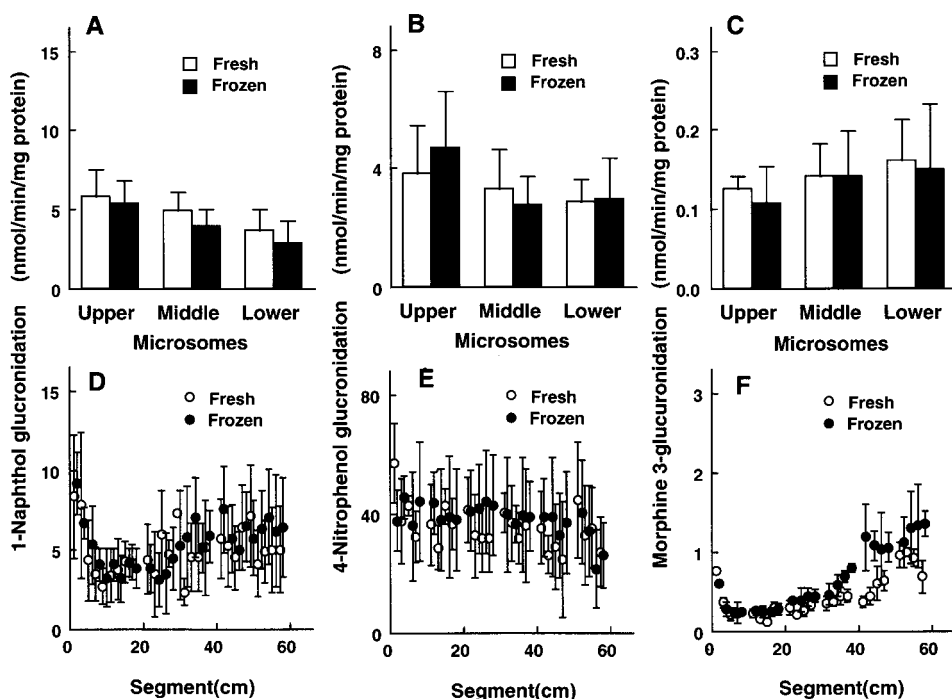


Figure 3. Glucuronidation activities of 1-naphthol (A, D), 4-nitrophenol (B, E) and morphine (C, F) catalysed by intestinal microsomes (A–C) and everted sacs (D–F) from rats. The Triton N-101 activated intestinal microsomes or everted sacs that were untreated (open symbols) or frozen and thawed once (closed symbols) were incubated with the substrate and UDP-GA (3 mM). Data are the mean $\pm$ SD from five rats.

microsomes or everted sacs on these glucuronidation activities were not observed. Morphine 6-glucuronidation was not detected in intestinal microsomes and everted sacs from rats. The average activities of 1-naphthol glucuronidation and morphine 3-glucuronidation in everted sacs were similar to those in the intestinal microsomes obtained from the upper parts (table 2).

Immunoblot analyses in intestinal microsomes

No immunoreactive bands in the rat intestinal microsomes could be detected using anti-rat CYP1A1 (figure 4A), anti-rat CYP2C6, anti-rat CYP2D1 and anti-rat CYP2E1 as well as anti-human UGT1A and UGT2B7 (data not shown). On the other hand, immunoreactive bands could be observed by anti-rat CYP2B1- (figure 4B) and CYP3A2 antibodies (figure 4C) in microsomes from the upper and middle intestine regions, these bands exhibiting the same migration as the bands of liver microsomes.

Discussion

In general, the preparation of intestinal microsomes is relatively difficult compared with the preparation of hepatic microsomes because intestinal micro-

somes are exposed to an abundance of proteases during preparation. In the preparation of intestinal microsomes from humans, monkeys and rodents, different laboratories have used various combinations of protease inhibitors (Rosenberg and Mankowski 1994, Kashfi *et al.* 1995). We recently reported that combinations of protease inhibitors as well as glycerol (buffer A in this study) were necessary to obtain adequate testosterone 6 β -hydroxylation activities in mouse intestinal microsomes (Emoto *et al.* 2000b). Previously, we demonstrated that the everted sacs of the small intestine are useful for the study of drug metabolism (Emoto *et al.* 2000a). In this latter study, we investigated the catalytic activities in rat intestinal microsomes in the presence of combinations of protease inhibitors as well as of glycerol. Intestinal microsomes from rats prepared with buffer A have lower testosterone 6 β -hydroxylation activities (42 ± 2 pmol min⁻¹ mg⁻¹ protein, mean $\pm$ SD, $n = 5$) than those from mice (170 ± 29 pmol min⁻¹ mg⁻¹ protein, $n = 3$) (Emoto *et al.* 2000a). Similarly, the testosterone 6 β -hydroxylation activities (89 ± 19 pmol min⁻¹ mg⁻¹ protein) in rat everted sacs are lower than those in mice everted sacs (930 ± 73 pmol min⁻¹ mg⁻¹ protein) (Emoto *et al.* 2000a). Therefore, it was deduced that CYP3A activities in rat intestine may be lower than the equivalent of those in mice. It should be mentioned that the testosterone 6 β -hydroxylation activities were abolished by freezing and thawing treatment (table 1 and figure 1). Thus, this may be one of the reasons that considerably low CYP3A activities had been reported in rat intestine (Rich *et al.* 1989, Zhang *et al.* 1997).

High rates of intestinal drug glucuronidation have been reported (Grams *et al.* 2000) as well as the fact that glucuronidation activities depend on detergent for microsomal UGTs (Fisher *et al.* 2000). However, in our preliminary experiments, glucuronidation activities in everted sacs were not changed in the presence or absence of detergents. We detected 1-naphthol, 4-nitrophenol and morphine glucuronidation activities in rat intestinal microsomes in the present study (table 2 and figure 3). It has been reported that the glucuronidation of 1-naphthol, 4-nitrophenol, and morphine are catalysed mainly by UGT1A6 (Vargas and Franklin 1997), UGT1A1 (Ghabrial *et al.* 1995, Vargas and Franklin 1997), and UGT2B1 and UGT2B12 (Mackenzie *et al.* 1997) in rats, respectively. 1-Naphthol and 4-nitrophenol glucuronidation activities in rat intestinal microsomes similar to those of the present study have been reported (Vargas and Franklin 1997, Ghabrial *et al.* 1995). In the intestinal microsomes or everted sacs, morphine 3-glucuronide was detected, but 6-glucuronide was not observed, as previously reported with microsomes (Lear *et al.* 1991). The present morphine 3-glucuronidation activities were also similar to the reported activities (Lear *et al.* 1991). It has been reported that 1-naphthol glucuronidation and morphine 3-glucuronidation activities appear to decrease from the upper to lower regions of the intestine in isolated mucosal cells (Koster *et al.* 1985), in contrast with the present results using microsomes or everted sacs (figure 3C, F). In the present study, it was demonstrated that morphine 3-glucuronidation activities were about 10 times higher in the lower part of the intestine compared with those activities in the upper region. The discordance of these data for morphine 3-glucuronidation activities remains to be clarified. It is also very interesting that the everted sacs gave about 10 times higher activities of 4-nitrophenol glucuronidation (by UGT1A1) than microsomal preparations from rat intestine (table 2). The UGT1A1 enzyme will very unstable in

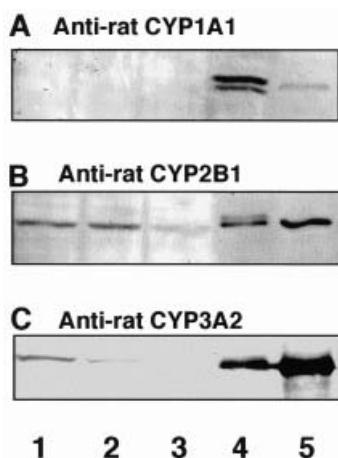


Figure 4. Immunoblot analyses of rat intestinal and hepatic microsomes. (A) Immunoblot analysis of rat intestinal and hepatic microsomes were performed using anti-rat CYP1A1 antibodies. Lanes 1–3, intestinal microsomes (30 μ g) from the upper, middle, and lower parts of rat small intestine, respectively; 4, rat hepatic microsomes (2 μ g) pretreated with β -naphthoflavone; 5, recombinant rat CYP1A2 (0.25 pmol). (B) Immunoblot analysis was performed using anti-rat CYP2B1 antibodies. Lanes 1–3 were the same as those in (A); 4, rat hepatic microsomes (0.2 μ g) pretreated with phenobarbital; 5, recombinant rat CYP2B1 (0.25 pmol). (C) Immunoblot analysis was performed using anti-rat CYP3A2 antibodies. Lanes 1–3 were the same as those in (A); 4, rat hepatic microsomes (2 μ g); 5, recombinant rat CYP3A1 (0.25 pmol).

the microsome preparation procedure and the mechanism of the phenomena remains to be clarified.

Several xenobiotic oxidation activities were detected in the upper part of the small intestine from rats (table 1) and the inhibitory effects of typical P450 inhibitors in rat intestinal microsomes were determined (figure 2). These xenobiotic oxidation activities in rat intestine would be catalysed by the same P450 enzymes as in livers (except for 7-ethoxycoumarin). However, immunoreactive bands of CYP1A, CYP2C, CYP2D and CYP2E1 enzymes were not detected in rat intestine, and only CYP2B and CYP3A proteins were clearly detected using anti-rat CYP2B and CYP3A antibodies (figure 4). Since ANF did not inhibit 7-ethoxycoumarin *O*-deethylation in intestinal microsomes (figure 2B), this reaction appears to be mainly catalysed by CYP2B, presumably because of the abundance of CYP3A in rat intestine. Concerning UGT, the reason why there were no immunoreactive bands of UGT1A and UGT2B in the rat hepatic and intestinal microsomes may be the poor reactivities of antibodies against human UGTs for rat UGT forms. These results suggest that the determination of catalytic activities might have higher sensitivity than immunoblot analyses for characterizing microsomal P450 or UGT enzymes in rat intestine.

In conclusion, freezing and thawing treatment of rat intestinal microsomes should be avoided as much as possible to maintain the catalytic activities of rat CYP3A enzymes. Our results also demonstrated that the upper part of the rat small intestine serves as the major site for intestinal P450 mediated first-pass metabolism but glucuronidation activities are not dependent on the intestinal region. Everted sacs of the small intestine are also useful for the study of drug metabolism involving P450 and UGT enzymes.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture of Japan. We thank Professor Kazuta Oguri for providing morphine glucuronides and Mr Brent Bell for critically reading the manuscript.

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Studies on the inhibition of human cytochromes P450 by selenocysteine *Se*-conjugates

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Received 1 May 2002

1. To investigate whether cytochrome P450 (P450) inhibition can contribute to the chemopreventive activity of selenocysteine *Se*-conjugates (SeCys conjugates), 21 SeCys conjugates were screened for their inhibitory potency towards seven of the most important human P450s.

2. The majority of the SeCys conjugates produced near complete inhibition of CYP1A1 at a concentration of 250 μM . The most potent inhibitor, *Se*-benzyl-L-selenocysteine, displayed an IC_{50} of $12.8 \pm 1.2 \mu\text{M}$. CYP2C9, -2C19 and -2D6 were moderately (50–60%) inhibited by the SeCys conjugates. CYP1A2, -2E1 and -3A4 were least inhibited.

3. Studies on the susceptibility of CYP1A1 to SeCys conjugates implicated a thiol-reactive intermediate, as evidenced by reduced inhibition levels in the presence of glutathione and *N*-acetyl cysteine. Uncoupling of the P450-catalytic cycle was of no importance as ROS scavengers did not influence inhibition levels.

4. P450 inhibition by two physiologically relevant metabolite classes of SeCys conjugates was also studied. *N*-acetylation of SeCys conjugates consistently increased the inhibitory potency towards CYP1A2, -2C19, -2E1 and -3A4. Beta-lyase catalysed bioactivation of alkyl-substituted SeCys conjugates or *Se*-benzyl-L-selenocysteine produced little or no additional inhibition of P450 activity. For *Se*-phenyl-L-selenocysteine, however, significant increases in P450 inhibition were obtained by beta-lyase pre-incubation.

5. It is concluded that the potent and relatively selective CYP1A1 inhibition exerted by SeCys conjugates may contribute to their chemopreventive activity.

Introduction

Over the past decades, organoselenium compounds have received increasing attention because they display a wide range of pharmacological activities, including chemoprevention, chemoprotection and antitumour activity (Ip 1998, Ganther 1999, reviewed in Mughesh *et al.* 2001). One of the first studies demonstrating the chemopreventive effect of organoselenium compounds was by El-Bayoumy (1985), where both 4-methoxybenzeneselenol and benzyl selenocyanate were shown effectively to inhibit benzo[*a*]pyrene-induced forestomach tumorigenesis in the mouse. Subsequent animal studies showed that aliphatic and several benzylic selenocyanates also inhibited chemically induced tumour development in various other organs such as the lung, mammary glands and colon (El-Bayoumy *et al.* 1995). As reflected by a substantial decrease of DNA-adducts formed after

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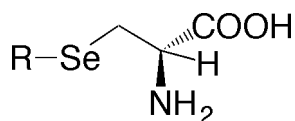


Figure 1. General structure of R-substituted selenocysteine *Se*-conjugates.

exposure to 7,12-dimethyl-benz[*a*]anthracene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, organic selenocyanates appear capable of modulating events in the initiation phase of carcinogenesis (Ip *et al.* 1995, Prokopczyk *et al.* 1996). An *in vitro* study using rat and human liver microsomes and recombinant CYP1A1, -1A2 and -1B1 reported that chemopreventive selenocyanates were very potent inhibitors of various human cytochromes P450 (P450s) (Shimada *et al.* 1997). Concentrations required to cause 50% inhibition of activation of procarcinogens by CYP1A and -1B were $<0.5 \mu\text{M}$. Based on these observations, it was proposed that inhibition of P450s may contribute to the chemopreventive activity of selenocyanates (Shimada *et al.* 1997).

Another class of organoselenium compounds that has recently gained interest is selenocysteine *Se* (SeCys) conjugates (figure 1). It has been demonstrated that a number of SeCys conjugates, including *Se*-methyl-, *Se*-(*n*-propyl)- and *Se*-allyl-selenocysteine, possess chemopreventive activity in animal tumour models (Lu *et al.* 1996, Schaffer *et al.* 1996, Ip *et al.* 1999, Jiang *et al.* 1999). Dietary *Se*-allyl-selenocysteine decreases tumorigenesis in a rat mammary tumour model by almost 90%, making it one of the most potent chemopreventive agents known to date (Ip *et al.* 1999). The mode of action of chemopreventive SeCys conjugates is yet to be elucidated, however. It has been proposed that local beta-lyase-mediated activation to chemopreventive selenols may explain the antitumour activity in renal tissues (Ip *et al.* 1999). SeCys conjugates may therefore be useful as prodrugs of pharmacologically active selenols (Andreadou *et al.* 1996, Rooseboom *et al.* 2000). The combination of active uptake of SeCys conjugates and high activity of beta-lyases in proximal tubular cells may be applied to target selenols with antitumour activity to the kidney in order to treat renal cell carcinoma.

The inhibition characteristics of SeCys conjugates towards P450s have hitherto not been the subject of investigation. Recently, we demonstrated that microsomal selenoxidation of SeCys conjugates is catalysed by flavin-containing monooxygenases, but not by P450s (Rooseboom *et al.* 2001). Similar results were previously obtained with cysteine *S*-conjugates, the sulphur-analogues of SeCys conjugates (Sausen and Elfarra 1990, Ripp *et al.* 1997). Whether the absence of P450-mediated selenoxidation and sulfoxidation results from an inability to bind to P450s is not clear. Cysteine *S*-conjugates of isothiocyanates are potent inhibitors of rat CYP2E1, -1A1, -1A2 and -2B1 (Conaway *et al.* 1996, Jiao *et al.* 1996). These effects, however, were attributed to the release of inhibitory isothiocyanates. *S*-allylmercaptocysteine was suggested to act as an inhibitor of CYP2E1, thus explaining its protective effect against acetaminophen-induced liver injury in mice (Sumioka *et al.* 1998). *N*-acetyl cysteine *S*-conjugates of several halogenated alkenes, such as *N*-acetyl-*S*-(pentachlorobutadienyl)-L-cysteine, have been shown to undergo sulfoxidation by CYP3A1 and -3A2 (Werner *et al.* 1995, 1996) and are therefore also likely to inhibit P450s.

Given the recent observation that several organoselenium compounds can act as potent inhibitors of P450s, the aim of the present study was to investigate whether SeCys conjugates also interact with members of this superfamily. Knowledge of the inhibitory properties of SeCys conjugates may elucidate whether inhibition of P450-mediated bioactivation reactions contributes to their chemopreventive activity. Because P450s are involved in the metabolism of virtually all drugs marketed today (Spatzenegger and Jaeger 1995), inhibition of these enzymes may also lead to drug–drug interactions.

In the current study, we have investigated the inhibitory capacity of 21 SeCys conjugates and two cysteine *S*-conjugates towards seven of the most important human P450s. For selected compounds, the underlying inhibitory mechanism was investigated in more detail. As metabolites of SeCys conjugates may also contribute to P450 inhibition, the inhibitory potency of a selected set of compounds belonging to two classes of metabolites has additionally been studied. They include selenols, resulting from enzymatic beta-elimination of SeCys conjugates, and *N*-acetyl SeCys conjugates.

Material and methods

Chemicals

The SeCys conjugates investigated were synthesized as described (Andreadou *et al.* 1996, Rooseboom *et al.* 2000), as was *N*-methyl 7-methoxy-4-(aminomethyl)-coumarin (MMAMC) (Venhorst *et al.* 2000). 3-Cyano-7-ethoxy-coumarin (CEC) was obtained from Molecular Probes (Eugene, OR, USA), whereas 7-methoxy-4-(trifluoromethyl)-coumarin (MTFC) was from Sigma Chemical Co. (St Louis, MO, USA). 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BTFC) was from Gentest Corp. (Woburn, MA, USA). NADPH was from Applichem (Darmstadt, Germany). Pyruvate was from Aldrich (Beerse, Belgium). Mannitol was from J. T. Baker (Deventer, The Netherlands). All other chemicals were obtained from Sigma.

Enzymes

Heterologously expressed human CYP1A1, –1A2, –2C9, –2C19, –2D6, –2E1 and –3A4 were all purchased from Gentest. Glutamine transaminase K/cysteine conjugate beta-lyase (GTK/ β -lyase) was purified 1036-fold from rat kidney cytosol by the procedure of Yamauchi *et al.* (1993) to a concentration of 81 $\mu\text{g ml}^{-1}$. Superoxide dismutase (SOD) from bovine blood and peroxidase type I from horseradish were obtained from Sigma. Catalase from beef liver was purchased from Roche (Mannheim, Germany), as was glucose 6-phosphate dehydrogenase.

Synthesis of *N*-acetyl SeCys conjugates

To a solution of 0.13 mmol SeCys conjugate in 2.5 ml 2 M potassium carbonate solution (pH $\approx$ 11), 0.10 ml acetic anhydride was added at 0°C every 10 min until pH $\approx$ 6. The reaction mixture was subsequently acidified to pH 2, extracted with ethylacetate, dried over sodium sulphate and evaporated under vacuum after filtration. The product was further dried to give a yellow solid. Purities as determined by ^1H -NMR and HPLC were $> 98\%$.

N-acetyl-*Se*-(2-methylphenyl)-L-selenocysteine (yield 69%) ^1H -NMR ($\text{D}_2\text{O}/\text{Na}_2\text{CO}_3$): δ (ppm) 1.8 (3H, s, $\text{CH}_3\text{-C(O)}$), 2.3 (3H, s, $\text{CH}_3\text{-Ar}$), 3.1–3.5 (2H, m, $\text{CH}_2\text{-CH}$), 4.4 (1H, m, $\text{CH}_2\text{-CH}$), 7.2–7.6 (4H, m, ArH).

N-acetyl-*Se*-(4-methylphenyl)-D-selenocysteine (yield 62%) ^1H -NMR ($\text{D}_2\text{O}/\text{Na}_2\text{CO}_3$): δ (ppm) 1.8 (3H, s, $\text{CH}_3\text{-C(O)}$), 2.3 (3H, s, $\text{CH}_3\text{-Ar}$), 3.1–3.4 (2H, m, $\text{CH}_2\text{-CH}$), 4.4 (1H, m, $\text{CH}_2\text{-CH}$), 7.2 (2H, d, ArH), 7.5 (2H, d, ArH).

N-acetyl-*Se*-(4-methoxyphenyl)-L-selenocysteine (yield 70%) ^1H -NMR ($\text{D}_2\text{O}/\text{Na}_2\text{CO}_3$): δ (ppm) 1.8 (3H, s, $\text{CH}_3\text{-C(O)}$), 3.1–3.5 (2H, m, $\text{CH}_2\text{-CH}$), 3.9 (3H, s, $\text{CH}_3\text{-O-Ar}$), 4.4 (1H, t, $\text{CH}_2\text{-CH}$), 7.0 (2H, d, ArH), 7.7 (2H, d, ArH).

N-acetyl-*Se*-(4-methoxybenzyl)-L-selenocysteine (yield 72%) ^1H -NMR ($\text{D}_2\text{O}/\text{Na}_2\text{CO}_3$): δ (ppm) 1.9 (3H, s, $\text{CH}_3\text{-C(O)}$), 3.0 (2H, d, $\text{CH}_2\text{-CH}$), 3.9 (5H, s, $\text{CH}_3\text{-O-Ar-CH}_2$), 4.4 (1H, t, $\text{CH}_2\text{-CH}$), 7.0 (2H, d, ArH), 7.3 (2H, d, ArH).

Table 1. Apparent K_m of the fluorescent probes serving as the substrate for the P450s studied. (See www.gentest.com and Venhorst *et al.* (2000) for further details on the assays.)

P450	Substrate	K_m (μ M)
CYP1A1	CEC	1.6
CYP1A2	CEC	3.5
CYP2C9	MTFC	78
CYP2C19	CEC	29
CYP2D6	MMAMC	5.1
CYP2E1	MTFC	200
CYP3A4	BTFC	38

Competition experiments

All competition studies were carried out in Costar 3595 96-well plates using a 100 mM phosphate buffer (pH 7.4) and a total volume of 200 μ l. The final concentrations of the different fluorescent probes, serving as the substrate for the P450s studied, were approximately equal to their K_m . K_m (table 1) and rates of metabolism of the probes were as reported by Gentest (<http://www.gentest.com>) and Venhorst *et al.* (2000). Details of the fluorimetric assays have been described by Crespi and co-workers (1997, 1999, 2000) and by Venhorst *et al.* (2000). All analyses were performed on a Victor² 1420 multilabel counter (Wallac Oy, Turku, Finland) equipped with a plate heater set at 37°C. Initial screening of the inhibitory potencies of the SeCys conjugates toward the P450s investigated was performed at an inhibitor concentration of 250 μ M. This single high concentration enabled rapid identification of the P450s and SeCys conjugates which gave rise to low inhibition levels, and were therefore not relevant for further studies on the potential drug–drug interactions and chemopreventive activity exerted by SeCys conjugates.

In the case of CYP1A1, 0.5 pmole enzyme and 2 μ M CEC were pre-incubated for 10 min in the presence or absence of (seleno)cysteine conjugate or *N*-acetyl SeCys conjugate. The reaction was started by the addition of a pre-incubated NADPH-regenerating system, resulting in final concentrations of 1.3 mM NADPH, 3.3 mM glucose 6-phosphate and 0.4 U ml⁻¹ glucose 6-phosphate dehydrogenase in a final volume of 200 μ l. Metabolite formation was subsequently followed in time by measuring the increase in fluorescence for 20 min at intervals of 5 min. The excitation wavelength was 405 nm (bandwidth 8 nm) and the emission wavelength was 460 nm (bandwidth 30 nm). Incubations to investigate the effects of nucleophiles, radical scavengers and antioxidant enzymes on P450 inhibition were carried out analogously. Tested chemicals included ascorbic acid (2 and 0.5 mM), glutathione (1 mM), *N*-acetyl-L-cysteine (1 mM), mannitol (1 mM), SOD (400 U ml⁻¹) and catalase (800 U ml⁻¹).

The incubations with CYP1A2, –2C19 and –2D6 were carried out analogous to CYP1A1, but with the following adaptations. For CYP1A2 and –2C19, a CEC concentration of 5 and 75 μ M, respectively, was used. The fluorescent probe used for CYP2D6 was MMAMC, at a final concentration of 5 μ M (Venhorst *et al.* 2000). The amount of protein used per well was 1 pmole for CYP2C19 and 2 pmole for CYP2D6, and for these P450s the incubation time was 45 min. To reduce interference from background fluorescence of NADPH, a final concentration of this cofactor of 0.1 mM was used in the case of CYP2D6. This did not have an effect on the kinetics of MMAMC *O*-dealkylation (Venhorst *et al.*, 2000).

The fluorescent substrate used in the case of CYP2C9 and CYP2E1 was MTFC, at a concentration, respectively, of 75 and 100 μ M. BTFC (50 μ M) was used as the fluorescent probe for CYP3A4. The amount of protein used in the case of these P450s was 1 pmole for CYP2C9 and CYP3A4, and 4 pmole for CYP2E1. Analyses were performed by means of an end-point measurement, due to the low fluorescent yield of the metabolites at physiological pH. After 30 (CYP3A4) or 45 min (CYP2C9 and CYP2E1), the reaction was stopped by the addition of 10 μ l 5 M trichloroacetic acid. Before analysis, 100 μ l of a 1.5 M Tris/HCl solution (pH 9.0) was added to increase the fluorescent yield of the product formed. The metabolites were detected at an excitation and emission wavelength of 405 nm (bandwidth 8 nm) and 535 nm (bandwidth 30 nm), respectively. All other incubation conditions were as described for CYP1A1.

Assessment of uncoupling of CYP1A1 by SeCys conjugates

Formation of ROS by uncoupling of electron transfer and oxygen reduction in the catalytic cycle of CYP1A1 reportedly can be stimulated by NADPH or ligand binding, leading to inactivation of the enzyme (Puntarola and Cederbaum 1998, Schleizinger *et al.* 1999). To investigate whether uncoupling also plays a role in the presently observed inhibition of CYP1A1, the effect of *Se*-benzyl-L-selenocysteine on CYP1A1-mediated ROS formation was investigated by measuring the oxidation of *p*-

hydroxyphenylacetate by H_2O_2 and horseradish peroxidase to the fluorescent dimer 2,2'-dihydroxybiphenyl-5,5'-diacetate (Hyslop and Sklar 1984). In a 100 mM phosphate buffer (pH 7.4) and total volume of 1 ml, 10 nM CYP1A1 was incubated in the presence of 0.5 mM NADPH, 222 U ml^{-1} SOD, 5 U ml^{-1} horseradish peroxidase and 0.91 mM *p*-hydroxyphenylacetate at room temperature. SOD was added to the incubations to convert superoxide anions to hydrogen peroxide. The background increase in fluorescence was followed continuously for 2 min, after which 5 μl 50 mM *Se*-benzyl-L-cysteine in DMSO was added. The effect on 2,2'-dihydroxybiphenyl-5,5'-diacetate formation was subsequently followed for 5 min. To the control sample, 5 μl DMSO was added. Fluorescence was measured on a Perkin Elmer 3000 fluorescence spectrometer (Beaconsfield, UK). An excitation and emission wavelength of, respectively, 323 and 400 nm (slits 10 nm) was used.

P450 inhibition by GTK/ β -lyase bioactivated SeCys conjugates

To investigate whether bioactivation by beta-elimination leads to formation of more potent P450-inhibitors, inhibition levels induced by SeCys conjugates after 18 min of bioactivation by GTK/ β -lyase were compared with those without bioactivation. To this end, 1 mM of the investigated SeCys conjugate was pre-incubated in a 12.5-mM sodiumborate buffer (pH 8.6) in the presence of 1 $\mu\text{g ml}^{-1}$ GTK/ β -lyase and 0.5 mM α -keto- γ -methiolbutyrate at 37°C in a final volume of 3.5 ml. The reaction was terminated at $t = 0$ and 18 min by the addition of the potent GTK inhibitor aminooxyacetic acid to a final concentration of 0.5 mM. The incubation mixtures were subsequently added to the P450-assays described above, resulting in a fourfold dilution of selenium compounds. The reaction products formed in parallel to the selenols, i.e. pyruvate and methionine, as well as α -keto- γ -methiolbutyrate and aminooxyacetic acid, had no significant effect on any P450 enzyme activity in concentrations up to 125 μM (data not shown). The amount of selenol formed in the pre-incubation phase with GTK was quantified by measuring pyruvate, which is formed at equimolar amounts (Andreadou *et al.* 1996). To this end, samples of 100 μl were drawn at $t = 0$ and 18 min and added to 500 μl 0.14% *o*-phenylenediamine dissolved in 3 N HCl. The samples were heated for 60 min at 60°C to derivatize pyruvate with *o*-phenylenediamine under acidic conditions and subsequently centrifuged for 10 min at 2500g. The supernatant was analysed as described by Rooseboom *et al.* (2000). Pyruvate was quantified using a standard curve.

Data analyses

IC_{50} were determined with the non-linear least-square fitting method for one site binding, as implemented in Prism 2.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were performed using the unpaired Student's *t*-test. Values were considered significant when $p < 0.05$.

Results

Extent and selectivity of P450 inhibition by SeCys conjugates

Varying degrees of inhibition, ranging from no inhibition to complete inhibition, were obtained for the seven P450s investigated when incubated in the presence of 250 μM SeCys conjugate. As shown in table 2, CYP1A2, CYP2E1 and CYP3A4 enzyme activities were least affected by the SeCys conjugates, with inhibition levels on average $< 30\%$. Intermediate levels of inhibition were generally observed for CYP2C19 (average $49 \pm 20\%$), CYP2C9 ($46 \pm 12\%$) and CYP2D6 ($40 \pm 15\%$). For the latter P450s, the alkyl-substituted SeCys conjugates were least capable of inhibiting enzyme activities, followed by the phenyl- and benzyl-substituted SeCys conjugates.

In sharp contrast to the relatively low inhibition observed for the P450s described above, inhibition levels of CYP1A1-mediated CEC *O*-dealkylation activity $> 80\%$ for the majority of compounds (table 2). Most marked was the inhibitory potency of the benzylic SeCys conjugates, which all exerted complete or near complete inhibition of CYP1A1 activity at 250 μM . More extensive competition studies on the most potent inhibitor, *Se*-benzyl-L-selenocysteine, revealed an $\text{IC}_{50} = 12.8 \pm 1.2 \mu\text{M}$. Considerable levels of CYP1A1 inhibition were also

Table 2. Inhibition (%) of P450 in the presence of 250 μ M R-substituted L-selenocysteine *Se*-conjugates listed for CYP1A1, -1A2, -2C9, -2C19, -2D6, -2E1 and -3A4.

R	CYP1A1	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4
Methyl	45 $\pm$ 5	13 $\pm$ 2	36 $\pm$ 8	35 $\pm$ 1	13 $\pm$ 2	42 $\pm$ 2	9 $\pm$ 1
Ethyl	45 $\pm$ 3	29 $\pm$ 1	30 $\pm$ 5	23 $\pm$ 5	20 $\pm$ 3	40 $\pm$ 1	n.d.
<i>n</i> -Propyl	83 $\pm$ 1	22 $\pm$ 1	29 $\pm$ 11	28 $\pm$ 2	20 $\pm$ 3	14 $\pm$ 3	11 $\pm$ 7
<i>n</i> -Butyl	52 $\pm$ 2	37 $\pm$ 4	28 $\pm$ 5	51 $\pm$ 2	21 $\pm$ 3	34 $\pm$ 8	10 $\pm$ 5
Allyl	88 $\pm$ 1	n.d.	52 $\pm$ 7	37 $\pm$ 3	32 $\pm$ 2	6 $\pm$ 4	-19 $\pm$ 3
Alkyl average	63 $\pm$ 21	21 $\pm$ 14	35 $\pm$ 10	35 $\pm$ 11	21 $\pm$ 7	27 $\pm$ 16	2 $\pm$ 13
Phenyl	56 $\pm$ 1	14 $\pm$ 4	35 $\pm$ 5	10 $\pm$ 3	32 $\pm$ 6	29 $\pm$ 7	30 $\pm$ 5
4-CH3-phenyl	69 $\pm$ 1	18 $\pm$ 5	46 $\pm$ 7	47 $\pm$ 3	31 $\pm$ 2	24 $\pm$ 4	26 $\pm$ 4
4-CH3-phenyl*	78 $\pm$ 1	25 $\pm$ 8	50 $\pm$ 15	42 $\pm$ 3	30 $\pm$ 7	23 $\pm$ 1	40 $\pm$ 2
4-MeO-phenyl	94 $\pm$ 0	50 $\pm$ 6	54 $\pm$ 12	86 $\pm$ 2	62 $\pm$ 3	n.d.	30 $\pm$ 1
4-Cl-phenyl	51 $\pm$ 2	18 $\pm$ 7	44 $\pm$ 11	56 $\pm$ 2	29 $\pm$ 4	21 $\pm$ 1	30 $\pm$ 6
2-CH3-phenyl	86 $\pm$ 1	30 $\pm$ 6	68 $\pm$ 3	66 $\pm$ 1	33 $\pm$ 3	n.d.	23 $\pm$ 2
2-MeO-phenyl	88 $\pm$ 1	24 $\pm$ 6	49 $\pm$ 1	25 $\pm$ 1	48 $\pm$ 2	n.d.	23 $\pm$ 4
2-Cl-phenyl	75 $\pm$ 1	12 $\pm$ 2	37 $\pm$ 13	32 $\pm$ 1	61 $\pm$ 5	n.d.	45 $\pm$ 3
Phenyl average	75 $\pm$ 15	24 $\pm$ 12	48 $\pm$ 10	46 $\pm$ 24	41 $\pm$ 14	12 $\pm$ 13	31 $\pm$ 8
Benzyl	99 $\pm$ 0	19 $\pm$ 4	39 $\pm$ 3	61 $\pm$ 1	60 $\pm$ 2	n.d.	9 $\pm$ 1
4-CH3-benzyl	87 $\pm$ 1	28 $\pm$ 8	49 $\pm$ 2	57 $\pm$ 2	50 $\pm$ 12	n.d.	n.d.
4-MeO-benzyl	92 $\pm$ 0	29 $\pm$ 3	46 $\pm$ 6	56 $\pm$ 2	53 $\pm$ 1	n.d.	12 $\pm$ 10
4-Cl-benzyl	92 $\pm$ 2	44 $\pm$ 3	54 $\pm$ 14	87 $\pm$ 1	52 $\pm$ 6	37 $\pm$ 6	44 $\pm$ 5
2-CH3-benzyl	86 $\pm$ 1	25 $\pm$ 5	67 $\pm$ 3	55 $\pm$ 2	49 $\pm$ 4	n.d.	n.d.
2-Cl-benzyl	85 $\pm$ 1	12 $\pm$ 2	46 $\pm$ 6	48 $\pm$ 2	39 $\pm$ 6	n.d.	n.d.
2-NO2-benzyl	88 $\pm$ 1	22 $\pm$ 3	64 $\pm$ 7	48 $\pm$ 2	54 $\pm$ 4	22 $\pm$ 8	n.d.
3,4-diCl-benzyl	85 $\pm$ 1	45 $\pm$ 2	40 $\pm$ 8	76 $\pm$ 1	48 $\pm$ 2	38 $\pm$ 1	62 $\pm$ 3
Benzyl average	89 $\pm$ 5	28 $\pm$ 12	51 $\pm$ 10	61 $\pm$ 14	51 $\pm$ 6	12 $\pm$ 17	16 $\pm$ 24
Total average	77 $\pm$ 17	25 $\pm$ 12	46 $\pm$ 12	49 $\pm$ 20	40 $\pm$ 15	16 $\pm$ 16	18 $\pm$ 20

* D-Isomer
Values are the mean $\pm$ SD ($n = 3$); n.d., not detected

observed for the alkyl-substituted SeCys conjugates: 63 $\pm$ 21% on average. The proven chemopreventive compounds *Se*-(*n*-propyl)-L-selenocysteine and *Se*-allyl-L-selenocysteine inhibited CYP1A1 activity by 83 and 88% respectively when incubated at a concentration of 250 μ M.

Inhibitory potency of L-selenocysteine Se-conjugates versus L-cysteine S-conjugates

To establish whether the selenium atom is a factor contributing to the inhibitory potency of SeCys conjugates, the P450 inhibition induced by *Se*-(4-methoxyphenyl)-L-selenocysteine and *Se*-benzyl-L-selenocysteine was compared with that induced by the corresponding sulphur-analogues, namely *S*-(4-methoxyphenyl)-L-cysteine and *S*-benzyl-L-cysteine. As shown in figure 2, *Se*-(4-methoxyphenyl)-L-selenocysteine was a more potent inhibitor of P450-activities than *S*-(4-methoxyphenyl)-L-cysteine for all P450s studied, except CYP2E1. Most pronounced were the effects of selenium substitution for CYP1A1 and CYP2C19, with differences in inhibitory potency of > 60%. Interestingly, CYP2E1 was inhibited by *S*-(4-methoxyphenyl)-L-cysteine but not by *Se*-(4-methoxyphenyl)-L-selenocysteine. The trend of higher inhibition of CYP2E1 by the sulphur-containing analogue was also seen for *Se*-benzyl-L-selenocysteine and *S*-benzyl-L-cysteine. In the case of CYP1A2 and CYP3A4, these analogues produced

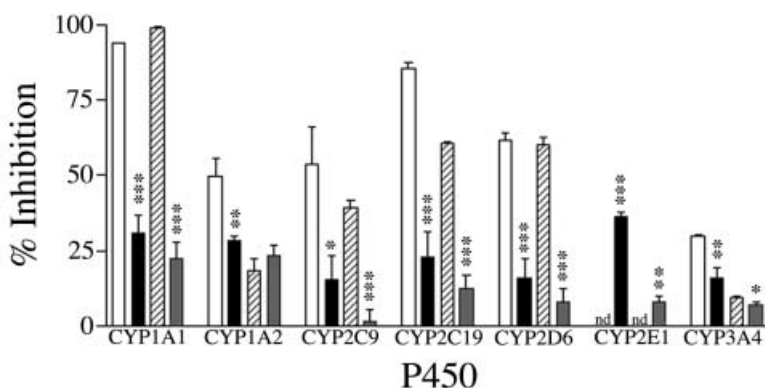


Figure 2. Comparison of the inhibitory potency of *Se*-(4-methoxyphenyl)- (white bars) and *Se*-benzyl-L-selenocysteine (striped bars) with the inhibition capacity of their sulphur-containing analogues, i.e. respectively *S*-(4-methoxyphenyl)- (black bars) and *S*-benzyl-L-cysteine (grey bars), towards CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Inhibition was measured as the relative reduction in metabolite formation of the reference substrate by the presence of 250 μ M of conjugate. Statistical significance of the difference in inhibition between selenium- and sulphur-containing analogues is indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are the mean $\pm$ SD of three determinants.

a similar degree of inhibition. However, a considerably larger reduction in enzyme activity was detected for CYP1A1, CYP2C9, CYP2C19 and CYP2D6 in the presence of *Se*-benzyl-L-selenocysteine compared with *S*-benzyl-L-cysteine. CYP1A1 again displayed a difference in potency between the selenium and sulphur-containing analogues > 60%.

Role of reactive oxygen species (ROS) in CYP1A1 inhibition by SeCys conjugates

Further examination of the inhibition of CYP1A1 mediated CEC *O*-dealkylation by SeCys conjugates showed that it was time-dependent (figure 3). To investigate whether ROS formation was involved in the inhibition mediated by SeCys conjugates, the effects of several antioxidants on CYP1A1 inhibition were studied for *Se*-methyl-, *Se*-phenyl- and *Se*-benzyl-L-selenocysteine. As illustrated in figure 4, addition of ascorbic acid at a concentration of 0.5 or 2 mM did not reduce CYP1A1 inhibition by the investigated compounds. Contrary to the effects of ascorbic acid, 1 mM glutathione (GSH) significantly reduced CYP1A1 inhibition levels induced by the SeCys conjugates. In the case of *Se*-methyl-L-selenocysteine, the inhibition was completely abolished by GSH. The inhibitory potency of *Se*-phenyl-L-selenocysteine was furthermore reduced to a mere 10%. GSH, however, had a minor effect on the inhibition exerted by *Se*-benzyl-L-selenocysteine, with only a 10% increase in enzyme activity. The combined addition of 1 mM GSH and 0.5 mM or 2 mM ascorbic acid generally resulted in slightly smaller reductions in inhibition levels when compared with the addition of GSH alone.

In addition to ascorbic acid and GSH, the influence of mannitol (1 mM), *N*-acetyl cysteine (1 mM), SOD (400 U ml⁻¹) and catalase (800 U ml⁻¹) on the CYP1A1 inhibition produced by *Se*-benzyl-L-selenocysteine was investigated. The hydrophilic hydroxyl radical scavenger mannitol had no effect on the inhibitory potency of the SeCys conjugate (data not shown). SOD and catalase,

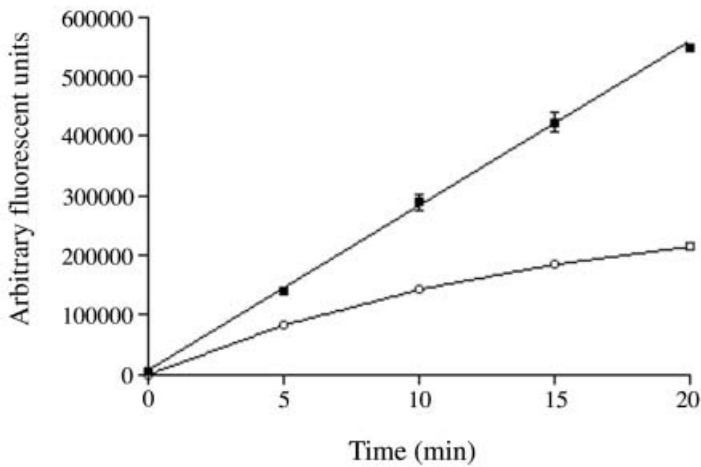


Figure 3. Time-dependency of CYP1A1 inhibition illustrated for 250 μ M *Se*-phenyl-L-selenocysteine (circles). The linearity of CEC *O*-dealkylation is also shown for the control samples (squares). Data are the mean $\pm$ SD of three determinants.

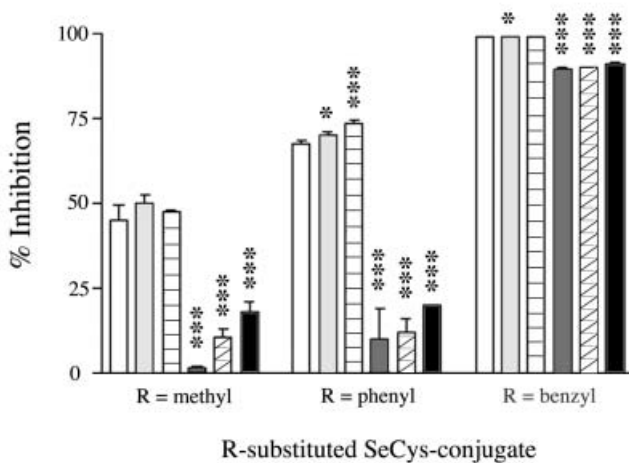


Figure 4. Observed inhibition of CYP1A1 by 250 μ M *Se*-methyl-, *Se*-phenyl and *Se*-benzyl-L-selenocysteine in the presence of 100 mM phosphate buffer (white bars), 0.5 mM ascorbic acid (light grey bars), 2 mM ascorbic acid (horizontally striped bars), 1 mM GSH (dark grey bars), 0.5 mM ascorbic acid + 1 mM GSH (double arced bars), and 2 mM ascorbic acid + 1 mM GSH (black bars). The statistical significance of the difference in inhibition between the control sample (250 μ M SeCys conjugate plus buffer) and samples containing ascorbic acid and/or GSH is indicated by asterisks: * p < 0.05; ** p < 0.01; *** p < 0.001. Data are the mean $\pm$ SD of three determinants.

enzymes acting on superoxide anion radicals and H_2O_2 , respectively, similarly had no effect (data not shown). CYP1A1 inhibition was, however, attenuated by 1 mM *N*-acetyl cysteine to the same extent as by addition of 1 mM GSH.

CYP1A1 uncoupling leading to the formation of superoxide anion radicals and H_2O_2 in the presence of 250 μ M *Se*-benzyl-L-selenocysteine was investigated by following the time course of dimerization of *p*-hydroxyphenylacetate to 2,2'-dihydroxybiphenyl-5,5'-diacetate (Hyslop and Sklar 1984). The latter reaction is

catalysed by horseradish peroxidase and requires H_2O_2 . SOD was added to convert all superoxide anion radicals to H_2O_2 . *Se*-benzyl-L-selenocysteine was found not to increase the background formation of 2,2'-dihydroxybiphenyl-5,5'-diacetate (data not shown), indicating that *Se*-benzyl-L-selenocysteine did not stimulate ROS formation by uncoupling of CYP1A1.

Inhibitory potency of N-acetylated SeCys conjugates

A physiologically important metabolic pathway of SeCys conjugates may be *N*-acetylation by cysteine conjugate *N*-acetyl transferases. Incubations of male Wistar rat hepatocytes with SeCys conjugates were shown to produce high levels of *N*-acetyl conjugates (data not shown). To investigate how this biotransformation route may affect the P450 inhibition by SeCys conjugates, the capacity of a selection of *N*-acetyl SeCys conjugates to inhibit P450s was determined. The results listed in table 3 show that inhibition levels for CYP1A2, CYP2C19, CYP2E1 and CYP3A4 were consistently and considerably increased by *N*-acetylation. The most marked effect was observed in the case of CYP3A4, with inhibition levels > 85% for all *N*-acetyl SeCys conjugates investigated. *N*-acetylation of SeCys conjugates both increased and decreased CYP2C9 inhibition levels, whereas extensive inhibitory potency was retained in the case of CYP1A1. Only small effects on the inhibitory potency of SeCys conjugates upon *N*-acetylation were found for CYP2D6, with the exception that *N*-acetyl-*Se*-(2-methylphenyl)-L-selenocysteine substantially reduced MMAMC *O*-dealkylation by an additional 30% compared with the non-*N*-acetylated analogue.

N-acetyl-*Se*-(4-methoxybenzyl)-L-selenocysteine induced virtually complete inhibition (> 90%) of five of the investigated P450s, i.e. CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP3A4 (table 3). As shown in table 4, the IC_{50} 's of this compound were in the low micromolar range in the case of CYP1A1, CYP1A2 and CYP2C19, ranging between 6.4 ± 1.1 and $17.0 \pm 0.8 \mu\text{M}$. CYP2C9 and CYP3A4 displayed somewhat higher IC_{50} 's of 56.5 ± 12.7 and $31.1 \pm 9.8 \mu\text{M}$, respectively.

Table 3. Inhibition (%) of P450 in the presence of $250 \mu\text{M}$ R-substituted *N*-acetyl L-selenocysteine *Se*-conjugates for CYP1A1, -1A2, -2C9, -2C19, -2D6, -2E1 and -3A4.

R	CYP1A1	CYP1A2	CYP2C9	CYP2C1g	CYP2D6	CYP2E1	CYP3A4
4-CH ₃ -phenyl*	78 ± 1 (+ 1)	69 ± 2 (+ 44)	72 ± 8 (+ 22)	69 ± 2 (+ 27)	21 ± 1 (- 8)	36 ± 1 (+ 12)	90 ± 1 (+ 50)
4-MeO-phenyl	99 ± 0 (+ 5)	84 ± 1 (+ 34)	72 ± 12 (+ 18)	96 ± 0 (+ 10)	63 ± 2 (+ 2)	37 ± 5 (+ 37)	88 ± 1 (+ 58)
2-CH ₃ -phenyl	71 ± 1 (- 16)	66 ± 1 (+ 35)	38 ± 3 (- 29)	88 ± 1 (+ 22)	64 ± 1 (+ 31)	30 ± 2 (+ 30)	86 ± 1 (+ 62)
4-MeO-benzyl	96 ± 0 (+ 4)	97 ± 0 (+ 68)	91 ± 2 (+ 45)	94 ± 1 (+ 37)	52 ± 9 (- 1)	65 ± 2 (+ 65)	96 ± 1 (+ 84)

* D-Isomer.

Values are the mean ± SD ($n = 3$).

The difference in potency (% inhibition) compared with the corresponding underivatized SeCys conjugates (table 2) is given in parentheses

Table 4. IC₅₀ of *Se*-benzyl-L-selenocysteine and *N*-acetyl-*Se*-(4-methoxybenzyl)-L-selenocysteine for a selective set of P450s.

P450	IC ₅₀ (μM) <i>Se</i> -benzyl-L-selenocysteine
CYP1A1	12.8 ± 1.2
P450	IC ₅₀ (μM) <i>N</i> -acetyl- <i>Se</i> -(4-methoxybenzyl)-L-selenocysteine
CYP1A1	17.0 ± 0.8
CYP1A2	6.4 ± 1.1
CYP2C9	56.5 ± 12.7
CYP2C19	6.8 ± 0.5
CYP3A4	31.1 ± 9.8

Values are the mean ± SD (*n* = 3)

Inhibitory potency of GTK/β-lyase bioactivated SeCys conjugates

Aside from *N*-acetylation, another important metabolic pathway for SeCys conjugates is beta-elimination to selenols by beta-lyases. The effects of selenol formation on P450 inhibition were therefore assessed by comparing inhibition levels induced by four selected SeCys conjugates upon bioactivation by GTK/β-lyase (*t* = 18 min) to those without GTK/β-lyase bioactivation (*t* = 0 incubation).

The 18-min pre-incubation of *Se*-methyl-, *Se*-(*n*-propyl)-, *Se*-phenyl- and *Se*-benzyl-L-selenocysteine in the presence of GTK/β-lyase resulted, as determined from pyruvate formation, in respective selenol concentrations of 37.5, 37, 20 and 15 μM in the P450-assays. GTK/β-lyase mediated metabolism of *Se*-methyl-L-selenocysteine only affected the inhibition of CYP2C19 (figure 5A). A significant decrease in P450 inhibition was observed, indicating that the metabolic products formed were less potent inhibitors of CYP2C19 than the parent compound *Se*-methyl-L-selenocysteine. Pre-incubation of *Se*-(*n*-propyl)-L-selenocysteine with GTK/β-lyase similarly had no or little effect on the inhibition of the P450s (figure 5B). Bioactivation of *Se*-phenyl-L-selenocysteine significantly affected the inhibition of all seven P450s (figure 5C). Most pronounced was the increase in inhibition of CYP1A1 and CYP2C9 by 23 and 35%, respectively, and the decrease in CYP2E1 inhibition by 29%. GTK/β-lyase mediated metabolism of *Se*-benzyl-L-selenocysteine resulted in a slight but significant increase in inhibition of several P450s (figure 5D). An increase in inhibition of CYP1A1 could not be measured because the parent compound, *Se*-benzyl-L-selenocysteine, already produced complete inhibition. However, in the presence of 1 mM GSH, CYP1A1 inhibition increased from 93.1 ± 0.2% to 95.7 ± 0.2% by pre-incubation with GTK/β-lyase.

Discussion

Recently, it was reported that SeCys conjugates possess potent chemopreventive activity in animal models for chemically induced carcinogenesis (Ip *et al.* 1999). It is currently still unknown whether inhibition of bioactivating enzymes contributes to this chemopreventive activity. In the present study, we investigated whether SeCys conjugates and/or their metabolites, i.e. selenols and *N*-acetyl SeCys conjugates, exert inhibition on the most important human P450 enzymes. CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were

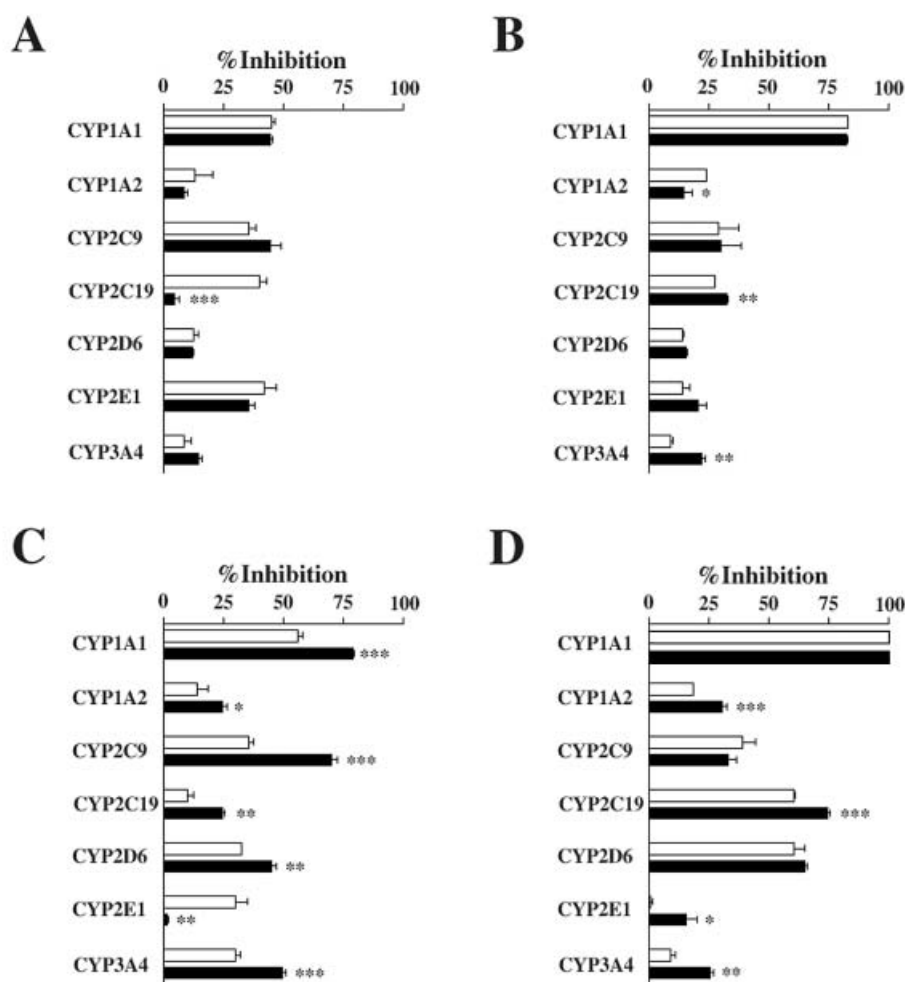


Figure 5. P450 inhibition induced by GTK/ β -lyase pre-incubated ($t = 18$ min; black bars) and non-pre-incubated ($t = 0$ min; white bars) samples shown for CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Pre-incubations were performed with (A) *Se*-methyl-L-selenocysteine, (B) *Se*-(*n*-propyl)-L-selenocysteine, (C) *Se*-phenyl-L-selenocysteine and (D) *Se*-benzyl-L-selenocysteine, resulting in, respectively, 37.5 μ M methylselenol, 37 μ M *n*-propylselenol, 20 μ M phenylselenol and 15 μ M benzylselenol. CYP1A1 activity was completely abolished in the presence of *Se*-benzyl-L-selenocysteine in both the $t = 0$ - and 18-min samples. The statistical significance of the difference in P450 inhibition between corresponding $t = 18$ - and 0-min samples is indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are the mean $\pm$ SD of three determinants.

selected because these P450s are known to be involved in the biotransformation of the majority of pharmaceutical drugs and/or in the bioactivation of carcinogens (Guengerich and Shimada 1991, Ingelman-Sundberg *et al.* 1999). The results may indicate whether drug–drug interactions can occur in multidrug treatment involving SeCys conjugates, and whether P450 inhibition may contribute to the reported chemopreventive effect.

Distinctly different levels in inhibition of the human P450s were observed in the presence of 250 μ M SeCys conjugate (table 2). Inhibition levels depended both

on the P450 and the *Se*-bound substituent, indicating that binding of SeCys conjugates to P450s is dictated by the active site topologies of the latter. When comparing the influence of the different substituent groups, it is clear that the alkyl-substituted SeCys conjugates exhibited the lowest inhibitory potency towards all P450s except CYP2E1, followed by the phenyl- and benzyl-substituted SeCys conjugates. Apart from the nature of the *Se*-bound substituent, the selenium atom itself was found to influence significantly the capacity of the compounds to inhibit P450s. With the exception of CYP2E1, all P450s showed higher inhibition levels in the presence of selenium-containing SeCys conjugates when compared with the corresponding sulphur-containing analogues. One explanation for this observation may be that the selenium atom interacts more favourably with active site residues or possibly with the haem-group of the different P450s.

When comparing the individual P450s, CYP1A1 was most susceptible to inhibition by SeCys conjugates, with a total average of 77% inhibition (table 2). The other P450s showed, on average, much lower degrees of inhibition. CYP1A1 is generally not expressed in the human liver and is not regarded as a major enzyme involved in the metabolism of pharmaceutical drugs (Spatzenegger and Jaeger 1995, Kawajiri and Hayashi 1997). The occurrence of significant drug-drug interactions by administration of SeCys conjugates, therefore, seems unlikely. However, CYP1A1 is involved in the bioactivation of numerous procarcinogens, and upon exposure to ligands is highly inducible in the liver (Schwartz *et al.* 2001). As such, CYP1A1 is a particularly important target for chemopreventive agents (Ioannides and Parke 1993). With IC_{50} 's in the low micromolar range as observed for *Se*-benzyl-L-selenocysteine ($12.8 \pm 1.2 \mu M$), the inhibitory potency of SeCys conjugates may seem somewhat low compared with the IC_{50} ($< 0.5 \mu M$) previously observed for selenocyanates by Shimada *et al.* (1997). However, because of their resemblance to cysteine conjugates, it can be anticipated that SeCys conjugates will be taken up actively by amino acid transporters (Commandeur *et al.* 1995). Therefore, it may well be envisaged that inhibition of CYP1A1 by SeCys conjugates contributes to the chemopreventive activity of this class of compounds. Especially interesting to note in this respect is the high inhibitory potency of *Se*-(*n*-propyl)- and *Se*-allyl-L-selenocysteine towards CYP1A1. For these compounds, significant chemopreventive activity has been demonstrated in animal models (Ip *et al.* 1999).

A possible explanation for the time-dependent inhibition of CYP1A1 by SeCys conjugates may be ligand-induced uncoupling of the CYP1A1 catalytic cycle, resulting in the formation of ROS *in situ*, and consequently causing oxidative inactivation of the enzyme (Goeptar *et al.* 1995). This mechanism has recently been described for the inhibition of CYP1A1 by 3,3',4,4'-tetrachlorobiphenyl (Schlezinger *et al.* 1999). Addition of radical scavengers or antioxidant enzymes, however, did not have any effect on the inhibition levels induced by the SeCys conjugates. Furthermore, basal H_2O_2 formation by CYP1A1 in the presence of SOD was not increased upon addition of *Se*-benzyl-L-selenocysteine. Thus, uncoupling of the monooxygenation cycle and subsequent formation of ROS appear not to play a role in CYP1A1 inactivation in the case of SeCys conjugates.

Another explanation for time-dependent inhibition may be mechanism-based inhibition, where a reactive metabolite (an electrophile or oxidative species) formed by P450 inactivates the enzyme (Kent *et al.* 2001, Hollenberg 2002).

This mechanism is supported by the observed inhibition of inactivation by addition of the nucleophiles GSH and *N*-acetyl-cysteine. The fact that inhibition levels were reduced by addition of 1 mM GSH or *N*-acetyl-cysteine suggests that an electrophilic or oxidative species released from the CYP1A1 active site may be involved in the observed inhibition. A possible metabolic pathway of SeCys conjugates leading to the inactivation of P450s may be selenoxidation or the formation of reactive intermediates from selenoxidation (Rooseboom *et al.* 2001). Selenoxides of SeCys conjugates are thiol-oxidizing species that can degrade spontaneously into reactive selenenic acid, ammonia and pyruvate. Pyruvate formation, however, was not detected when 250 μM *Se*-benzyl-L-selenocysteine was incubated for 20 min in the presence of either 10 or 100 nM CYP1A1. Similar results were also previously obtained for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Rooseboom *et al.* 2001). An explanation may be that the selenoxide has been reduced by protein thiols and as a result does not decompose to pyruvate.

Previously, it has been reported that *N*-acetyl cysteine *S*-conjugates of halogenated alkenes are sulphoxidated by CYP3A enzymes (Werner *et al.* 1995, 1996). Because of their structural resemblance, it was anticipated that *N*-acetyl SeCys conjugates also interact with human CYP3A4 and possibly other P450s. Screening of four *N*-acetylated SeCys conjugates not only showed that this was indeed the case, but also that *N*-acetylation resulted in consistently higher inhibitory potencies of the compounds towards CYP1A2, CYP2C19, CYP2E1 and CYP3A4 (table 3). *N*-acetyl SeCys conjugates, therefore, are more likely to inhibit these P450s than the non-*N*-acetylated compounds. $\text{IC}_{50} > 6 \mu\text{M}$ were obtained for the most potent inhibitor *N*-acetyl *Se*-(4-methoxybenzyl)-L-selenocysteine (table 4). Whether SeCys conjugates and their *N*-acetyl analogues are effective inhibitors *in vivo* as well depends on the physiological concentration of these compounds. As described above, SeCys conjugates may reach relatively high concentrations by active uptake. This may also be true for *N*-acetyl SeCys conjugates, as transporters for active uptake have also been described for the structurally related *N*-acetyl cysteine *S*-conjugates (Commandeur *et al.* 1995). However, the latter are also actively excreted from cells.

Selenols have recently been implicated as the species responsible for the chemopreventive activity of SeCys conjugates (Ip 1998). Therefore, it was also studied whether pre-incubation of the SeCys conjugates in the presence of GTK/ β -lyase increased the levels of P450 inhibition. Bioactivation of alkyl-substituted SeCys conjugates and *Se*-benzyl-L-selenocysteine by GTK/ β -lyase, respectively resulting in 37 μM alkylselenol and 15 μM benzylselenol as determined from stoichiometric pyruvate detection, did not inhibit the P450s investigated to any large extent. Given the fact that both *Se*-methyl- and *Se*-(*n*-propyl)-L-selenocysteine exhibit chemopreventive activity, it can be concluded that the biological activity of these SeCys conjugates is not likely to be exerted by selenol induced P450 inhibition. The products formed by GTK/ β -lyase-mediated metabolism of *Se*-phenyl-L-selenocysteine were found more capable of inhibiting various P450s. The observed additive increase in P450 inhibition, on top of that induced by the parent-compound, at a respective selenol concentration of 20 μM , indicates that potentiation of P450 inhibition can occur by bioactivation of (phenylic) SeCys conjugates. This suggests that aromatic selenols may contribute to the chemopre-

ventive activity of SeCys conjugates. Indeed, 4-methoxybenzeneselenol has been demonstrated to possess chemopreventive activity (El-Bayoumy 1985).

In summary, this study has shown that SeCys conjugates display potent and relatively selective inhibition of CYP1A1. This inhibition is time-dependent and appears to involve a thiol-reactive intermediate, possibly a selenoxide. The CYP1A1 inhibition exerted by SeCys conjugates may contribute to their chemopreventive activity, as CYP1A1 is responsible for the bioactivation of several procarcinogens (e.g. benzo[*a*]pyrene). However, P450 inhibition is probably not the only mechanism involved in chemoprevention as other mechanisms have also been implicated. These include induction of apoptosis in various wild-type and non-functional p53 cell lines possibly by selenol-mediated release of cysteinylthiolate-bound zinc from caspases (Ganther 1999), inhibition of angiogenesis by inhibiting key attributes (proliferation, survival and matrix degradation) of endothelial cells (Jiang *et al.* 1999), and induction of phase II enzymes mediated by the antioxidant responsive element (Talalay *et al.* 1995, Ip and Lisk 1997, 't Hoen *et al.* 2002). *N*-acetylation and beta-elimination can further increase the inhibitory potency of SeCys conjugates towards P450s. As such, these metabolites may also contribute to the chemopreventive activity of SeCys conjugates. Finally, the relatively low inhibition of P450s other than CYP1A1 by SeCys conjugates suggests a low potential of drug–drug interactions.

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CYP2A13-catalysed coumarin metabolism: comparison with CYP2A5 and CYP2A6

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Received 8 May 2002

1. We investigated the total metabolism of coumarin by baculovirus (BV)-expressed CYP2A13 and compared it with metabolism by BV-expressed CYP2A6. The major coumarin metabolite formed by CYP2A13 was 7-hydroxycoumarin, which accounted for 43% of the total metabolism. The product of 3,4-epoxidation, *o*-hydroxyphenylacetaldehyde (*o*-HPA), accounted for 30% of the total metabolites.

2. The K_m and V_{max} for CYP2A13-mediated coumarin 7-hydroxylation were $0.48 \pm 0.07 \mu M$ and $0.15 \pm 0.006 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ CYP, respectively. The V_{max} of coumarin 7-hydroxylation by CYP2A13 was about 16-fold lower than that of CYP2A6, whereas the K_m was 10-fold lower.

3. In the mouse, there were two orthologues for CYP2A6: CYP2A4 and CYP2A5, which differed by only 11 amino acids. However, CYP2A5 is an efficient coumarin 7-hydroxylase, whereas CYP2A4 is not. We report here that BV-expressed CYP2A4 metabolizes coumarin by 3,4-epoxidation. Two products of the 3,4-epoxidation pathway, *o*-HPA and *o*-hydroxyphenylacetic acid (*o*-HPAA), were detected by radioflow HPLC.

4. The K_m and V_{max} for the coumarin 3,4-epoxidation by CYP2A4 were $8.7 \pm 3.6 \mu M$ and $0.20 \pm 0.04 \text{ nmol min}^{-1} \text{ nmol}^{-1}$ CYP, respectively. Coumarin 7-hydroxylation by CYP2A5 was more than 200 times more efficient than 3,4 epoxidation by CYP2A4.

Introduction

CYP2A13 is one of three members of the human CYP2A gene family (Fernandez-Salguero *et al.* 1995). The other members are CYP2A6, a hepatic coumarin 7-hydroxylase, and CYP2A7, reported to be a non-functional enzyme (Yamano *et al.* 1990). A number of regions in the CYP2A13 sequence are similar to CYP2A7, therefore CYP2A13 was also predicted to be non-functional (Ding *et al.* 1995). However, Su *et al.* (2000) recently expressed CYP2A13 in *Spodoptera frugiperda* (Sf9) insect cells and reported that it is in fact a functional enzyme that metabolizes several compounds which are substrates for CYP2A6. The coumarin 7-hydroxylase activity by CYP2A13 was previously measured at a single coumarin concentration. At $100 \mu M$ NNK, CYP2A13 catalysed the 7-hydroxylation of coumarin at about 10% of the rate of CYP2A6 (Su *et al.* 2000).

The highest level of CYP2A13 expression was found in the nasal mucosa; however, 2A13 is also expressed in significant levels in human lung and trachea (Su *et al.* 2000). CYP2A6 is primarily hepatic, constituting 1–10% of total CYP in the human liver (Yun *et al.* 1991, Shimada *et al.* 1994). CYP2A6 has been detected in small amounts in nasal mucosa and there are conflicting results about whether or not CYP2A6 is present in the human lung (Crawford *et al.* 1998, Koskela *et al.*

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1999, Su *et al.* 2000). Koskela *et al.* did not detect any CYP2A6 transcripts in human lung using RT-PCR. In contrast, Crawford *et al.* detected CYP2A6 mRNA in human bronchial epithelial cells. The discrepancy observed might be due to the detection of CYP2A13 with the primers used by Crawford *et al.* The recognition site for these primers in CYP2A13 differs from 2A6 by only one nucleotide. Using sequence-specific primers and competitive RT-PCR, Su *et al.* (2000) reported that the amount of CYP2A13 mRNA in the lung is eight times higher than that of CYP2A6.

CYP2A6 metabolizes the carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosornicotine (NNN), which are tobacco-specific nitrosamines (Patten *et al.* 1996, 1997). NNK is a potent lung carcinogen in laboratory animals and a likely human lung carcinogen (Hecht 1998). NNN primarily induces oesophageal tumours in animals and is believed to play a role in tobacco-induced oral and oesophageal cancer in humans. The metabolism of NNN by CYP2A6 is both specific and efficient (Patten *et al.* 1997); however, CYP2A6-catalysed NNK metabolism is much less efficient (Patten *et al.* 1996). CYP2A13 catalyses the activation of NNK by α -hydroxylation at a significantly higher rate than does 2A6 (Su *et al.* 2000). The $V_{\max}/K_m$ for NNK methylene hydroxylation by 2A6 is 0.008 compared with 0.36 for 2A13 (Patten *et al.* 1996, Su *et al.* 2000). Since CYP2A13 is present in human lung, it is likely to catalyse NNK activation in this tissue and therefore play an important role in tobacco related lung cancers.

In the mouse there are two orthologues of CYP2A6: CYP2A4 and 2A5 (Honkakoski and Negishi 1997). These two enzymes are 98% homologous, differing by only 11 amino acids; however, they catalyse NNK metabolism with strikingly different efficiencies (Felicia *et al.* 2000). CYP2A4 catalyses NNK α -hydroxylation with a 54-fold lower efficiency ($V_{\max}/K_m$) than does CYP2A5. However, the rate of CYP2A5-catalysed NNK α -hydroxylation is 1000-fold greater than that of CYP2A6 at 1 μM NNK (Felicia *et al.* 2000).

CYP2A5 and 2A6 are well-characterized coumarin 7-hydroxylases. However, CYP2A4 has been reported to have little or no coumarin 7-hydroxylase activity (Lindberg and Negishi 1989, Negishi *et al.* 1989, Felicia *et al.* 2000). Coumarin 7-hydroxylation is one of several pathways of coumarin metabolism (figure 1). In all species studied to date, the predominant pathway is either 7-hydroxylation or the formation of the ring-opened products *o*-hydroxyphenylacetaldehyde (*o*-HPA) and *o*-hydroxyphenylacetic acid (*o*-HPAA) (Shilling *et al.* 1969, Egan and O'Kennedy 1992, Fentem and Fry 1993). *o*-HPA is generated non-enzymatically from the unstable coumarin 3,4-epoxide, whereas *o*-HPAA is an oxidation product of *o*-HPA (figure 1) (Born *et al.* 1997, 2000). 7-Hydroxylation is the predominant coumarin metabolism pathway in humans, due to the presence of CYP2A6 in the liver (Miles *et al.* 1990). However, other human CYPs metabolize coumarin by pathways other than 7-hydroxylation. *o*-HPA is the major metabolite formed by CYP1A1, 1A2, 2B6, 2E1 and 3A4, although the rate of metabolism is quite low (Zhuo *et al.* 1999).

In the current study, the coumarin 7-hydroxylase activity of CYP2A13 was compared with that of CYP2A6. The kinetic parameters for CYP2A13-mediated coumarin 7-hydroxylation were determined and other CYP2A13-catalysed pathways of coumarin metabolism were quantified. Total coumarin metabolism by the mouse CYP, 2A4, was also investigated and compared with the metabolism of

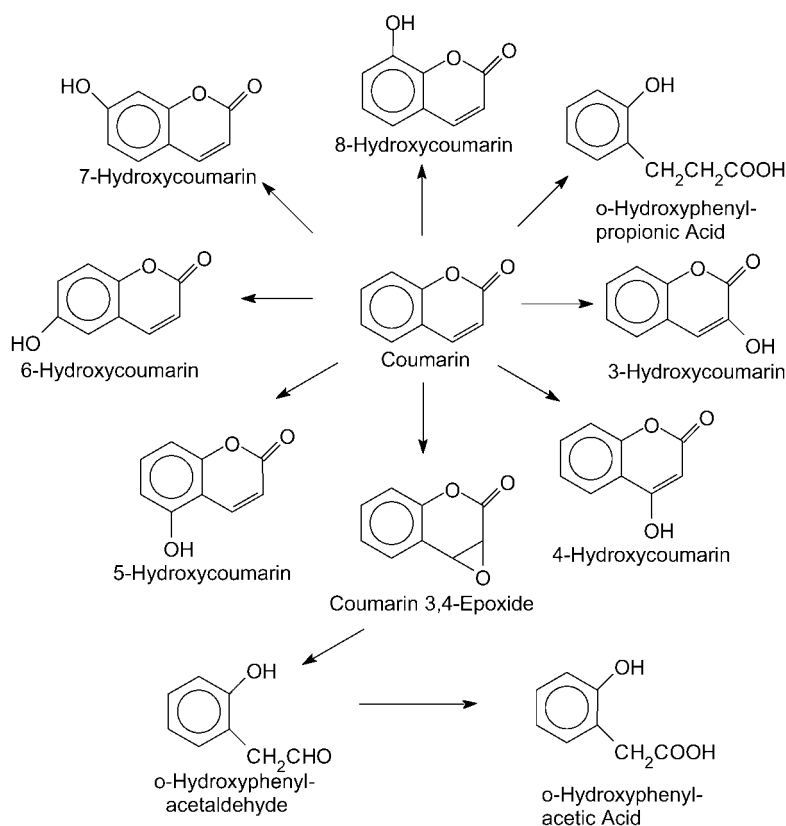


Figure 1. Pathways of coumarin metabolism.

coumarin by the highly homologous CYP, 2A5, as well as the human CYPs, 2A6 and 2A13.

Materials and methods

Chemicals

[U- ^{14}C -benzyl] coumarin (58 mCi mmol^{-1}), purity > 99.5% by HPLC analysis, was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). 4-Hydroxycoumarin, 2-hydroxyphenylacetic acid, *o*-coumaric acid and 6,7-dihydroxycoumarin were purchased from Fluka Chemical Co. (Ronkonkoma, NY, USA). 3-Hydroxycoumarin was obtained from Indofine Chemical Co. (Sommerville, NJ, USA). *o*-Hydroxyphenylacetaldehyde was a gift from Dr Lois D. Lehman-McKeeman of Procter & Gamble (Cincinnati, OH, USA) and 5-hydroxycoumarin was a gift from Dr Takashi Harayama (Okayama University, Japan). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) or Aldrich Chemical Co. (Milwaukee, WI, USA).

Metabolism of ^{14}C -coumarin

Homogenate of baculovirus (BV)-expressed CYP2A13 (Su *et al.* 2000) was provided by Dr Xinxin Ding (Wadsworth Center, Albany, NY, USA). CYP2A4, CYP2A5 and CYP2A6 were expressed in Sf9 cells using the BV-expression system (Felicia *et al.* 2000), and microsomes were prepared as described. Microsomes from BV-expressed CYP2A4 (33.9 pmol), CYP2A5 (1.8 pmol), CYP2A6 (1.8 pmol) and CYP2A13 cell homogenate (7.2 pmol) were incubated with BV-expressed human P450:NADPH oxidoreductase, $5 \mu\text{M}$ [U- ^{14}C -benzyl]-coumarin, and an NADPH-generating system (0.4 mM NADP^+ , 100 mM glucose 6-phosphate, and $0.4 \text{ units ml}^{-1}$ glucose 6-phosphate dehydrogenase) in a $200 \mu\text{l}$ total volume of 50 mM Tris buffer (pH 7.4) for 30 min at 37°C . A ratio of 1:5, CYP to

oxidoreductase, was used in all experiments. All analyses were performed in duplicate. The reactions were terminated by the addition of 20 μ l 15% trichloroacetic acid and then centrifuged. The supernatant was mixed with standards and analysed by reverse-phase HPLC with radioflow detection. The standards added were *o*-HPA, *o*-HPAA, *o*-hydroxyphenylpropionic acid, *o*-coumaric acid, 5-, 6-, 7-, and 8-hydroxycoumarin, coumarin, and 6,7-dihydroxycoumarin. A Phenomenex (Torrance, CA, USA) Bondclone C₁₈ column (3.9 $\times$ 300 mm, 10 μ m) was used for the analysis. The mobile phase consisted of 1% acetic acid in water (A) and 1% acetic acid in methanol (B). The metabolites were eluted isocratically with 29% B and 71% A. The flow rate was 1 ml min⁻¹. The metabolism of coumarin was linear with time and protein for all CYP enzymes. For the determination of kinetic parameters, CYP2A4 (18.4 pmol) was incubated with 0.5–20 μ M ¹⁴C-coumarin as described above.

Coumarin 7-hydroxylation by CYP2A13

CYP2A13 (4.15 pmol) was incubated with 0.1–20 μ M coumarin (non-radioactive) for 10 min at 37°C as described above. The 7-hydroxycoumarin formed was analysed by HPLC with fluorescence detection (excitation wavelength 350 nm, emission wavelength 453 nm) as described by von Weymarn *et al.* (1999). Quantification was obtained by comparison to a standard curve of 7-hydroxycoumarin versus peak area.

Kinetics and statistical analysis

K_m and V_{max} for coumarin metabolism by CYP2A13 and 2A4 were determined using the EZ-Fit 5 kinetics program from Perrella Scientific (Amherst, NH, USA). It uses a non-linear regression method of curve fitting and the Runs test of residuals to determine statistically whether experimental data are randomly distributed around the curve with 95% confidence (Perrella 1988).

Results and discussion

The metabolism of ¹⁴C-coumarin by CYP2A4, CYP2A5, CYP2A6 and CYP2A13 was analysed by reverse-phase HPLC with radioflow detection (figures 2 and 4). The use of coumarin labelled with ¹⁴C in the benzene ring allows the detection of other metabolites, as well as 7-hydroxycoumarin, and we previously developed an HPLC system to separate most coumarin metabolites (von Weymarn and Murphy 2001). The major metabolite of CYP2A6-catalysed coumarin metabolism eluted at 20.3 min (figure 2A), namely 7-hydroxycoumarin. As expected, this is the only metabolite detected (table 1) (Miles *et al.* 1990, Yamano *et al.* 1990). However, in contrast to CYP2A6, several radioactive metabolites of CYP2A13-catalysed coumarin metabolism were detected (figure 2B). The major metabolite was 7-hydroxycoumarin (20.3 min), accounting for 43% of the total metabolism (table 1). However, a product of 3,4-epoxidation, *o*-HPA, (14.2 min), was also formed at a significant rate accounting for 30% of the total metabolism. Two hydroxylated coumarins, 6-hydroxy (6-OH, 15.6 min) and 8-hydroxycoumarin (8-OH, 16.4 min), were also detected. The formation of all of these products was linear with enzyme and time under the conditions studied (data not shown). The early eluting peak (5 min) was present in control samples and dependent on the presence of Sf9 microsomes (figure 2C). The metabolism of coumarin by CYP2A13 is clearly not as specific as metabolism by CYP2A6 despite the 94% sequence homology between the two enzymes.

The kinetic parameters for 7-hydroxycoumarin formation by CYP2A13 were determined using reverse-phase HPLC with fluorescence detection. The formation of 7-hydroxycoumarin followed simple Michaelis–Menten kinetics (figure 3). The K_m for coumarin 7-hydroxylation was $0.48 \pm 0.07 \mu$ M, the V_{max} 0.15 ± 0.006 nmol min⁻¹ nmol⁻¹ CYP. This K_m is similar to that of BV-expressed CYP2A5 (1.2 μ M; Felicia *et al.* 2000) and about 10-fold lower than that of BV-expressed CYP2A6 (6 μ M; Zhuo *et al.* 1999). The V_{max} for CYP2A13-catalysed

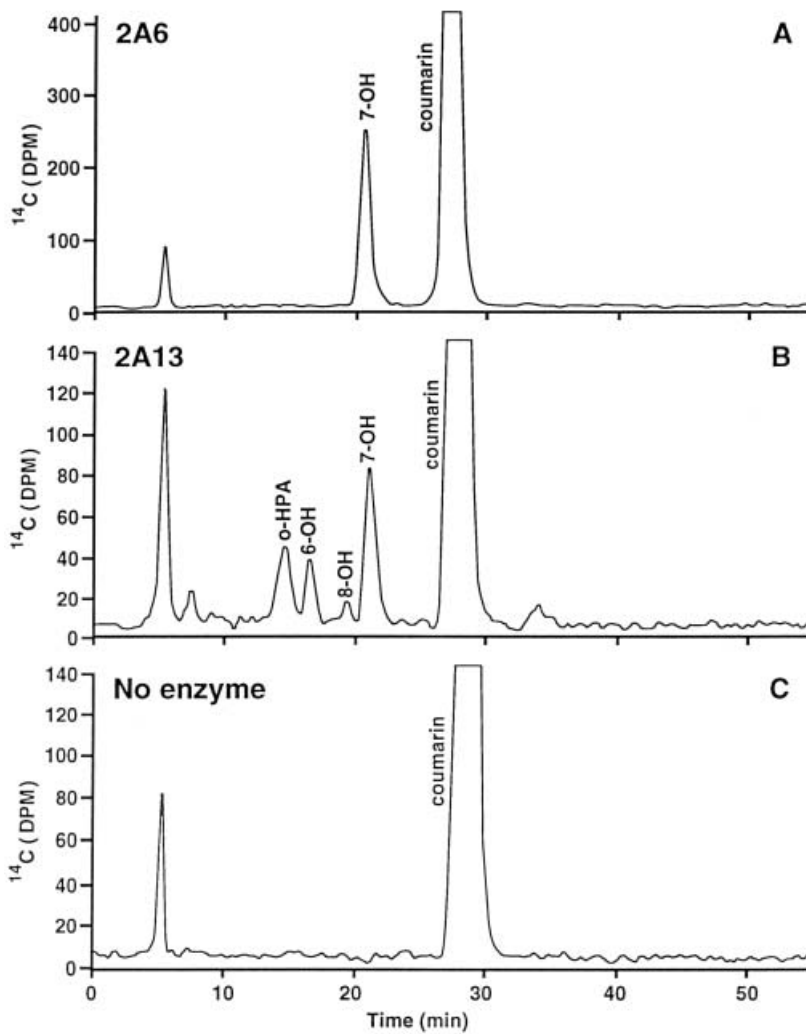


Figure 2. Radioflow HPLC analysis of coumarin metabolites. ^{14}C -coumarin ($5\ \mu\text{M}$) was incubated for 30 min with 1.8 pmol CYP2A6 (A), 7.2 pmol CYP2A13 (B) or without enzyme (C) as described in the Materials and methods. The products of the reaction were co-injected with standards on a C_{18} HPLC column.

Table 1. Relative metabolism of coumarin by two major pathways.^a

	Metabolites (% of total) ^b			
	Mouse		Human	
	CYP2A4	CYP2A5	CYP2A6	CYP2A13
7-Hydroxylation	0	79	100	43
3,4-Epoxidation ^c	98	7	0	30

^a CYP2A4 (33.9 pmol), CYP2A5 (1.8 pmol), CYP2A6 (1.8 pmol) or CYP2A13 (7.2 pmol) were incubated for 30 min with $5\ \mu\text{M}$ coumarin as described in the Materials and methods. The products were analysed by radioflow HPLC.

^b Averages of duplicate determinations.

^c Values determined for the 3,4-epoxide pathway are the sum of *o*-HPA and *o*-HPAA.

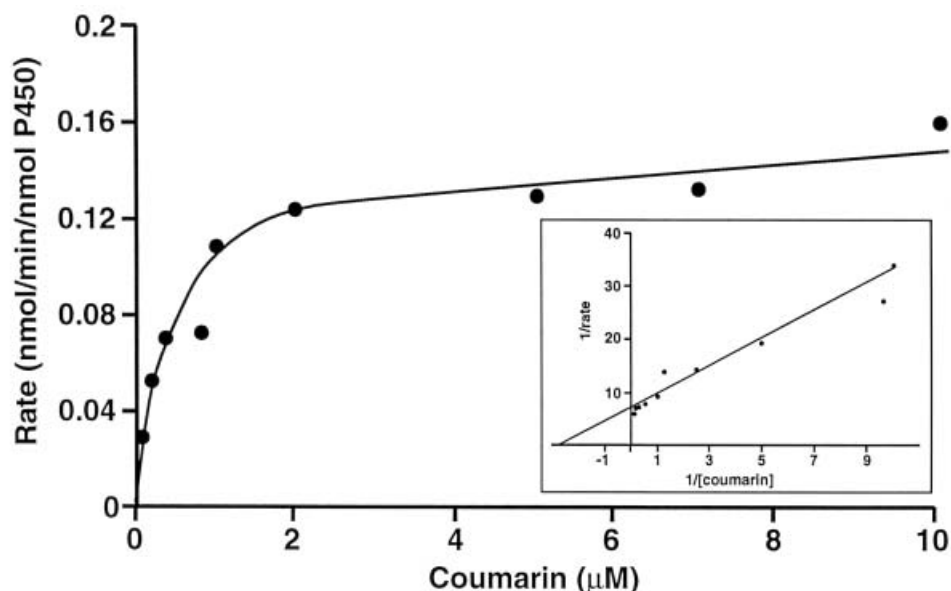


Figure 3. Kinetic analysis of 7-hydroxycoumarin formation by CYP2A13. Coumarin (0.5–10 μM) was incubated for 10 min with 4.15 pmol CYP2A13 as described in the Materials and methods. The formation of 7-hydroxycoumarin was determined by HPLC analysis with fluorescence detection. Curves were graphed by non-linear regression kinetic parameters determined using EZ-Fit 5. The insert is a Lineweaver-Burk plot of the kinetic analysis. The data points are the average of two sets of duplicate determinations.

coumarin 7-hydroxylation is about 16-fold less than that of CYP2A5 and CYP2A6 (Zhuo *et al.* 1999, Felicia *et al.* 2000).

Coumarin metabolism by CYP2A4 and 2A5, two mouse orthologues of CYP2A6, was next investigated using ^{14}C -coumarin (figure 4). The major metabolites of CYP2A4-mediated coumarin metabolism were *o*-HPA (13.5 min, figure 4B) and *o*-HPAA (12.1 min), products of the coumarin 3,4-epoxide pathway. 7-Hydroxycoumarin was not detected (limit of detection < 0.1 pmol) (figure 4A and table 1). The formation of products from the 3,4-epoxide pathway accounted for 98% of the coumarin metabolism by CYP2A4 (table 1). An unknown metabolite, eluting at 7.5 min, accounted for the remaining 2%. The radioactive peak eluting at 5 min, as noted above, is dependent on the presence Sf9 membranes. This peak is greater in the CYP2A4 than the CYP2A5 sample illustrated in figure 4 since more CYP2A4 was used, and therefore more Sf9 membrane proteins are present. The kinetic parameters for the 3,4-epoxidation of coumarin by CYP2A4 were determined. The K_m was $8.7 \pm 3.6 \mu\text{M}$, the $V_{\max}$ $0.20 \pm 0.04 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ CYP}$. The product distribution did not change with coumarin concentration.

The major product of CYP2A5-catalysed coumarin metabolism detected was 7-hydroxycoumarin (figure 4B), consistent with the previous characterizations of 2A5 as a coumarin 7-hydroxylase (Negishi *et al.* 1989, Felicia *et al.* 2000). Small amounts of three other metabolites, *o*-HPA, 6-hydroxy and 8-hydroxy coumarin, were also detected (figure 4B). Compared with the kinetic parameters of coumarin 7-hydroxylation by CYP2A5, CYP2A4-catalysed coumarin 3,4-epoxidation has a ninefold higher K_m and a 40-fold lower $V_{\max}$ (Felicia *et al.* 2000). Relative to

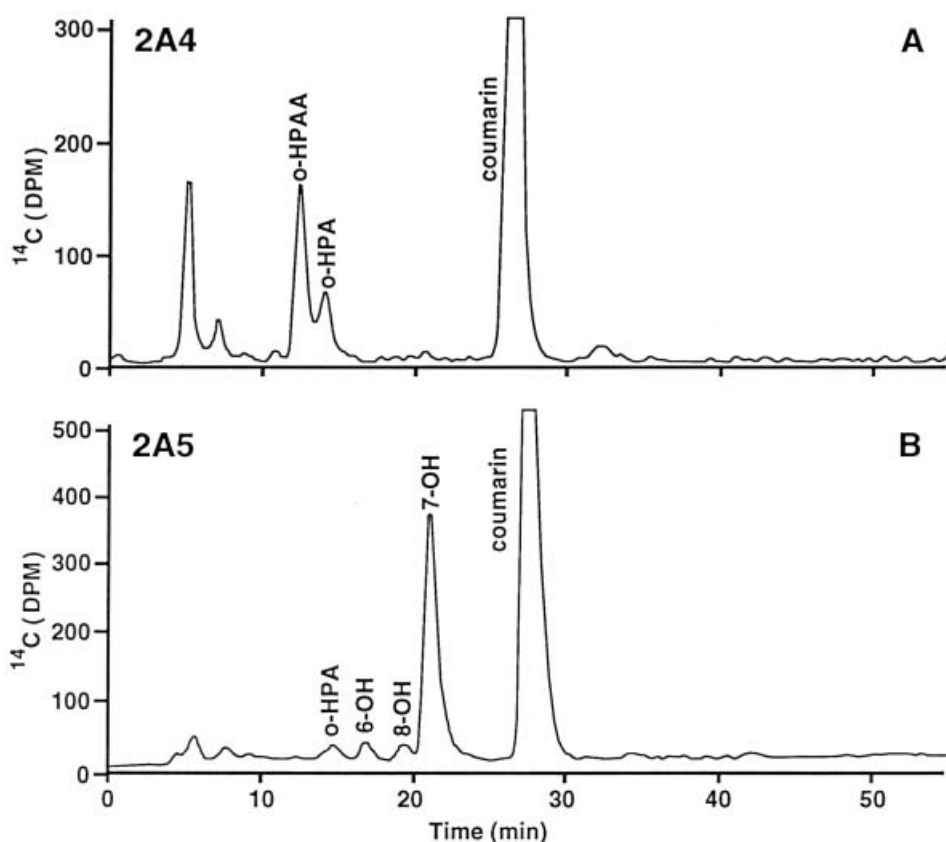


Figure 4. Radioflow HPLC analysis of coumarin metabolites. ^{14}C -coumarin ($5\text{ }\mu\text{M}$) was incubated for 30 min with 33.9 pmol CYP2A4 (A) or 1.8 pmol CYP2A5 (B) as described in the Materials and methods. The products of the reaction were co-injected with standards on a C_{18} HPLC column.

CYP2A5-catalysed coumarin 7-hydroxylation, CYP2A4 is not a very efficient catalyst of coumarin 3,4-epoxidation. This is also true compared to the rates of coumarin 3,4-epoxidation by the rat and human P450s CYP1A1, CYP1A2 and CYP2E2 (Born *et al.* 2002). Recombinant human CYP1A1 and rat CYP1A2 catalyse coumarin 3,4-epoxidation at a rate of 6.26 and $22.2\text{ nmol min}^{-1}\text{ nmol}^{-1}$ CYP, respectively (Born *et al.* 2002). These rates are 31- and 111-fold higher than the rate of CYP2A4-mediated coumarin 3,4-epoxidation.

Coumarin 3,4-epoxide is reactive and could bind to cellular protein. We determined here that 3,4-epoxidation is the major pathway of coumarin metabolism catalysed by CYP2A4. Negishi *et al.* (1989) reported that even though CYP2A4 did not metabolize coumarin by 7-hydroxylation, coumarin did inhibit CYP2A4-mediated testosterone 15α -hydroxylation. Both enzyme modification by coumarin 3,4-epoxide and competitive inhibition may account for the inhibition observed by Negishi *et al.* (1989).

The striking difference in the efficiency of coumarin 7-hydroxylation by CYP2A4 and CYP2A5 has been extensively studied by Honkakoski and Negishi (1997), Lindberg and Negishi (1989) and Negishi *et al.* (1989). By using site-directed mutagenesis, they changed three amino acids in CYP2A4 to their

counterparts in CYP2A5. The resulting enzyme had coumarin 7-hydroxylase activity of equal efficiency to that of CYP2A5 (Lindberg and Negishi 1989). The mutated amino acids were 117, 209 and 365. These three amino acids have been indicated to play a role in coumarin metabolism by several CYP2A enzymes (Honkakoski and Negishi 1997). The CYP2A enzymes capable of catalysing the 7-hydroxylation of coumarin all have a phenylalanine at position 209. CYP2A4, which is not a coumarin 7-hydroxylase, has a leucine at position 209. CYP2A13 and CYP2A4 both have an alanine at position 117 compared with valine in 2A5 and 2A6, which may contribute to the low coumarin metabolism observed with 2A4 and 2A13. With respect to amino acids 117, 209 and 365, CYP2A13 is identical to rat CYP2A3. CYP2A3 is an efficient coumarin 7-hydroxylase (von Weymarn *et al.* 1999). However, CYP2A3, like CYP2A13, also catalyses the 3,4-epoxidation of coumarin.

A number of CYP2A enzymes in different species have sequence homology > 85% (Honkakoski and Negishi 1997), however their substrate specificities differ significantly. This is clearly illustrated by the differences observed in coumarin and NNK metabolism by CYP2A4, CYP2A5, CYP2A6 and CYP2A13. CYP2A6 and CYP2A13 are 94% homologous, they differ by only 32 amino acids (Su *et al.* 2000). We report here that compared with CYP2A6, CYP2A13 is not a specific coumarin 7-hydroxylase. Su *et al.* (2000) reported that CYP2A13 efficiently metabolizes NNK, however 2A6 is significantly less efficient at catalysing NNK metabolism (Patten *et al.* 1996). A different relationship exists between the closely related CYP2A enzymes in the mouse. CYP2A4 is not a catalyst of coumarin 7-hydroxylation and is a poor catalyst of NNK hydroxylation while CYP2A5 is a very efficient catalyst of both reactions.

While amino acid residues 117, 309 and 365 are clearly important in determining the substrate specificity of CYPs 2A4 and 2A5, other amino acids must play a role in CYP2A13 and other CYP2A enzymes. Complete characterization of coumarin metabolism by CYP2A enzymes should help to identify amino acid residues important in defining the active site of these enzymes. This information, in turn, should contribute to our understanding of the role specific amino acids play in determining the specificity of nitrosamine metabolism by these enzymes.

Acknowledgements

The authors thank Xinxin Ding for providing CYP2A13. The study was supported by Grant CA-74913 from the National Cancer Institute.

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Sulphoxidation of ethyl methyl sulphide, 4-chlorophenyl methyl sulphide and diphenyl sulphide by purified pig liver flavin-containing monooxygenase

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Received 19 June 2002

1. The biotransformation of ethyl methyl sulphide (EMS), 4-chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) to their corresponding sulfoxides by purified flavin-containing monooxygenase (FMO) is described.

2. Purified pig liver flavin-containing monooxygenase catalysed the sulfoxidation of EMS, CPMS and DPS to their corresponding sulfoxides and the reactions followed single enzyme Michaelis–Menten kinetics.

3. The apparent K_m and V_{max} for the sulfoxidation of EMS were 1.38 ± 0.05 mM and 78.74 ± 3.9 nmoles mg^{-1} protein min^{-1} , respectively. The apparent K_m and V_{max} for the sulfoxidation of CPMS were 0.185 ± 0.03 mM and 103 ± 5.0 nmoles mg^{-1} protein min^{-1} , respectively. The apparent K_m and V_{max} for the sulfoxidation of DPS were 0.068 ± 0.002 mM and 49.26 ± 2.05 nmoles mg^{-1} protein min^{-1} , respectively.

4. A significant reduction of the sulfoxidation of these simple sulphides was observed with addition of 1-naphthylthiourea in the incubation medium. On the other hand, incorporation of catalase and superoxide dismutase into the incubation media produced no appreciable inhibition of the observed sulfoxidation of the sulphides.

5. These results suggest that FMO is responsible, at least in part, for the sulfoxidation of nucleophilic sulphides as well as for the oxidation of sulphur atoms that reside within or adjacent to aromatic systems.

Introduction

Sulphide and sulfoxide functionalities are frequently encountered in agricultural chemicals and medicinal agents and may determine or influence the biological fate of the parent molecule (Ziegler 1980). Sulphides are readily converted to sulfoxides *in vitro* and *in vivo* due to the readily accessible lone pair of electrons on the divalent sulphur atom (Ziegler 1990). The psychoactive phenothiazine drugs, for example, are metabolized to their corresponding sulfoxides in various animal species and in man (Traficante *et al.* 1979). Sulfoxides, on the other hand, may be oxidized to the corresponding sulphones or reduced to sulphides. The sulfoxide drug, sulphinpyrazone, undergoes both reduction to the corresponding sulphide and oxidation to the sulphone after parenteral administration in rats (Renwick 1982).

The mammalian flavin-containing monooxygenase (FMO) is a family of enzymes mainly distributed in the liver, lung and kidney of mammals (Ziegler

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1988). FMOs exhibit broad substrate specificity for catalysing sulphur and nitrogen functional groups, which are common in therapeutic drugs (Ziegler 1990). Additionally, FMO is a major contributor to the metabolism of agricultural chemicals and may be responsible for producing biologically active metabolites (Ziegler 1988). To date, five distinct members of this protein family, designated FMO1–5, have been identified in mammals including humans. FMO1, the dominant form of FMO in pig liver, has been reported to S-oxygenate a number of nucleophilic sulfur-containing substrates (Lawton *et al.* 1994). There is a considerable amount of information available on the pig liver FMO1 compared with the human form. However, the substrate specificity of human FMO1 appears to be considerably more restricted than that of the pig FMO1. Therefore, differences in the types of nucleophiles accepted must be considered in attempting to extrapolate the extensive structure–activity studies available for the pig FMO1 to the human FMO1 (Kim and Ziegler 2000). Interestingly, the specific role of the FMOs in oxidative metabolism of xenobiotics in mammals is not well defined due to lack of specific and selective inhibitors for the FMO forms.

Ethyl methyl sulphide (EMS), 4-chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) (figure 1) are simple sulphides that are encountered in petroleum distillates and in the environment. These compounds are also intermediates in the manufacture of other xenobiotics. EMS, CPMS and DPS are biotransformed to their corresponding sulfoxides *in vitro* and *in vivo* (Nnane and Damani 1999, 2002). The cytochrome P450 or the FMO enzyme systems are

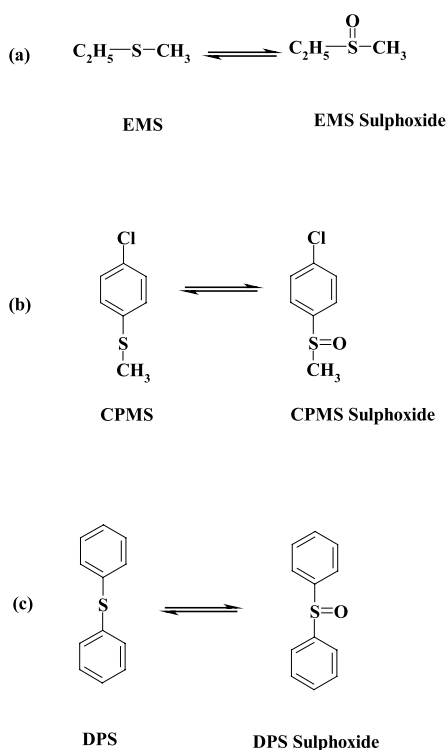


Figure 1. Sulphoxidation of (a) ethyl methyl sulphide (EMS), (b) 4-chlorophenyl methyl sulphide (CPMS) and (c) diphenyl sulphide (DPS) to their corresponding sulfoxides.

thought to mediate these S-oxidation reactions depending on the nucleophilicity of the substrates (Poulsen and Ziegler 1995). It has been reported that FMO is responsible for the sulphoxidation of the more nucleophilic aliphatic sulphides to their corresponding sulphoxides while both cytochrome P450 and the FMO are involved in the oxidation of sulphur atoms that reside within or adjacent to aromatic or heterocyclic ring systems (Hoodi and Damani 1984, Nnane and Damani 2002). Previous studies have also reported that the metabolism of a simple dialkyl sulphide such as EMS to its sulphoxide is predominantly mediated by FMO whereas sulphoxidation of an alkylaryl sulphide such as CPMS is mediated by both cytochrome P450 and the FMO in rat liver microsomes (Nnane and Damani 1999, 2002). These studies also indicate that the sulphoxidation of DPS to its sulphoxide is mediated almost exclusively by cytochrome P450 in rat liver microsomes. The aim of the present study was to determine the specific role of purified pig liver FMO in the sulphoxidation of these simple sulphides to their corresponding sulphoxide metabolites. The information obtained on the sulphoxidation of these simple sulphides by the pig liver FMO may be extrapolated to S-oxidation reactions involving more complex xenobiotics bearing the thioether functionality.

Materials and methods

Chemicals

Ethyl methyl sulphide (98%), 4-chlorophenyl methyl sulphide (98%), diphenyl sulphide (98%), *n*-propyl sulphone, 1-naphthylthiourea, 4-chlorophenyl phenyl sulphone, nicotinamide adenine dinucleotide (NADP^+), glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD) superoxide dismutase and catalase (11 000 units mg^{-1} protein) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The sulphoxides of EMS, CPMS and DPS were synthesized in our laboratory by methods described by Madesclaire (1986). The purity of each sulphoxide was >98%. All other reagents and organic solvents of HPLC or analytical grades were purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified pig liver FMO, which gave an electrophoretically single protein band, was kindly supplied by Dr D. M. Ziegler of the University of Texas, Austin.

Incubation procedures

Purified pig liver FMO was reconstituted in phosphate buffer (2 mM, pH 7.4) at a protein concentration of 4 mg ml^{-1} . Incubations were carried out in duplicate with the reconstituted enzyme in 25 ml Erlenmeyer flasks at 37°C using a shaking water bath in three separate occasions ($n = 3$). A typical incubation mixture consisted of a cofactor solution (0.5 ml), reconstituted enzyme (1 ml, 0.1 mg ml^{-1} protein) and substrate (20 μl) in acetone plus 0.480 μl distilled water (0–5 mM) in a total volume of 2 ml. The cofactor solution consisted of NADP^+ (1 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 unit) and magnesium chloride (10 mM, 50% w/w aqueous solution) in sodium phosphate buffer (0.2 M, pH 7.4). Metabolism was initiated by the addition of the reconstituted enzyme and terminated by adding NaOH (0.5 ml, 0.1 M) to the incubation mixture. The purified enzyme was omitted in control incubations. In another series of incubation flasks, EMS, CPMS and DPS were incubated with the purified enzyme and a modified cofactor solution containing superoxide dismutase (1000 units/flask) and catalase (11 000 units mg^{-1} protein). The metabolic inhibition reactions were carried out at 37°C in a shaking water bath using the optimum incubation time of 15 min for EMS, CPMS and DPS. A saturating substrate concentration of 2.5, 0.5 and 0.25 mM for EMS, CPMS and DPS, respectively, was used routinely for the metabolic inhibition studies. The biologically formed sulphoxides were extracted and measured by chromatographic assay methods.

Sample preparation and construction of calibration curves

The levels of the sulphoxide of EMS in the incubation mixture were monitored by a gas chromatographic assay method. To the incubation samples, sodium hydroxide (50 μl , 0.1 M), the internal standard, *n*-propyl sulphone (10 μl , 1 mg ml^{-1}) and sodium chloride (0.1 g) were added. The samples were then extracted with dichloromethane (2 $\times$ 5 ml). The pooled extracts were evaporated to dryness, reconstituted in 20 μl methanol and aliquots (5 μl) of the reconstituted extracts injected onto the

GLC column. The calibration curves for quantitation of EMSO were constructed by spiking varying amounts ($0\text{--}50\text{ }\mu\text{g ml}^{-1}$) into extraction tubes containing inactivated enzyme preparation, EMS (2.5 mM), cofactors and the internal standard, *n*-propyl sulphone ($10\text{ }\mu\text{l}$, 1 mg ml^{-1}), in a total volume of 2 ml of incubation mixture.

The calibration curves for quantitation of CPMSO were constructed by spiking varying amounts ($0\text{--}50\text{ }\mu\text{g ml}^{-1}$) into extraction tubes containing inactivated enzyme preparation (1 ml), CPMS (0.5 mM), cofactors and the internal standard, CPPSO₂ ($20\text{ }\mu\text{l}$, 1 mg ml^{-1}), in a total volume of 2 ml incubation mixture. The analytes were extracted with diethyl ether ($2 \times 5\text{ ml}$) using a mechanical bench test tube shaker for 10 min followed by centrifugation at 400g for 10 min. The organic layers were evaporated to dryness in a water bath at 40°C. The extracts were reconstituted in a small volume ($50\text{ }\mu\text{l}$) of methanol and an aliquot ($10\text{ }\mu\text{l}$) of the concentrate was injected onto the HPLC column. The ratios of the peak area of the sulphoxide divided by the peak area of the internal standard were plotted against actual concentrations of the sulphoxide in the mixture. Similarly, the calibration curves for DPSO were constructed by spiking varying amounts ($0\text{--}50\text{ }\mu\text{g ml}^{-1}$) into extraction tubes containing inactivated enzyme preparation (1 ml), DPS (0.5 mM), cofactors and the internal standard, CPPSO₂ ($20\text{ }\mu\text{l}$, 1 mg ml^{-1}), in a total volume of 2 ml incubate. The mixtures were extracted with diethyl ether ($2 \times 5\text{ ml}$) using a mechanical bench test tube shaker for 10 min followed by centrifugation at 400g for 10 min. After HPLC analysis, the peak areas for DPSO and the internal standard were measured for the construction of calibration curves as described for CPMSO above.

GC method

Gas chromatography was performed on an HP 5840A gas chromatograph (Hewlett-Packard Co., Avondale, PA, USA), equipped with a flame ionization detector and a glass column ($200 \times 0.4\text{ mm}$). Separation of EMSO and *n*-propyl sulphone was achieved on a glass column packed with 10% Carbowax 20 M on Chromosorb W (Hewlett-Packard). The optimum working conditions for analysis of EMSO and *n*-propyl sulphone were: nitrogen flow rate, 30 ml min^{-1} , injection port temperature, 200°C, column temperature, 170°C; and detector temperature, 250°C.

HPLC method

The high-performance liquid chromatographic (HPLC) system used consisted of an hp[®] HPLC system (series 1050) equipped with a multichannel solvent delivery system coupled to an autosampler and a photodiode array detection system operated at 260 nm (Hewlett Packard). Chromatographic separation and quantitation of CPMS, DPS and their corresponding sulphoxides was achieved using a reversed-phase Spherisorb[®] 5ODS column ($15 \times 0.46\text{ cm}$) obtained from HPLC technology (Macclesfield, UK). The analytical column was protected by a guard column packed with pellicular ODS packing material. The mobile phase was composed of water and tetrahydrofuran (THF) (45:55 v/v) at a flow rate of 0.5 ml min^{-1} for the study of CPMS sulphoxidation. For DPS sulphoxidation studies, the mobile phase composition was changed to THF/water (50:50 v/v) at a flow rate of 0.5 ml min^{-1} .

Data analysis

Least-squares regression analysis on SigmaPlot[®] for Windows[®] (SPSS Science Chicago, IL, USA) was used to evaluate calibration curves and the enzyme kinetics. The Lineweaver–Burk transformation of the Michaelis–Menten rate equation was used to evaluate the enzyme kinetics in this study:

$$\frac{1}{V} = \left(\frac{K_m}{V_{\max}} \right) \frac{1}{[S]} + \frac{1}{V_{\max}},$$

where $[S]$ is the concentration of the substrate, V is the rate of sulphoxide formation, $V_{\max}$ is the maximum rate of sulphoxide formation and K_m is the substrate concentration when one-half of the maximum rate of sulphoxide formation is attained. K_m , $V_{\max}$ and K_i were calculated by least-squares fit to the kinetic equations. One-way analysis of variance (ANOVA) on SigmaStat[®] for Windows was used to obtain probabilities (p) and compare different treatment groups. The Bonferroni *post-hoc* test was used for the determination of the level of significance. $p < 0.05$ was considered as statistically significant.

Result and discussion

EMSO ($R_t = 3.9\text{ min}$) and the internal standard, *n*-propyl sulphone ($R_t = 7.5\text{ min}$) were separated satisfactorily by using the 10% Carbowax 20 M on Chromosorb W column and the calibration curve for EMSO was linear and reproducible over the range examined ($r \geq 0.997$). The inter- and intraday vari-

ation at the lower concentrations was $<10\%$, and $<5\%$ at the higher concentrations.

Satisfactory separation of CPMSO ($R_t = 4.8$ min) and the internal standard, CPPSO₂ ($R_t = 9.6$ min), was achieved by using the reversed-phase HPLC conditions described. Similarly, satisfactory separation of DPSO ($R_t = 5.6$ min) and the internal standard, CPPSO₂ ($R_t = 10.2$ min), was achieved by using the reversed-phase HPLC conditions described. The calibration curves for measuring the sulfoxide of CPMS or DPS were linear ($r \geq 0.995$) in the range $0\text{--}50\text{ }\mu\text{g ml}^{-1}$ and the assay methods were reproducible with low inter- and intra-assay variation of $<13.5\%$. The assay methods were simple, fairly rapid and allowed accurate estimation of enzymically formed sulfoxides of CPMS and DPS *in vitro*.

The purified pig liver FMO catalysed the sulphoxidation of the model sulphides, EMS, CPMS and DPS to their corresponding sulfoxides under the incubation conditions used. The sulphoxidation of EMS, CPMS and DPS increased linearly with respect to time ($0\text{--}30$ min) and protein concentration ($0.02\text{--}0.2\text{ mg ml}^{-1}$) and followed a typical Michaelis–Menten profile with respect to substrate concentration ($0\text{--}5$ mM). A Lineweaver–Burk plot of EMS sulphoxidation was linear (figure 2). The apparent K_m and V_{max} for the sulphoxidation of EMS were 1.38 ± 0.05 mM and 78.74 ± 3.9 nmoles mg^{-1} protein min^{-1} , respectively. Similarly, Lineweaver–Burk plots of CPMS and DPS sulphoxidation were linear (figures 3 and 4). The estimated apparent K_m and V_{max} for the sulphoxidation of CPMS were 0.185 ± 0.03 mM and 103 ± 5.0 nmoles mg^{-1} protein min^{-1} , respectively. The estimated apparent K_m and V_{max} for the sulphoxidation of DPS were 0.068 ± 0.002 mM and 49.26 ± 2.05 nmoles mg^{-1} protein min^{-1} , respectively.

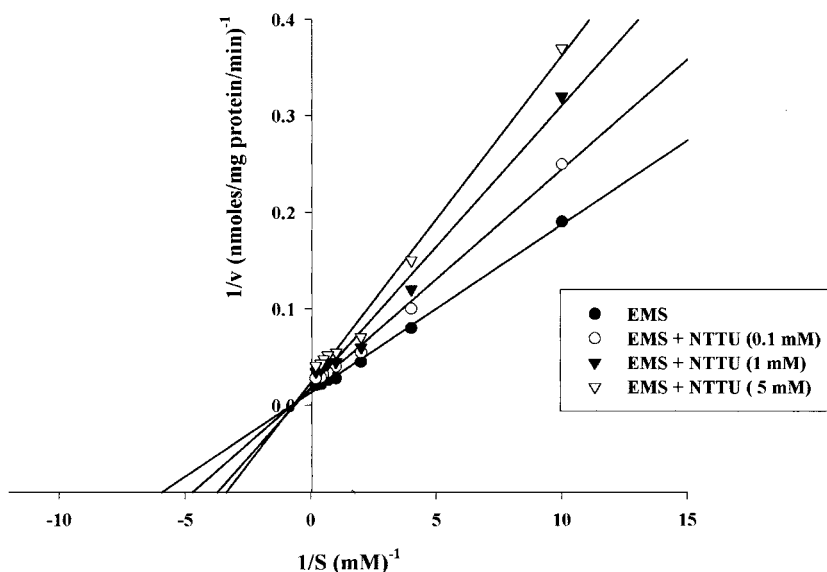


Figure 2. Lineweaver–Burk plots of ethyl methyl sulphide (EMS) sulphoxidation and the inhibitory effects of 1-naphthylthiourea (NTTU) on EMS sulphoxidation by purified pig liver flavin-containing monooxygenase. The incubation conditions were protein content = 0.1 mg ml^{-1} ; incubation time = 15 min and EMS concentrations = $0.1\text{--}5$ mM. Inhibitor concentrations were 0.1, 1 and 5 mM. Values are the means of three separate experiments ($n = 3$). The spread was 5–10% of the means.

A significant ($p < 0.05$) and concentration-dependent reduction of the sulphoxidation of these simple sulphides was observed with the addition of 1-naphthylthiourea, a known inhibitor of FMO, in the incubation medium (figures 2–4). The results indicate that 1-naphthylthiourea competitively inhibited the sulphoxidation of EMS by purified pig liver FMO with a $K_i = 8.49 \pm 0.51$ mM (figure 2). Analysis of the sulphoxidation of CPMS and DPS by purified pig liver FMO in the presence of 1-naphthylthiourea suggest that the inhibitor non-competitively inhibited the sulphoxidation of CPMS and DPS with K_i of 10.95 ± 0.78 and 6.8 ± 0.34 mM, respectively (figures 3 and 4). On the other hand, incorporation of catalase and superoxide dismutase into the incubation media produced no appreciable inhibition ($p > 0.05$) of the observed sulphoxidation of these simple sulphides (table 1). Non-enzymatic oxidation of the sulphides was negligible since the incorporation of catalase and superoxide dismutase into the incubation media produced no statistically significant inhibition of the observed sulphoxidation of the compounds. If reactive oxygen species played a significant role in the oxidation of these substrates then the inclusion of catalase and superoxide dismutase into the incubation medium would have markedly inhibited the metabolic reactions.

Previous studies have reported that the sulphoxidation of EMS by rat liver microsomes is mediated predominantly by the flavin-containing monooxygenase (Nnane and Damani 1999). Our present findings with purified pig liver FMO support the contention that FMO is responsible for the sulphoxidation of the more nucleophilic aliphatic sulphide, such as EMS where the sulphur atom resides adjacent to alkyl groups, to its corresponding sulphoxide. In a separate report, it

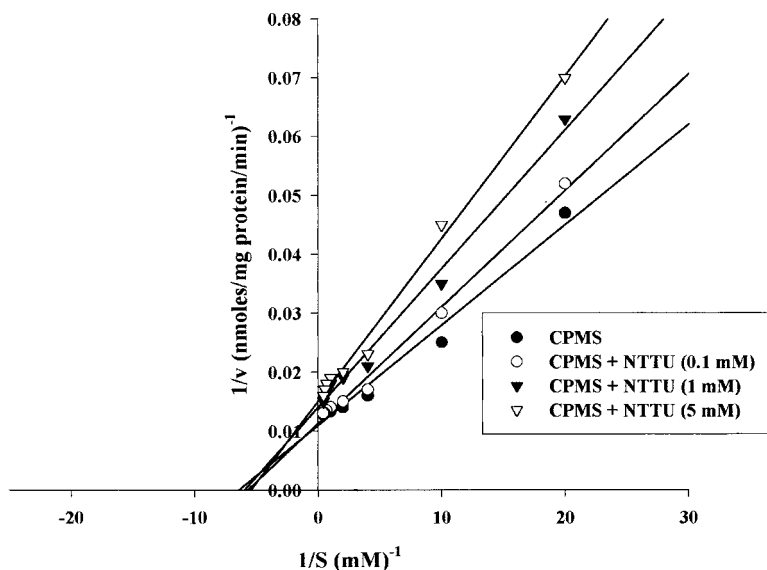


Figure 3. Lineweaver–Burk plots of 4-chlorophenyl methyl sulphide (CPMS) sulphoxidation and the inhibitory effects of 1-naphthylthiourea (NTTU) on the sulphoxidation of CPMS by purified pig liver flavin-containing monooxygenase. The incubation conditions were protein content = 0.1 mg ml^{-1} ; incubation time = 15 min and CPMS concentrations = $0.05\text{--}2.5 \text{ mM}$. Inhibitor concentrations were 0.1, 1 and 5 mM. Values are the means of three separate experiments ($n = 3$). The spread was 5–10% of the means.

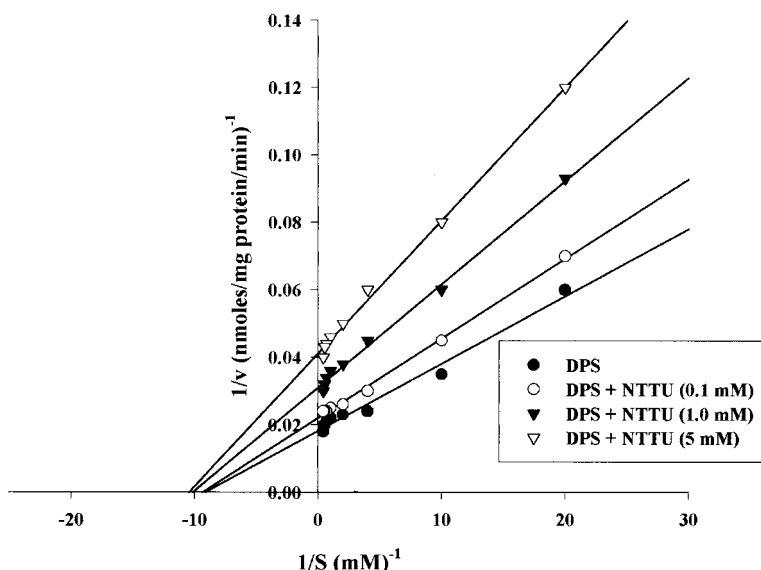


Figure 4. Lineweaver-Burk plots of the sulphoxidation of diphenyl sulphide (DPS) and the inhibitory effects of 1-naphthylthiourea (NTTU) on the sulphoxidation of DPS by purified pig liver flavin-containing monooxygenase. The incubation conditions were protein content = 0.1 mg ml^{-1} ; incubation time = 15 min and DPS concentrations = $0.05\text{--}0.25 \text{ mM}$. Inhibitor concentrations were 0.1, 1 and 5 mM. Values are the means of three separate experiments ($n=3$). The spread was 5–10% of the means

Table 1. Effects of catalase and superoxide dismutase on the sulphoxidation of ethyl methyl sulphide (EMS), 4-chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) by purified pig liver flavin-containing monooxygenase.

Substrate	Enzyme activity ($\text{nmoles mg}^{-1} \text{ protein min}^{-1}$)	
	Purified FMO	Purified FMO + catalase/superoxide dismutase
EMS	44.5 ± 4.08	$40.95 \pm 3.28^*$
CPMS	73.88 ± 5.20	$67.97 \pm 5.44^*$
DPS	41.77 ± 2.10	$40.12 \pm 2.40^*$

Incubation conditions were protein content = 0.1 mg ml^{-1} ; incubation time = 15 min. Substrate concentrations were 2.5 mM for EMS, 0.5 and 0.25 mM for DPS metabolism studies.

* $p > 0.05$. Data are the means ($\pm$ SD) from three separate experiments ($n=3$).

was observed that the sulphoxidation of CPMS is mediated by both cytochrome P450 and FMO in rat liver microsomes (Nnane and Damani 2001). This finding was in agreement with another study where it was reported that the sulphoxidation of a simple alkylaryl sulphide was mediated by both cytochrome P450 and the flavin-containing monooxygenase (Waxman *et al.* 1982). Thus, the findings in the present study with purified pig liver FMO demonstrate that FMO has a role in the sulphoxidation of sulphides of intermediate nucleophilicity such as CPMS, where the sulphur atom resides adjacent to both alkyl and aryl groups.

The formation of the sulfoxide of DPS was also dependent on the presence of the purified pig liver FMO. The effects of catalase and superoxide dismutase on the S-oxidation of DPS by pig liver FMO was evaluated to rule out the participation of reactive oxygen species in this metabolic reaction. The sulfoxidation of DPS by pig liver FMO was not significantly ($p > 0.05$) affected by the addition of catalase or superoxide dismutase in the incubation medium (table 1). These results indicate that reactive oxygen species were not involved in the oxidation of DPS. On the other hand, addition of with 1-naphthylthiourea, an inhibitor of FMO, in the incubation mixture caused a significant decrease ($p < 0.05$) in DPS sulfoxidation in a concentration-dependent manner (figure 4). These results suggest that DPS is catalysed to its corresponding sulfoxide by purified pig liver FMO. Previous studies have indicated that the S-oxidation of simple diaryl sulphides was mediated mainly by cytochrome P450 monooxygenase (Hoodi and Damani 1984, Nnane and Damani 2002). In contrast, the present study clearly demonstrates a significant role for the FMO in the sulfoxidation of a diaryl sulphide such as DPS. Therefore, it would appear that the FMO contributes to the sulfoxidation of less nucleophilic thioethers such as DPS, where the sulphur atom resides adjacent to aromatic groups, in addition to its established role in the S-oxidation of nucleophilic compounds bearing the sulphur atom.

In conclusion, our studies demonstrate the capacity of purified pig liver FMO to catalyse the oxidation of both nucleophilic substrates such as EMS and substrates of intermediate nucleophilicity such as CPMS and also contributes to the S-oxidation of electrophilic substrates such as DPS. These simple sulphides may be used as probes for assessing the relative contribution of the microsomal monooxygenases to sulfoxidation reactions and the information generated on the sulfoxidation of these simple sulphides by the pig liver FMO may be used to predict the S-oxidation of more complex xenobiotics bearing the thioether functional groups in humans.

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Oxidative activation of acylguanidine prodrugs: intestinal presystemic activation in rats limits absorption and can be inhibited by co-administration of ketoconazole

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Received 17 April 2002

1. The disposition of acyl prodrugs was studied to improve the delivery of a guanidine-containing parent compound with poor membrane permeability and poor absorption.

2. The prodrugs were evaluated *in vitro* and *in vivo* for conversion to drug. Prodrugs were evaluated for hydrolytic or oxidative bioactivation in intestinal homogenate and rat liver S9 or microsomes. The disposition of the prodrugs *in vivo* was monitored in bile duct-cannulated rats.

3. Compounds with *n*-alkylacyl groups were efficiently bioactivated, but were hydrolysed before absorption.

4. Hydrolytic bioactivation could be blocked *in vitro* by branching in the alkyl chain. These compounds showed modest improvements in absorption, despite favourable permeability. Experiments with liver microsomes demonstrated efficient NADPH-dependent oxidative bioactivation, which was proposed to occur through a CYP-mediated side chain oxidation followed by cyclization and release of parent compound. Ketoconazole co-administration yielded approximately a twofold increase in absorption.

5. The hydrolytically stable prodrugs were successful in increasing absorption of parent drug and were efficiently bioactivated, but they did not yield increased systemic levels of drug.

Introduction

There has been a great deal of effort in recent years in the synthesis of direct-acting thrombin inhibitors as new antithrombotic agents (Das and Kimball 1995, Hauptmann and Sturzebecher 1999, Rewinkel and Adang 1999). The ideal agent would be orally bioavailable and orally active, but attaining it has been a very elusive goal (Kimball 1995, Hauptmann 2002). Most potent inhibitors of thrombin contain a basic amino group that is generally detrimental to oral absorption. *N*-[[1-(aminoiminomethyl)-4-piperidiny]methyl]-1-[*N*-(methylsulfonyl)-*D*-phenylalanyl]-*L*-prolinamide (**1**) is one of the most potent direct-acting thrombin inhibitors from a series of guanidino-containing analogues and was thus chosen for *in vivo* studies. The compound had a low oral bioavailability (<15%) in mouse, rat, dog and cynomolgus monkey (unpublished data). Because the molecule is very

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hydrophilic ($c\log P = -1.5$) and contained a permanently charged guanidino group, it was postulated that the bioavailability was absorption-limited and likely due to poor membrane permeability. A dependence of membrane permeability on guanidino basicity has been demonstrated for other thrombin inhibitors (Kim *et al.* 1996).

For the above reasons, prodrugs were designed to mask the guanidino group and add lipophilicity to compound **1** to improve both membrane permeability and oral exposure. The purpose of this study was to explore the mechanisms of prodrug bioactivation and determine the effects on the oral delivery of compound **1**.

Materials and methods

Chemicals

N-[[1-(aminoiminomethyl)-4-piperidinyl]methyl]-1-[*N*-(methylsulfonyl)-D-phenyl-alanyl]-L-prolinamide (**1**), 1-[*N*-(methylsulfonyl)-D-phenylalanyl]-*N*-[[1-[(1-oxohexyl)-amino]iminoethyl]-4-piperidinyl]methyl-L-prolinamide (**2**), 1-[*N*-(methylsulfonyl)-D-phenylalanyl]-*N*-[[1-[(1-oxohexyl)-amino]iminoethyl]-4-piperidinyl]methyl-L-prolinamide (**3**), *N*-[[1-amino[(2-methyl-1-oxooctyl)imino]methyl]-4-piperidinyl]methyl]-1-[*N*-(methylsulfonyl)-D-phenylalanyl]-L-prolinamide (**4**), *N*-[[1-amino[(2,2-dimethyl-1-oxohexyl)imino]methyl]-4-piperidinyl]methyl]-1-[*N*-(methylsulfonyl)-D-phenylalanyl]-L-prolinamide (**5**), *N*-[[1-amino[(1-oxo-4-phenylbutyl)imino]methyl]-4-piperidinyl]methyl]-1-[*N*-(methylsulfonyl)-D-phenylalanyl]-L-prolinamide (**6**), *N*-[[1-amino[(2,2-dimethyl-3-hydroxy-1-oxohexyl)imino]methyl]-4-piperidinyl]methyl]-1-[*N*-(methylsulfonyl)-D-phenylalanyl]-L-prolinamide (**7**) and [^3H]-**1-5** were synthesized at Bristol-Myers Squibb Pharmaceutical Research Institute and were all >98% pure. γ -Phenyl- γ -butyrolactone was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other reagents were of high analytical grade.

Caco-2 cells (passage 17) were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium, non-essential amino acids, L-glutamine, penicillin-G and streptomycin were purchased from JHR Biosciences (Lenexa, KS, USA). Foetal bovine serum was obtained from Hyclone Lab., Inc. (Logan, UT, USA). Rat tail collagen-type I was purchased from Collaborative Research, Inc. (Bedford, MA, USA). HTS-Transwell[®] inserts (surface area 0.33 cm²) with a polycarbonate membrane (0.4 μm pore size) were purchased from Costar (Cambridge, MA, USA). Hank's balanced salt solution and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Tissue fractions

Rat tissue fractions were obtained from 250–300 g male Sprague–Dawley rats obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Mouse tissue fractions were obtained from 20–25 g male Swiss Webster mice obtained from Harlan Sprague Dawley. Rat and mouse liver S9 and microsomes were prepared by standard differential centrifugation methods (Raucy and Lasker 1991). Rat intestinal homogenate was prepared by homogenizing rinsed whole small intestines (duodenum to ileum) with a hand-held tissue homogenizer (Omni International, Inc., Warrenton, VA, USA) in 1 vol. (w/v) of 50 mM, pH 7.4, potassium phosphate buffer. Human liver S9 fraction was purchased from In Vitro Technologies (Baltimore, MD, USA). Protein concentrations for liver fractions were determined by the method of Lowry *et al.* (1951). Protein concentrations were not determined for the intestinal homogenate preparations.

In vitro incubations

Liver S9 incubation conditions for all species consisted of 50 mM potassium phosphate (pH 7.4) and between 1.5 and 12 mg protein and an incubation volume of 1 ml. A wide range of protein concentrations was necessary because of the wide range of hydrolysis rates observed. Liver microsome incubation conditions for all species consisted of 50 mM potassium phosphate (pH 7.4), 10 mM NADPH and between 0.8 and 2.0 mg microsomal protein in an incubation volume of 1 ml. Intestinal homogenate incubations consisted of 50 mM potassium phosphate (pH 7.4) plus 50% intestinal homogenate and an incubation volume of 1 ml. Reactions were initiated by the addition of substrate and carried out in a shaking water bath at 37 °C. Prodrug stock solutions were in 20% acetonitrile at 10 mM or at 2 mM in water and the compounds were added to a final concentration of 100 μM (acetonitrile final concentration was $\leq 1\%$). Incubations were terminated by the addition of an equal volume of acetonitrile and centrifugation. The supernatants after centrifugation were analysed by high-performance liquid

chromatography (HPLC) with no additional processing. Conditions were adjusted such that there was never more than 20% of the starting material consumed during the incubation so that the best estimates of initial reaction rates could be determined. Rates of metabolism were calculated by measuring peak areas of metabolite(s) and parent and determining the percent metabolism by dividing the total metabolite peak area by the total drug-related peak area. This method assumes that the molar extinction coefficient of the parent is the same as the metabolite. The percent of metabolism is then multiplied by the total drug in the incubation to determine the moles of compound metabolized.

HPLC peak isolation experiments were performed on the rat microsomal incubation mixtures of compound **5**. The isolation of compound **A** was accomplished by collecting a 1-min fraction beginning at a retention time of 25 min. The fraction was immediately placed on a SpeedVac evaporator (Savant, Holbrook, NY, USA) and evaporated to 200 μ l. Potassium phosphate buffer (50 μ l, 50 mM, pH 7.4) was added and the mixture placed in a water bath at 37 °C for 8 h. Aliquots (50 μ l) were withdrawn and analysed by HPLC at 0, 1, 2, 4 and 8 h.

Caco-2 cell permeability

Caco-2 cells were seeded onto a collagen-coated polycarbonate filter membrane at a density of 80 000 cells cm^{-2} . The cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, 100 U ml^{-1} penicillin-G and 100 $\mu\text{g ml}^{-1}$ streptomycin. The culture medium was replaced every 2 days and the cells maintained at 37 °C with 90% relative humidity and 5% CO_2 . Permeability studies were conducted with the monolayers between 14 and 21 days in culture, and the cell passage numbers were between 20 and 40.

The transport medium was modified Hank's balanced salt solution (MHBS). Each monolayer was washed twice with MHBS and 1.1 ml of pH 7.4 MHBS was placed on the basolateral side of the monolayer (receiver well). The permeability studies were initiated by adding 0.2 ml MHBS-containing compound (pH 7.4, initial target concentration of 200 μM) to the apical side of the monolayer. The monolayers were placed on an orbital shaker (50 cycles min^{-1}) and incubated for 4 h at 37 °C. Samples were taken from each receiver well and the apical compartment at the end of the 4 h.

Apical-to-basolateral permeability coefficients were calculated according to the following equation:

$$\text{Permeability coefficient (Pc)} = dA/(dtSC_o),$$

where dA/dt is the flux of compound across the monolayer (nmol s^{-1}), S is the surface area of the cell monolayer (0.33 cm^2) and C_o is the initial concentration (μM) in the apical compartment. The permeability coefficient values are expressed as nm s^{-1} .

Bile duct-cannulated rat

Bile duct-cannulated rats were used to determine the total absorption of compounds *in vivo* (for the purposes of the current studies, absorption is defined as the total amount of compound reaching the portal circulation). Rats with an indwelling bile duct and duodenal cannulae were prepared and allowed to recover for 48 h before experimentation. The cannulae were connected to form an external loop during the recovery period. They were fasted overnight before dosing. During the bile collection, control bile was infused into the duodenum at a rate of 1 ml h^{-1} . Dose solutions were administered through the intraduodenal cannula. Intraduodenal dosing was done for two reasons: (1) to minimize the stress of dose administration to the bile duct-cannulated animals, and (2) to allow for the efficient delivery of co-administered agents to the site of action. For experiments with compounds **2** and **3**, each rat received a dose of 6.0 mg (approximately 20 mg kg^{-1}) [^3H]-BMS compound (50 μCi total radioactivity) as a solution in 10% ethanol/corn oil. For experiments with compounds **4** and **5**, each rat received a dose of 0.6 mg (approximately 2 mg kg^{-1}) [^3H]-BMS compound (50 μCi total radioactivity) as a solution in sodium acetate buffer (50 mM, pH 5). Compound **1** was administered within each of the experiments with compounds **2–5** following the dosing protocol used for the compounds in that experiment. For ketoconazole co-administration experiments, the rats received a 10 mg kg^{-1} intraduodenal dose of ketoconazole (in ethanol-propylene glycol-water, 1:2:3 v/v) 30 min before receiving the test compound. Bile was either collected at 6-h intervals for 12 h or at 3-h intervals for 9 h. Urine was collected as either one 0–12-h sample or as one 0–9-h sample. After the termination of the experiment, the entire gastrointestinal (GI) tract of the animals was removed and homogenized with 3 vols water. The carcass was ground several times to assure a homogeneous sample. The total absorption of a compound is defined as the sum of the radioactivity in the bile, urine and carcass (after removal of the GI tract).

HPLC analysis

The supernatants from quenched *in vitro* incubations and urine samples were injected directly. Bile and GI tract homogenates were injected after the addition of 1 vol. acetonitrile and centrifugation. All HPLC analysis was conducted on a Waters Millennium HPLC system (Millipore Corp., Bedford, MA,

USA). Aliquots were injected onto a Waters Symmetry C₁₈ column (3.9 × 150 mm), which was eluted with a linear gradient of 15–85% aqueous acetonitrile (containing 0.1% trifluoroacetic acid) over 30 min at a flow rate of 1.0 ml min⁻¹. The effluent was monitored with UV detection at 215 nm. For radioactive samples, a Gilson autosampler was connected to the HPLC and 1-ml aliquots were collected directly into scintillation vials.

Analysis of samples from the Caco-2 cell monolayer experiments were done with a specific HPLC-UV assay. A C₁₈ YMC ODS AQ reverse-phase column (4.6 mm × 5 cm) was used. The mobile phase, consisting of solvent A (water–acetonitrile–trifluoroacetic acid, 95:5:0.1% v/v) and solvent B (water–acetonitrile–trifluoroacetic acid, 20:80:0.1% v/v), was programmed as a linear gradient. The flow rate was 1.0 ml min⁻¹ and the absorbance was monitored at 210 nm.

Mass spectrometry

The detection of γ -phenyl- γ -butyrolactone was accomplished with the following procedure. An aliquot of a rat microsomal incubation with compound **6** was injected onto an HPLC column using the conditions described above and the retention time corresponding to that of authentic γ -phenyl- γ -butyrolactone was collected. The eluate was placed directly onto a DEP probe and analysed by DCI (ammonia reagent gas) on a TSQ4600 MSDS Mass Spectrometer (Finnigan MAT, San Jose, CA, USA). γ -Phenyl- γ -butyrolactone (m/z , relative abundance), [M + H] (163, 5), [M + NH₃] (180, 100). HPLC isolate (m/z , relative abundance), [M + NH₃] (180, 100).

Quantitation of radioactivity

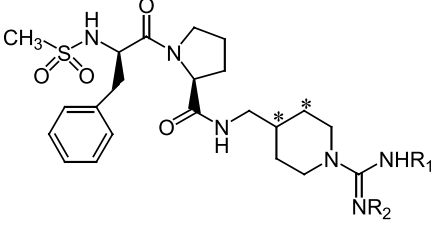
HPLC fractions were collected into vials and scintillation cocktail was added (EcoliteTM, ICN Radiochemicals, Irvine, CA, USA). The quantitation was done with a Packard Tricarb counter (Packard Corp., Meriden, CT, USA) with the appropriate quench curves. Bile and urine samples were counted directly. GI homogenate and carcass samples were counted after digestion with SolueneTM (Packard).

Results

In vitro studies with acyl prodrugs **2** and **3**

The *in vitro* hydrolysis of acylated or diacylated guanidines with straight-chain alkyl groups (see table 1 for structures) was examined. Compounds **2** and **3** were both efficiently bioactivated to **1** in *in vitro* incubations with the rat liver S9 fraction (table 2). The rate of hydrolysis of the first hexanoyl group from **3** was more rapid (3.8–50-fold) than the rate of hydrolysis of **2** in all species examined.

Table 1. Structure of compound **1** and prodrugs. * Sites of the radiolabel.



Compound no.	R ₁	R ₂
1	H	H
2	H	hexanoyl
3	hexanoyl	hexanoyl
4	H	2-methyl-octanoyl
5	H	2,2-dimethyl-hexanoyl
6	H	4-phenyl-butanoyl

Table 2. Hydrolytic bioactivation and permeability of compounds **2** and **3**.^a

Compound no.	Hydrolysis rate in liver S9 (pmol min ⁻¹ mg ⁻¹ protein)			Caco-2 cell monolayer permeability (Pc, nm s ⁻¹)
	Rat	Mouse	Human	
1	—	—	—	10 ^b
2	80	60	300	13
3 ^c	300	3000	5800	83

^aRate of hydrolysis in liver S9 was measured in singlicate for prodrugs **2** and **3**. The permeability of compounds **1–3** through Caco-2 cell monolayers was measured in duplicate and the average was found.

^bSimilar to mannitol.

^cRate of hydrolysis of one hexanoyl group.

The overall formation rate of **1** from **3** was similar to that from **2**, the rate-limiting step being the hydrolysis of the *mono*-acylated compound (data not shown).

Compounds **1–3** were studied in the Caco-2 cell monolayer system. Compound **1** had a permeability coefficient across Caco-2 cell monolayers similar to that of mannitol (table 2), a poorly permeable model compound with negligible *trans*-cellular permeability. Compound **2** showed limited improvement in permeability; however, compound **3** showed substantial improvement.

In vivo studies with acyl prodrugs 2 and 3

Compounds **1–3** were administered to bile duct-cannulated rats. The absorption, based on radioactivity in the bile, urine and carcass (after removal of the GI tract), was 10–15% for all three compounds. The GI tract homogenate was examined 12 h after administration of either [³H]-**1** or [³H]-**3** to the bile duct-cannulated rats. For both compounds, a large portion of the radioactivity (>70% of the dose) was present in the GI tract. After administration of **1**, the major radioactive peak eluted at a retention time corresponding to authentic **1**. The GI tract homogenate after administration of **3** showed the presence of a peak with a retention time of **1** and a major peak for a second more polar compound (figure 1). There was essentially no compound **3** remaining. The second compound was identified as a piperidine derivative of **1** generated by the loss of *bis*-hexanoyl urea (figure 2).

Hydrolytic bioactivation of hindered prodrugs

As seen in table 3, the substitution of one or two methyl groups at the α -carbon of the acyl side chain produced a marked decrease in the rate of prodrug hydrolysis in rat liver S9 and intestinal homogenate. The hydrolysis reaction was essentially blocked for the α,α -dimethyl compounds. In addition, this change was accompanied by a substantial increase in Caco-2 cell permeability that was closely related to the number of carbons of the prodrug side chain. Based on these results, prodrugs **4** and **5** were selected for further *in vivo* testing.

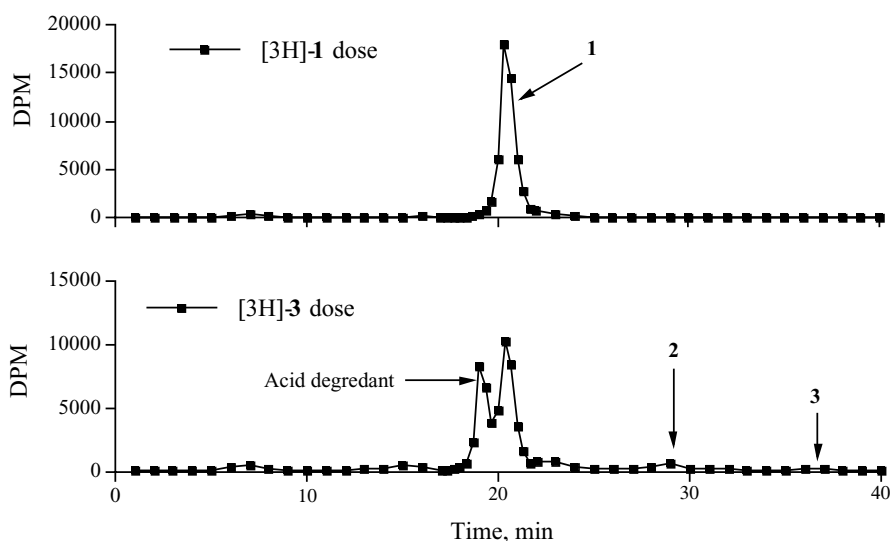


Figure 1. HPLC with radiochemical detection of the gastrointestinal tract homogenate after administration of either **1** (top) or **3** (bottom) to bile duct-cannulated rats. Rats were administered a 6-mg (approximately 20 mg kg^{-1}) dose of **1** or **3** and sacrificed after 12 h. The gastrointestinal tracts were then removed and homogenized. The retention times for **1–3** are denoted by the arrows.

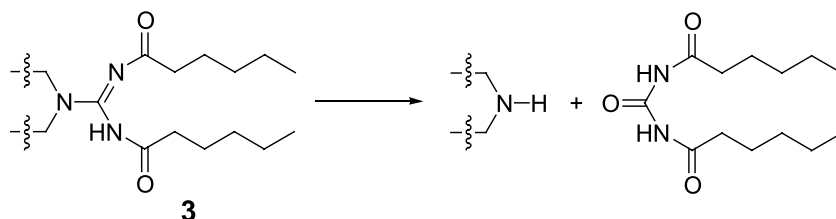


Figure 2. Breakdown of *bis*-acyl prodrugs to yield piperidine derivative of compound **1**.

Evaluation of compounds 4 and 5 in bile duct-cannulated rats

Rats with an indwelling bile duct and duodenal cannula were administered a 20 mg kg^{-1} dose of each compound intraduodenally. As seen in table 4, compounds **4** and **5** showed an increase in total absorption as compared with **1**. The GI tract homogenates of these animals were examined and most of the unabsorbed dose was present as **1** (figure 3), despite the low rate of *in vitro* enzymatic hydrolysis of **4** or **5** to produce **1** in intestinal S9 fractions (table 3).

Metabolism of 4 and 5 in rat liver microsomes

The formation of **1** in rat liver microsomes from either **4** or **5** was clearly increased when NADPH was present (table 3). The rate enhancements for the formation of **1** for the series of prodrugs in microsomes supplemented with NADPH ranged from 4- to >750 -fold (table 3). The NADPH-dependent formation of **1** from **5** was extensively inhibited (6% of the control value) in the presence of $10 \mu\text{M}$ ketoconazole (data not shown). In addition to **1** being formed in these incubations, there was also the formation of hydroxylated compounds.

Table 3. Bioactivation and permeability of hindered prodrugs.

R =	Compound no.	Rate of formation of 1 (pmol min ⁻¹ mg protein ⁻¹)				Caco-2 cell monolayer permeability
		Rat liver S-9	Rat intestinal homogenate	Rat liver microsomes		
				-NADPH	+NADPH	
Hexanoyl	2	60	60	n.d. ^b	n.d.	13
2-Methylhexanoyl		10	<8	20	2800	36
2,2-Dimethyl-hexanoyl	5	<4	<8	<2	1500	160
2-methyl-heptanoyl		8	<8	n.d.	n.d.	85
2,2-Dimethyl-heptanoyl		3	<8	n.d.	n.d.	153
Octanoyl		100	30	600	2600	45
2-Methyloctanoyl	4	7	<8	30	1600	120
2,2-Dimethyl-octanoyl		4	<8	<2	700	n.d.
4-Phenylbutanoyl	6	30	n.d.	100	1500	46
2-Methyl-4-phenylbutanoyl		4	<8	10	1400	89
2,2-Dimethyl-4-phenylbutanoyl		<4	n.d.	<2	1000	n.d.

The rate of formation of compound **1** in liver S9 or intestinal homogenate was measured in singlicate. The permeability of compounds **1–3** through Caco-2 cell monolayers was measured in duplicate and the average was found.

^bn.d. = not determined.

Table 4. Administration of compounds **1**, **4** or **5** to the bile duct-cannulated rat with or without ketoconazole.

Compound administered:	Recovery of the radioactive dose (%)				
	1 (<i>n</i> = 2)	4		5	
		(<i>n</i> = 2)	+ Keto ^a (<i>n</i> = 3)	(<i>n</i> = 5)	+ Keto (<i>n</i> = 6)
Bile (0–9 h)	1	14	31 ± 2	29 ± 9	53 ± 14
Urine (0–9 h)	3	4.2	4.0 ± 0.1	2.7 ± 1.2	10 ± 12
Gastrointestinal tract	93	70	61 ± 1	58 ± 6	33 ± 5
Carcass	1	2.5	2.7 ± 0.6	2.1 ± 0.8	2.2 ± 2.9
Total recovery	98	90	99 ± 1	92 ± 3	98 ± 5
Estimate of absorption ^b	5	21	37 ± 2	34 ± 10	65 ± 4 ^c

Bile duct-cannulated rats were administered a single 0.6-mg dose of **1**, **4** or **5** intraduodenally, and bile and urine were collected for 9 h. Results are either the average or mean ± SD.

^aKeto, ketoconazole.

^bEstimate of absorption is the sum of the percentage of the dose recovered in the bile, urine and carcass (after removal of the gastrointestinal tract).

A statistical difference in the mean absorption upon ketoconazole co-administration compared with administration of **5** alone (*p* < 0.01).

Mechanism of oxidative bioactivation

The product profile of the NADPH-catalysed microsomal incubation of **5** was examined further. The product profile for this compound clearly showed the presence of **1** as well as two other products (figure 5, peaks **A** and **7**). The mixture was analysed at various intervals after quenching and further incubation of the

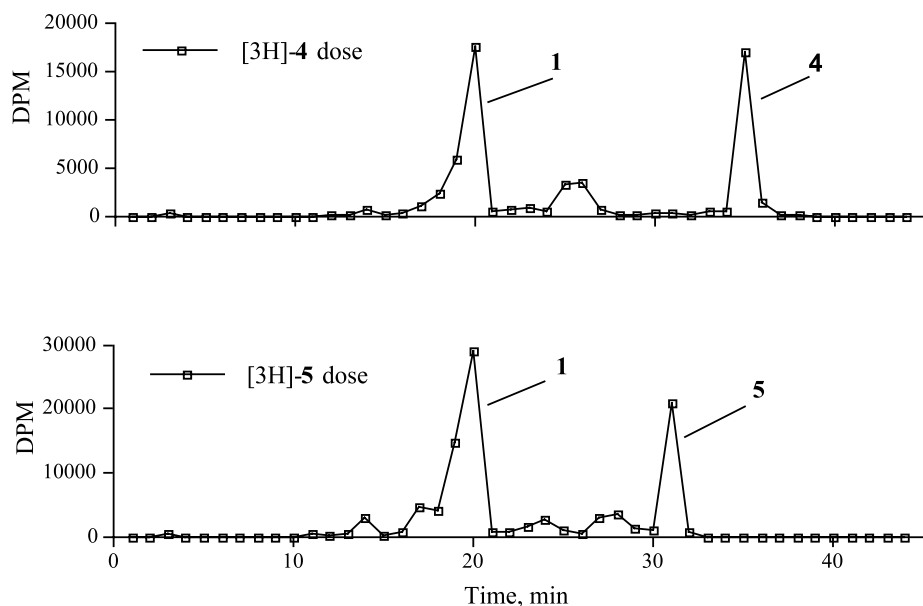


Figure 3. HPLC with radiochemical detection of the gastrointestinal tract homogenate after administration of either **4** (top) or **5** (bottom) to bile duct-cannulated rats. Rats were administered a 0.6-mg (approximately 2 mg kg^{-1}) dose of **4** or **5** and sacrificed after 9 h. The gastrointestinal tracts were then removed and homogenized. The retention times for **1**, **4** and **5** are denoted by the arrows.

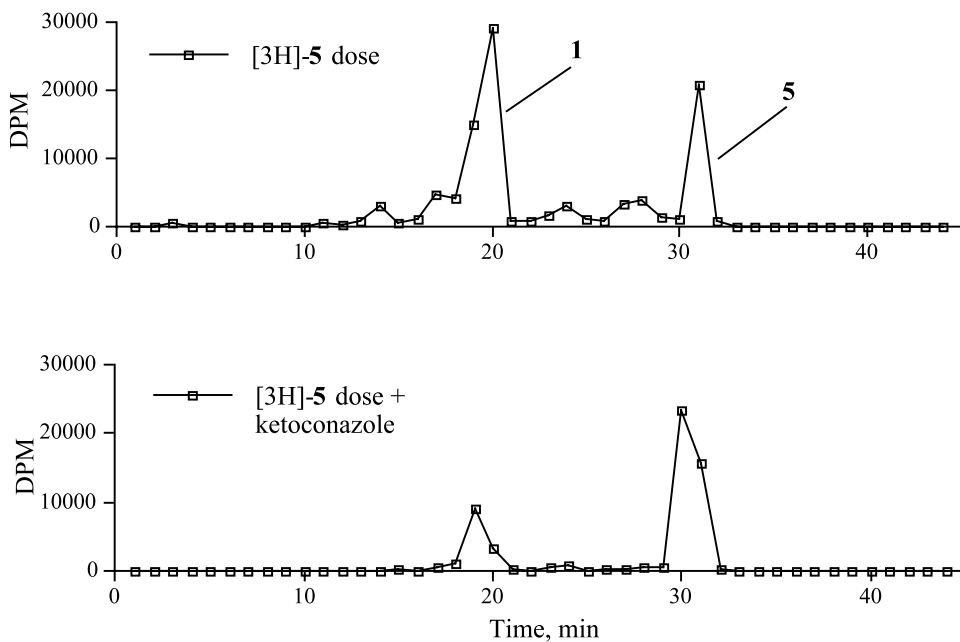


Figure 4. HPLC with radiochemical detection of the gastrointestinal tract homogenate after intra-duodenal administration of **5** to the bile duct-cannulated rat with (top) or without (bottom) co-administration of ketoconazole. Rats were administered a 0.6-mg (approximately 2 mg kg^{-1}) dose of **5** with or without pretreatment with ketoconazole (10 mg kg^{-1}) and sacrificed after 9 h. The gastrointestinal tracts were then removed and homogenized. The retention times for **1** and **5** are denoted by the arrows.

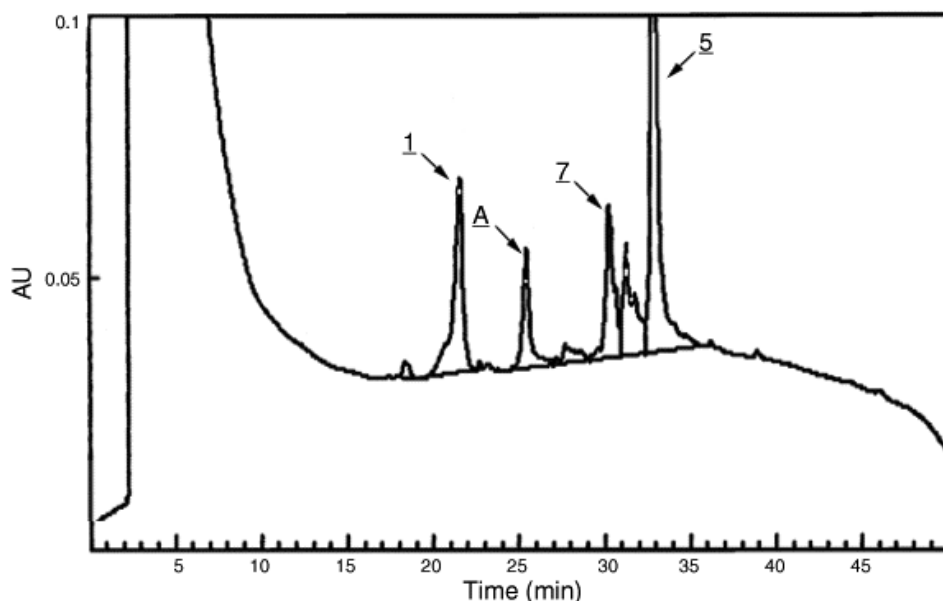


Figure 5. Compound **5** metabolism by rat liver microsomes. Rat liver microsomes (1.8 mg ml^{-1}) were incubated for 15 min with $100 \mu\text{M}$ of compound **5** in the presence of 10 mM NADPH. Chromatograms were obtained by HPLC with UV detection at 215 nm. Retention times for starting material (**5**) and products (**1**, **7**) are denoted. An additional product, compound **A**, was isolated for further characterization.

quenched material at 37°C . Under these conditions, the product profile shifted such that one of the product peaks fell in intensity while the peak of compound **1** gained in intensity. Compound **A** was subsequently isolated and incubated at 37°C and was cleanly converted to **1** with a reaction half-life of about 8 h (figure 6). The identity of the remaining major product peak, compound **7**, was elucidated by comparison with a synthetically prepared standard.

Further microsomal incubations were performed with compound **6**. It was used because the γ -lactone formed in the proposed mechanism was commercially available and readily detected. Authentic γ -phenyl- γ -butyrolactone was used as a standard to determine HPLC retention time as well as conditions for DCI-MS. An HPLC isolate from the microsomal incubations of **6** that corresponded to the retention time of the authentic material was analysed by DCI-MS and found to have a base peak at m/z 180, which was identical to the reference material.

Co-administration of ketoconazole and 4 or 5 to bile duct-cannulated rats

As can be seen from the preceding figures, these prodrugs are substrates for presystemic oxidative metabolism. To determine whether this was true *in vivo*, ketoconazole was co-administered with **4** or **5** to bile duct-cannulated rats. A dose of 10 mg kg^{-1} was administered 30 min before administration of **4** or **5**. This dose has been shown to produce significant inhibition of CYP enzymes when administered to rats (Houston *et al.* 1988). Total absorption of both **4** and **5** was approximately doubled when ketoconazole was co-administered (table 4). HPLC analysis of GI tract homogenates 12 h after the administration of **5** with or without

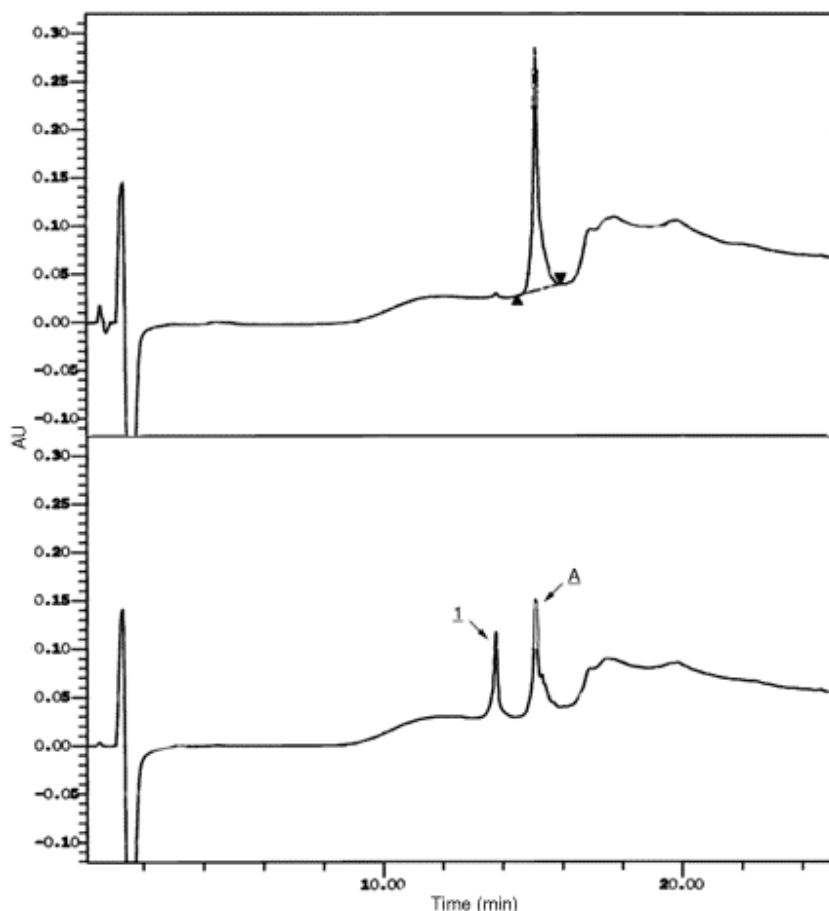


Figure 6. HPLC Chromatogram of compound **A** after isolation (top) or after 8 h in an aqueous solution (bottom). Isolated compound **A** was incubated in potassium phosphate buffer (50 mM, pH 7.4) at 37 °C for 8 h. Chromatograms were obtained by HPLC with UV detection at 215 nm. Retention times for compounds **A** and **1** are denoted.

ketoconazole shows that ketoconazole prevented the pre-absorptive conversion of **5** into **1** (figure 4). Of the dose remaining in the GI tract after 12 h, 66% was intact **5** when ketoconazole was co-administered as opposed to 30% when **5** was dosed alone.

Discussion

Compound **1** is a potent antithrombotic agent in animals when given systemically but not orally. This is likely due to poor intestinal absorption, as was demonstrated in the rat and in Caco-2 cell monolayer permeability studies described herein. The Caco-2 cell model has been extensively studied and demonstrated to be a useful system in the evaluation of intestinal permeability (Hidalgo *et al.* 1989, Artursson and Karlsson 1991) and compound **1** had a permeability similar to mannitol, which is used as a poorly permeable reference compound. Compound **1** is very hydrophilic ($c \log P = -1.5$), in part due to the charged guanidino group. Less basic analogues are more permeable (Kim *et al.*

1996), but the fully basic guanidino group is required for potency in this series. Since the target route of administration is oral, prodrugs were prepared to mask the guanidino group as well as to increase lipophilicity.

The first compounds evaluated were *mono*- and *bis*-prodrugs with *n*-alkylacyl side chains. These compounds showed promise because they were cleaved to the parent molecule *in vitro* and at least for the *bis*-prodrug series showed an improvement in Caco-2 cell monolayer permeability. The permeability coefficient in Caco-2 cells found for **3** was similar to the permeability found for reference compounds that have good absorption in humans (e.g. metoprolol, $P_c = 122 \text{ nm s}^{-1}$). However, when the *mono*- or *bis*-prodrugs were examined *in vivo*, neither compound gave any increase in the total absorption of **1** (as measured by the total radioactivity in the bile, urine and carcass after removal of the GI tract). The intestines of bile duct-cannulated rats administered **2** were examined and found to contain almost entirely parent drug **1**. The intestines of bile duct-cannulated rats administered **3** were examined and found to contain parent drug **1** plus a second compound later shown to be the piperidine derivative of compound **1** (figures 1 and 2). This transformation was found to occur readily at low pH and it is thought that the *in vivo* transformation was solely due to chemical degradation. As seen in figure 1, the guanidine cleavage product was still fairly abundant even though these animals were dosed through an intraduodenal cannula. These studies led to the conclusion that rapid pre-absorptive bioactivation occurred in the rat GI tract *in vivo* for both compounds **2** and **3** and that additionally the *bis*-prodrugs were susceptible to breakdown even at pH values close to neutral.

To attempt to overcome the two absorption-limiting problems, *bis*-prodrugs were abandoned completely and steric hindrance around the α -carbon of the acyl side chain was added to slow the enzymatic hydrolysis of the *mono*-prodrugs. This change did indeed lead to a slower rate of hydrolysis *in vitro* in intestinal and liver tissue (table 1) and the *mono*-prodrugs also showed improved behaviour in the Caco-2 cell monolayer assay. This approach led to an increase in net absorption when administered to bile duct-cannulated rats. However, compounds **4** and **5** were still subjected to significant presystemic metabolism that yielded parent compound (figure 3). This result was unexpected owing to the slow rate of enzymatic hydrolysis seen in the *in vitro* incubations with liver or intestinal tissue.

The mechanism by which the α -methyl or α,α -dimethyl compounds are converted to the parent compound was investigated in *in vitro* experiments with liver microsomes. Compound **1** was rapidly formed from compounds **4–6** in microsomes in the presence of NADPH. The NADPH-dependent formation of **1** from **5** could be extensively inhibited in the presence of $10 \mu\text{M}$ ketoconazole (6% of the control value in human liver microsomes, data not shown), a known inhibitor of the CYP3A family and other CYP enzymes (Maurice *et al.* 1992, Newton *et al.* 1995). We hypothesize that amide cleavage could be via an initial CYP-mediated hydroxylation of the alkyl side chain followed by an intramolecular lactonization that liberated the guanidine. This mechanism of prodrug release shares some similarities with the prodrugs first proposed by Bundgaard that take advantage of intramolecular cyclization reactions in a multistep prodrug release mechanism (Bundgaard 1985, Krogsgaard-Larsen and Bundgaard 1991, Bundgaard *et al.* 1986a, b, Shan *et al.* 1997). Johnson *et al.* (1988) reported that this mechanism was responsible for the intramolecular lactonization of an aliphatic alcohol and an amide; a situation analogous to that described here.

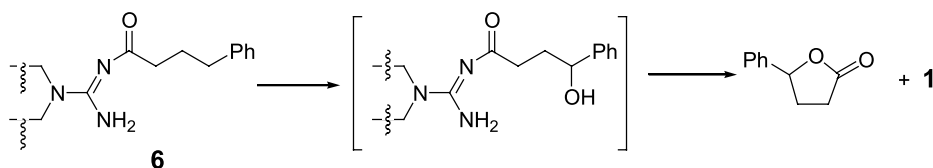


Figure 7. Oxidative biotransformation of compound **6** to yield γ -phenyl- γ -butyrolactone.

To pursue further the intramolecular cyclization hypothesis, experiments were done with alkylphenyl prodrug **6**. If the above mechanism was operative, this compound should liberate γ -phenyl- γ -butyrolactone (figure 7), a compound that could be detected by HPLC-UV chromatography. When the HPLC fraction corresponding to γ -phenyl- γ -butyrolactone was isolated from a microsomal incubation and analysed by MS, a peak was found in the spectra which corresponds to γ -phenyl- γ -butyrolactone. Further evidence for the intramolecular cyclization hypothesis was found in detailed studies with compound **5**. The product profile of the microsomal incubation of **5** showed that there were several hydroxylated products along with **1** when the mixture was assayed soon after completion of the incubation (figure 5). When one of the hydroxylated products was isolated and allowed to stand at 37°C at neutral pH, there was a gradual generation of **1** (figure 6). The half-life for this reaction was about 8 h at 37°C. This reaction is hypothesized to be due to initial ω -1 oxidation of the side chain followed by the formation of a δ -lactone. In addition, a major product in the microsomal mixture was the ω -3 hydroxylated product, compound **7**, which was confirmed by comparison with an authentic synthetic standard. This product does not liberate **1** because formation of a four-membered lactone ring would not be energetically favoured. This evidence taken together supports a mechanism as outlined in figure 8, in which the γ -lactone is formed very rapidly liberating **1** and the δ -lactone is formed slowly.

Based on the *in vitro* microsomal studies, the formation of **1** in the GI tract of bile duct-cannulated rats (figure 3) was hypothesized to be due to oxidative metabolism in the enterocytes, not by acyl bond hydrolysis. To explore this possibility further, ketoconazole was co-administered along with the prodrugs to examine how this affected absorption (entry into the portal circulation). The dose of ketoconazole administered has been shown to produce significant inhibition of CYP enzymes when given to rats (Houston *et al.* 1988). As seen in table 4, there was a marked effect when ketoconazole was present and absorption was approximately doubled for both **4** and **5**. Ketoconazole has effects on both CYP enzymes and intestinal P-glycoprotein enzymes and both systems could play a role in the disposition of **1** and the prodrugs. There is certainly an effect on CYP enzymes as seen by the increased percentage of intact **5** in the profiles of the GI tract homogenates shown in figure 4. The effect on net absorption could be due to the combined effects of decreased metabolism and inhibition of P-glycoprotein efflux.

The increased absorption of prodrug did not lead to an increased systemic exposure to drug. In fact, the systemic exposure to **1**, as measured by urinary excretion, was about the same when **4** or **5** were administered either with or without ketoconazole (table 4). There is a large difference in the per cent of absorbed dose excreted in the urine after administration of either **1** or the prodrugs (in all cases the majority of the radioactivity in urine is compound **1**, data not

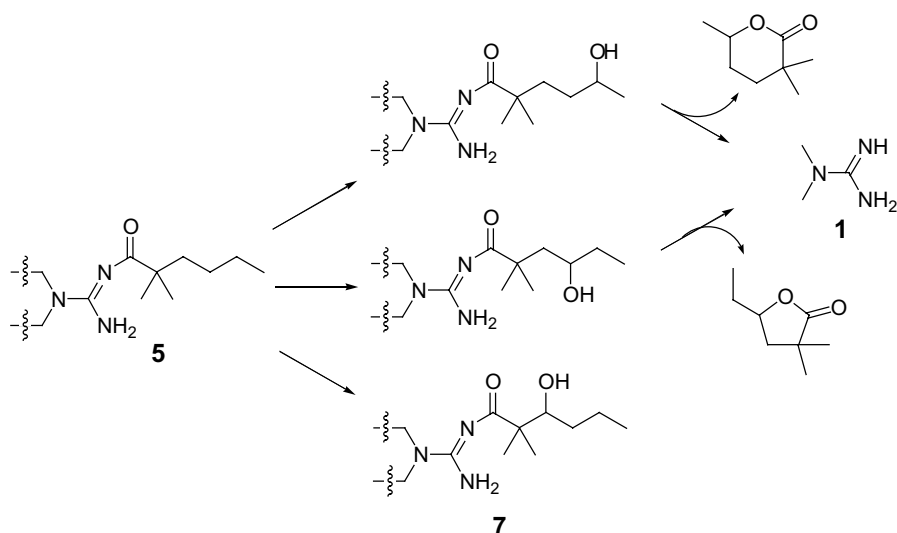


Figure 8. Proposed scheme for the oxidative metabolism of **5**.

shown). After administration of **1**, approximately 50% of the absorbed dose is excreted in the urine, but after prodrug administration the fraction drops to 8–21%. This is not due to inefficient bioactivation, indeed 61% of the material in bile is **1** after administration of **5** (data not shown). It is likely due to the differential processing of the prodrugs, and it is hypothesized that **1** is oxidatively formed from the prodrugs in the liver by CYP enzymes but is excreted in the bile before ever reaching the systemic circulation. Compound **1** has been shown to bind to human P-glycoprotein (data not shown), which provides some evidence for a potential efflux mechanism for this compound.

This study has described the unexpected oxidative bioactivation of acyl prodrugs of a guanidine-containing drug. The activation of these prodrugs was shown to be through CYP-mediated hydroxylation followed by intramolecular lactonization that liberated free compound **1**. These prodrugs were found to improve the absorption of the parent drug when given alone and to give large increases in absorption when given with ketoconazole. Unfortunately, the large absorption enhancements under any conditions did not translate into an increase in the systemic exposure of **1**, presumably because of two barriers: (1) a prodrug that undergoes oxidative conversion to **1** in the gut does not reach the portal circulation possibly because of an active efflux mechanism that pumps **1** out into the lumen; and (2) a prodrug that is oxidatively converted to **1** in the liver is efficiently secreted into the bile before the drug enters the systemic circulation. This approach to drug delivery via oxidative activation of prodrugs has received relatively little attention but could serve as a potential useful method of prodrug delivery, especially if the target of action for the drug is the liver.

Acknowledgements

The authors thank Marc Fancher and the members of the Technical Support Unit for their efforts in the completion of the *in vivo* experiments, and J. Kent

Rinehart for the preparation of radiolabelled compounds. A preliminary account of this work was presented at the 7th North American ISSX Meeting, San Diego, CA, abst 401.

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Time-dependent transcriptional induction of CYP1A1, CYP1A2 and CYP1B1 mRNAs by H⁺/K⁺-ATPase inhibitors and other xenobiotics

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Received 19 June 2002

1. Xenobiotic-mediated regulation of mRNA expression of all members of the human cytochrome P450 (CYP) 1 family has been measured by RT-PCR in the hepatoma cell line, HepG2. Besides the positive control β -naphthoflavone, the H⁺/K⁺-ATPase inhibitors omeprazole, lansoprazole, pantoprazole and rabeprazole and the anti-malaria drug primaquine were included in this study.

2. β -Naphthoflavone, primaquine, omeprazole and lansoprazole increased mRNA levels of CYP1A1, CYP1A2 and CYP1B1. Induction by rabeprazole was significant only for CYP1A1 and CYP1A2, whereas none of the CYP1 mRNAs was induced by pantoprazole. This result was confirmed in primary human hepatocytes.

3. Transcriptional regulation was proved by inhibition of induction with actinomycin D.

4. Increase of CYP1 mRNA was significant after 1 h and maximal after 4 h. CYP1B1, but not CYP1A1 or CYP1A2, was dramatically down-regulated between 4 and 24 h. This decrease was prevented by treatment of cells with actinomycin D after induction, indicating an active transcription-dependent mechanism of CYP1B1 mRNA degradation.

5. In conclusion, xenobiotics inducing CYP1A1 mRNA expression have been shown also to induce CYP1A2 and CYP1B1, differing only with regard to level and time course of induction.

Introduction

Expression of the cytochrome P450 (CYP) 1 enzymes can be modulated by a large variety of endogenous and exogenous substances, including drugs, and induction of CYP expression has been extensively analysed using animal models, mostly rat or mouse (reviewed in Dogra *et al.* 1998). Owing to considerable species-specific differences between rodents and human (Lu and Li 2001), the results derived from these animal studies do not reflect the human situation unrestrictedly. Therefore, a human-derived system is more appropriate to understand drug-related CYP induction in humans. Handling of primary cells in culture has been improved during the last few years, resulting in several studies on induction of CYP enzymes performed in primary human hepatocytes (Bowen *et al.* 2000, LeCluyse *et al.* 2000, Runge *et al.* 2000). However, extensive use of this

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approach is restricted due to high costs and limited availability of human liver material.

Several results are available concerning CYP1A1 induction in permanent cell lines by environmental pollutants as well as drugs (Chiarolini *et al.* 1997, Krusekopf *et al.* 1997, Frotschl *et al.* 1998). In addition to rat and mouse cell lines, human-derived cell lines are also suitable for such experiments (HepG2, HUH7 (liver), Caco2 (colon), A549 (lung)). Whereas induction of CYP1A1 has been studied extensively, the influence of xenobiotics on the expression of CYP1A2 and CYP1B1 has not been investigated in detail. Nevertheless, different regulation of CYP1A1 and CYP1B1 by TCDD has been described in human cell lines, including HepG2 cells (Spink *et al.* 1994, Hakkola *et al.* 1997, Kress and Greenlee 1997).

In previous studies, we have measured the induction of CYP1A1 mRNA, protein and enzyme activity (ethoxyresorufin-*O*-dealkylation, EROD) in the human hepatoma cell lines HepG2 and HUH7. The amounts of induced CYP1A1 mRNA, protein and enzyme activity corresponded with each other (Krusekopf *et al.* 1997, Kleeberg *et al.* 1999). For this enzyme, measurement of mRNA has proved a suitable way of determining xenobiotic-mediated induction.

In the current study, we examined whether drugs known to induce CYP1A1 are also inducers of CYP1A2 and CYP1B1. Induction of endogenous mRNA expression in HepG2 cells and primary human hepatocytes was measured by semiquantitative RT-PCR. Additionally, time dependence of induction of CYP1A1, CYP1A2 and CYP1B1 mRNAs was examined.

Materials and methods

All chemicals used were of the highest purity commercially available.

Cell culture and drug treatment

HepG2 cells (German Collection of Microorganisms and Cell Cultures, DSMZ) were grown in Dulbecco's modified Eagle medium supplemented with 5% foetal bovine serum at 5% CO₂ and 37°C. Cells were seeded in six-well dishes and grown to confluence. Primary human hepatocytes were from a rejected organ transplant of a male accident victim. Cells were seeded on collagen coated six-well dishes at a density of 6.7×10^5 cells per dish in Williams' medium supplemented with 10% foetal bovine serum.

Solutions of the inducers omeprazole (AntraTM, Astra), lansoprazole (Takeda), pantoprazole (PantozolTM, Byk Gulden), rabeprazole (Eisai), primaquine and β -naphthoflavone in dimethyl sulfoxide (DMSO; all Sigma-Aldrich) were added to the culture medium at the concentrations indicated. The concentration of DMSO was 0.1% in all experiments. β -Naphthoflavone was used as positive control for induction of CYP1 mRNA (100%), and other values are given relative to this control.

To exclude any toxic or stress effects, several concentrations of each substance were tested for toxicity (data not shown). The highest concentration of each substance that did not result in morphological changes of the cells within 48 h was selected for the subsequent experiments.

Harvest of cells and RNA preparation

Cells were washed twice with phosphate-buffered saline, scraped from culture dish in lysis buffer and homogenized by centrifugation through a Qiashreder (Qiagen). RNA preparation was performed using a Qiagen RNeasy Mini Kit according to the manufacturer's instructions. RNA concentration was determined photometrically. Human liver RNA (pool of one female and one male subject) was purchased from Clontech.

Reverse transcription, PCR

Reverse transcription was performed using MMLV reverse transcriptase (Life Technologies) and 2 μ g RNA in a 15- μ l assay. After establishing suitable cycle numbers for each pair of primers, cDNA equivalents of 12.5, 25 and 50 ng RNA were submitted to PCR. To allow quantification of the PCR

product, 2 kBq ^{32}P - αdCTP was added to each PCR reaction. After initial denaturation at 96°C for 1 min, PCR was performed as follows: $94^\circ\text{C}/40\text{ s}$, 50 to $56^\circ\text{C}/40\text{ s}$ (depending on the primers), $72^\circ\text{C}/50\text{ s}$ in a 20- μl assay, cycles as indicated, followed by an elongation reaction of 10 min/ 72°C . PCR products were separated on 2% agarose gels. Gels were dried and PCR products quantified with a phosphor-imager (raytest). The linearity of the PCR was proved by the quotient of the different concentrations. Except for the experiments presented in figure 4, values were normalized to actin, which was used as an internal control for the amounts of mRNA.

Specificity of primers and PCR products was confirmed by restriction analysis and sequencing. All CYP1 PCR products span exon-intron boundaries.

Results

Induction of CYP1A1, CYP1A2 and CYP1B1 in HepG2 cells

After treatment of HepG2 cells with the positive control β -naphthoflavone and with omeprazole, lansoprazole and primaquine for 4 h, amounts of all CYP1 mRNAs increased as compared with DMSO control (figure 1). These four substances did not differ in their CYP1A1 induction capacity at the concentrations tested. Induction of CYP1A2 by primaquine was significantly higher than by omeprazole and lansoprazole. Omeprazole and lansoprazole increased CYP1B1 mRNA less than β -naphthoflavone and primaquine. Induction by rabeprazole was significant only for CYP1A1 and CYP1A2. Rabeprazole induced CYP1A1 mRNA less than omeprazole, primaquine and β -naphthoflavone. The primaquine and β -naphthoflavone mediated increase of CYP1A2 mRNA was higher than the induction by rabeprazole. Pantoprazole did not induce any CYP1 mRNA.

Time-dependence of CYP1 induction

The mRNA content was determined at six time intervals (0, 30 min, 1, 2, 4 and 24 h) after adding omeprazole, lansoprazole or β -naphthoflavone to the culture medium (figure 2). The amount of CYP1A1, CYP1A2 and CYP1B1 mRNA produced after a 4-h treatment with β -naphthoflavone was defined as 100%. β -Naphthoflavone increased CYP1A1 64 times, CYP1A2 four times and CYP1B1 76 times over control levels. Induction of all CYP1 enzymes was significant after 1 h (except CYP1A2 after omeprazole and lansoprazole treatment) and reached a maximum after 4 h. CYP1A1 and CYP1A2 mRNA levels induced by β -naphthoflavone after 4 h remained unchanged within the following 20 h, whereas CYP1B1

Table 1. Gene-specific PCR-primers.

Gene	Accession number	Position	Sequence	Product (bp)
CYP1A1	K03191	899	AAC CTT TGA GAA GGG CCA CA	249
		1147	GAC CTG CCA ATC ACT GTG TC	
CYP1A2	Z00036	562	ACT TCG ACC CTT ACA ATC AG	285
		846	CAC TGT TCT TGT CAA AGT CC	
CYP1B1	U03688	1298	GAG AAC GTA CCG GCC ACT AT	516
		1813	GTG AGC CAG GAT GGA GAT GA	
AHR	L19872	2686	ATG TAT CAG TGC CAG CCA GA	340
		3025	CTC CAT GTG AAC TTG CTG AC	
Albumin	M12523	956	ATG ATG AGA TGC CTG CTG AC	486
		1441	GGA CCA CGG ATA GAT AGT CT	
β -Actin	X00351	486	ACT GGC ATC GTG ATG GAC TC	425
		319	CGG ATG TCC ACG TCA CAC TT	

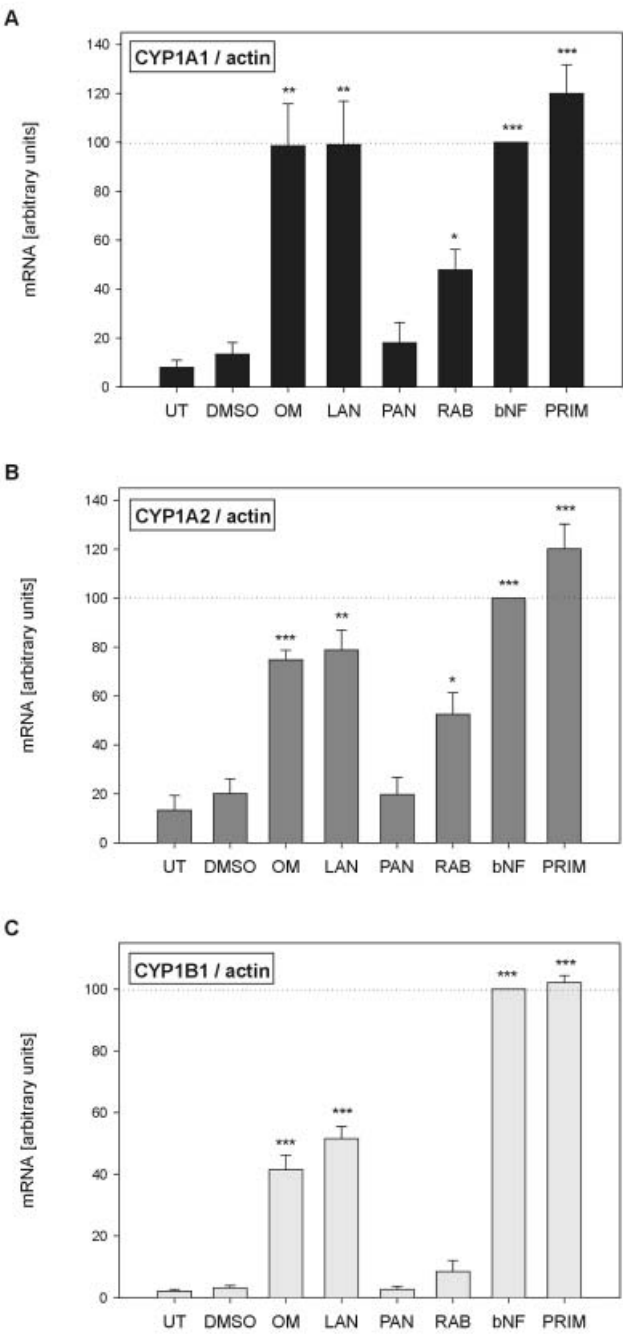


Figure 1. Induction of CYP1A1, CYP1A2 and CYP1B1 in HepG2 cells. HepG2 cells were untreated or incubated with vehicle (DMSO), 100 μ M omeprazole (OM), 50 μ M lansoprazole (LAN), 200 μ M pantoprazole (PAN), 50 μ M rabeprazole (RAB), 20 μ M β -naphthoflavone (β NF) or 50 μ M primaquine (PRIM) for 4 h. 2 kBq ^{32} P- α dCTP was added to each PCR (CYP1A1, 24 cycles; CYP1A2, 32 cycles; CYP1B1, 28 cycles; actin, 17 cycles). Columns represent the mean and SEM of four independent experiments. Marked columns are significantly different from DMSO control, determined by unpaired two-tailed Student's *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. (A) CYP1A1; (B) CYP1A2; (C) CYP1B1.

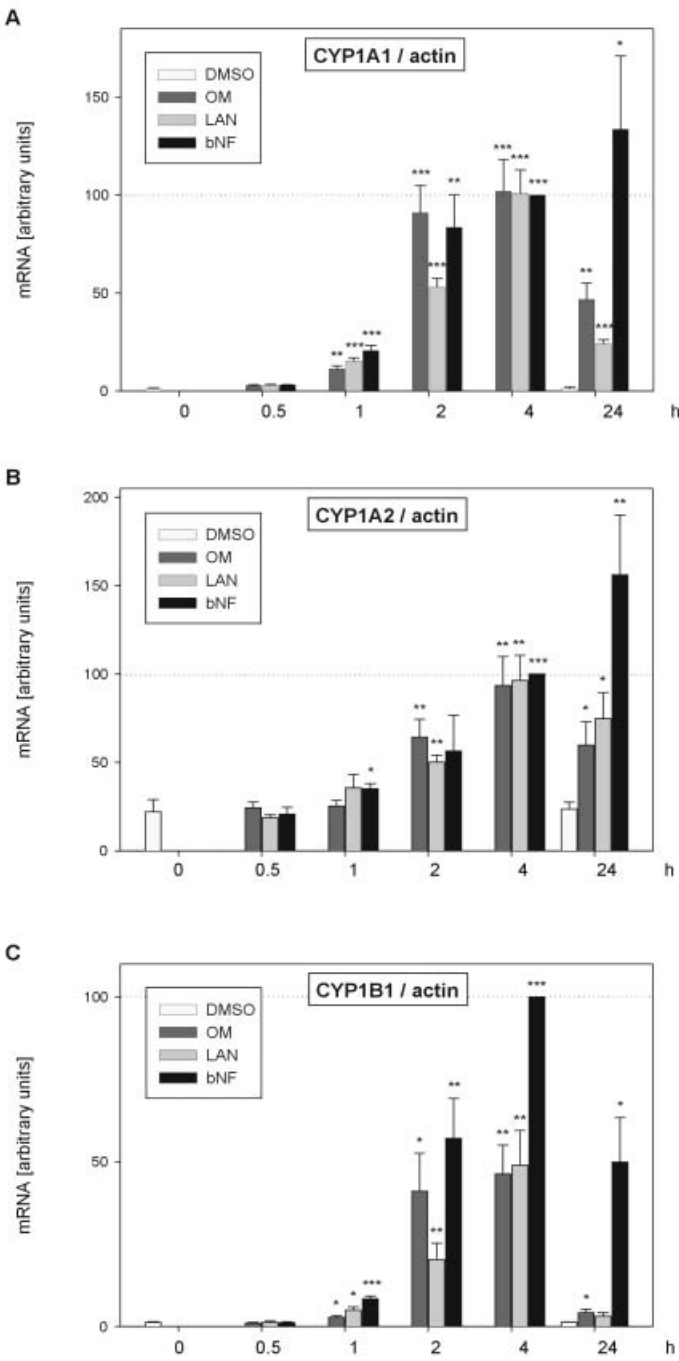


Figure 2. Time course of CYP1 induction. HepG2 cells were incubated with vehicle (DMSO), 100 μ M omeprazole (OM), 50 μ M lansoprazole (LAN) or 20 μ M β -naphthoflavone (β NF) for the indicated times. 2 kBq ^{32} P- α dCTP was added to each PCR (CYP1A1, 24 cycles; CYP1A2, 32 cycles; CYP1B1, 28 cycles; actin, 17 cycles). Columns represent the mean and SEM of four independent experiments. Marked columns are significantly different from DMSO control, determined by unpaired two-tailed Student's *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. (A) CYP1A1; (B) CYP1A2; (C) CYP1B1.

induced by β -naphthoflavone decreased to 50% of the 4-h value ($p < 0.01$). The amount of CYP1A1 mRNA decreased to 46% for omeprazole ($p < 0.05$) and to 24% for lansoprazole ($p < 0.001$) from 4 to 24 h of treatment. The change of CYP1A2 mRNA was not significant. From 4 to 24 h of incubation with each of the three substrates, CYP1B1 mRNA was reduced substantially (omeprazole $p < 0.01$, lansoprazole $p < 0.01$), leaving only 9% of omeprazole and 6% of lansoprazole-induced mRNA after 24 h. By this time, CYP1B1 mRNA levels of omeprazole and lansoprazole treated cells had decreased from 40-fold (4 h) to threefold of vehicle, and values of lansoprazole treated cells did not differ significantly from the DMSO control.

Inhibition of CYP1 induction by actinomycin D

To confirm that induction was due to new synthesis of mRNA, actinomycin D ($10 \mu\text{g ml}^{-1}$) was added to the medium 10 min before omeprazole (figure 3). Omeprazole-mediated induction of new CYP1A1, CYP1A2 and CYP1B1 mRNA synthesis was completely prevented by actinomycin D treatment.

Stabilization of CYP1 mRNA by actinomycin D

Treatment of cells with the inhibitor of transcription, actinomycin D, after induction with omeprazole was originally intended to determine the half-life of CYP1 mRNAs. This approach revealed the expected results for the aryl hydrocarbon receptor (AHR), included as a control (figure 4D), but not for the CYP1 mRNAs. Cells were incubated with vehicle (DMSO), actinomycin D or omeprazole for 24 h, or omeprazole for 2 h, or omeprazole for 24 h with actinomycin D added 2 h after starting the incubation with omeprazole. In contrast to the other experiments, values were not normalized to actin because actin mRNA amounts



Figure 3. Inhibition of CYP1 induction by actinomycin D. Pretreatment of HepG2 cells with $10 \mu\text{g ml}^{-1}$ actinomycin D or vehicle (DMSO) for 10 min was followed by addition of $100 \mu\text{M}$ omeprazole (OM) or vehicle (DMSO) and subsequent incubation of cells for 6 h. 25 ng total RNA was submitted to RT-PCR (CYP1A1, 25 cycles; CYP1A2, 32 cycles; CYP1B1, 30 cycles; actin, 17 cycles).

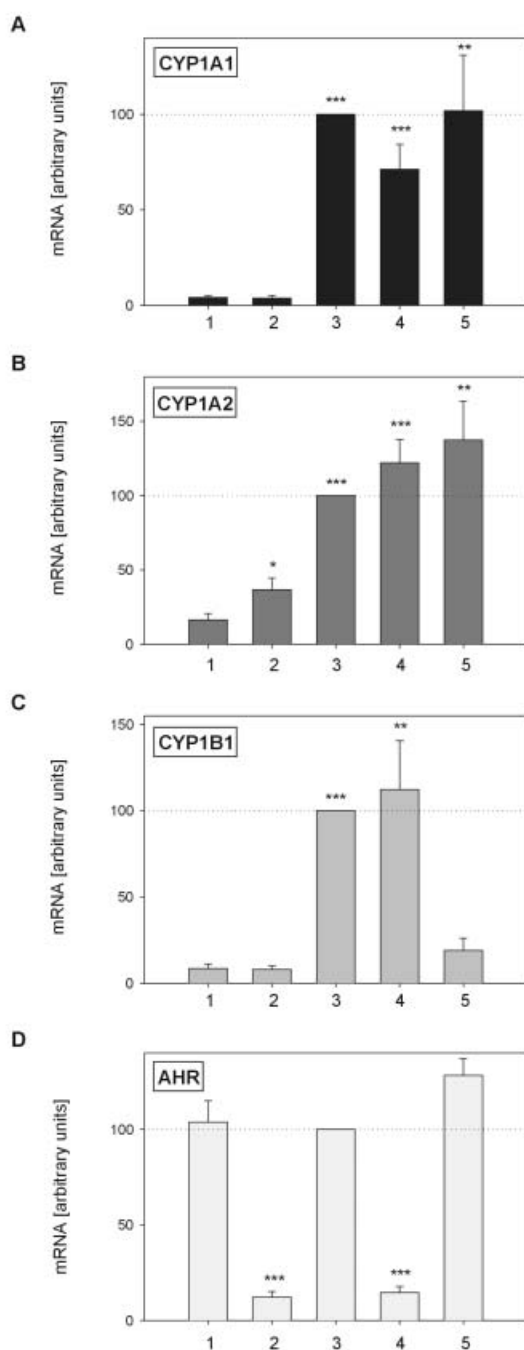


Figure 4. Stabilization of CYP1 mRNA by actinomycin D. HepG2 cells were treated with (1) DMSO for 24 h, (2) 10 $\mu\text{g ml}^{-1}$ actinomycin D for 24 h, (3) 100 μM omeprazole for 2 h, (4) 100 μM omeprazole for 24 h, addition of 10 $\mu\text{g ml}^{-1}$ actinomycin D after 2 h for the remaining 22 h, (5) 100 μM omeprazole for 24 h. PCR conditions were CYP1A1, 23 cycles; CYP1A2, 31 cycles; CYP1B1, 29 cycles; and AHR, 25 cycles. Results are the mean and SEM of six experiments. Marked columns are significantly different from vehicle control, determined by unpaired two-tailed Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A) CYP1A1; (B) CYP1A2; (C) CYP1B1; (D) AHR.

were decreased by actinomycin D (data not shown). Instead, values are based on equal mRNA concentration determined photometrically. Unexpectedly, the CYP1 mRNA synthesized within the 2-h omeprazole treatment was not degraded after the following 22-h incubation with actinomycin D. Only a small but not significant decrease of the amount of omeprazole-induced CYP1A1 mRNA was observed following incubation with actinomycin D (figure 4A). The amounts of CYP1A2 and CYP1B1 mRNA induced by omeprazole after 2 h were not decreased by actinomycin D. There was no significant difference between the CYP1A2 mRNA amounts obtained after 2 or 24 h of omeprazole-treatment of cells, nor did actinomycin D change these values. Incubation of cells with actinomycin D only resulted in a significant increase of CYP1A2 mRNA compared with vehicle ($p < 0.05$). As shown in figures 2C and 4C, CYP1B1 mRNA induced by omeprazole was substantially decreased between 2 and 24 h. This decrease was completely prevented by actinomycin D, if added 2 h after omeprazole for the remaining 22 h (figure 4C). Contrary to the effects of actinomycin D observed usually, this inhibitor of transcription stabilized CYP1B1 and also CYP1A2 mRNA.

Induction of CYP1 in primary human hepatocytes

Equal amounts of mRNA from human liver and primary human hepatocytes, determined photometrically, were applied to RT-PCR. A comparison between liver and untreated hepatocytes revealed a lower concentration of albumin and a higher concentration of actin and AHR mRNA in hepatocytes than in liver. Whereas CYP1A1 and CYP1B1 mRNAs were expressed at low levels both in hepatocytes and liver, CYP1A2 was down-regulated in primary hepatocytes (figure 5).

As shown for HepG2 cells, basal expression of CYP1A1, CYP1A2 and CYP1B1 was also low in primary human hepatocytes (figure 5). CYP1A1 and CYP1B1 were induced by omeprazole and lansoprazole and to a smaller extent by rabeprazole. No induction was observed for pantoprazole. Induction of CYP1A2 mRNA was lower than that of the other CYP1 mRNAs.

Discussion

The enzymes of the CYP1 family can be induced by a large variety of substances with completely different chemical structures (reviewed in Delescluse *et al.* 2000). Whereas much information exists about CYP1A1 induction, especially by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), much less data are available on the regulation of CYP1A2 and CYP1B1. The benzimidazole derivatives omeprazole and lansoprazole have been reported earlier to increase CYP1A1 in human cells of different origin, including hepatocytes (Diaz *et al.* 1990, Curi-Pedrosa *et al.* 1994, Daujat *et al.* 1996, Krusekopf *et al.* 1997, Frotschl *et al.* 1998). Another H^+/K^+ -ATPase inhibitor with benzimidazole-like structure, pantoprazole, has only a weak and usually insignificant influence on CYP1A1 expression (Krusekopf *et al.* 1997, Masubuchi *et al.* 1998), although it can induce transcription of CYP3A4 mRNA (Krusekopf *et al.* submitted). A fourth H^+/K^+ -ATPase inhibitor, rabeprazole, induces CYP1A1 significantly, but to a smaller extent than omeprazole and lansoprazole (Krusekopf *et al.* 1997). The antimalaria drug primaquine has

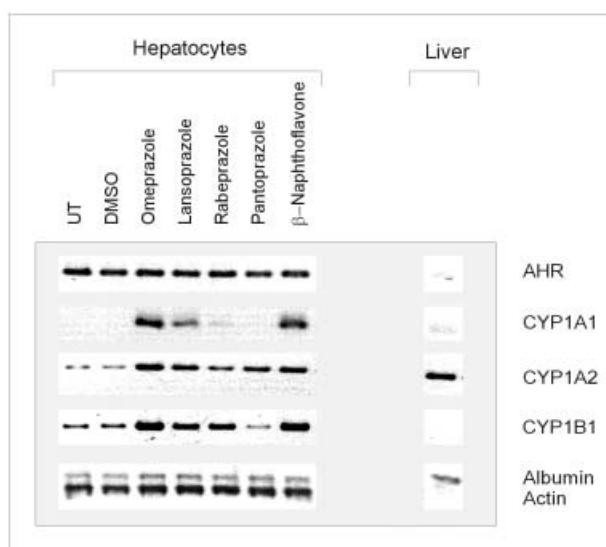


Figure 5. Induction CYP1 in primary human hepatocytes. Primary human hepatocytes were untreated or treated for 24 h with vehicle (DMSO), 100 μ M omeprazole, 50 μ M lansoprazole, 50 μ M rabeprazole, 200 μ M pantoprazole or 20 μ M β -naphthoflavone. RT-PCR was performed with 16 ng total RNA from hepatocytes and from human liver for comparison. PCR conditions were CYP1A1, CYP1A2, CYP1B1, AHR, 32 cycles; and albumin + actin, 20 cycles.

been shown to increase CYP1A1 expression in HepG2 cells and human hepatocytes considerably (Fontaine *et al.* 1999).

Here we used RT-PCR with enzyme-specific primers to measure induction of CYP1A1, CYP1A2 and CYP1B1 by these drugs. The results achieved for CYP1A1 correspond to the data reported earlier (Krusekopf *et al.* 1997).

In addition to an increase in CYP1A2 activity (measured by the caffeine breath test) in humans after treatment with omeprazole (Rost *et al.* 1994), induction of CYP1A2 mRNA and protein by benzimidazoles has been described by Diaz *et al.* (1990) (omeprazole) and Curi-Pedrosa *et al.* (1994) (omeprazole and lansoprazole), using primary human hepatocytes. This is in agreement with our results in HepG2 cells. Additionally, we found induction of CYP1A2 by rabeprazole and primaquine, but not by pantoprazole.

After 4-h treatment, endogenous CYP1B1 mRNA was induced in HepG2 cells by omeprazole, lansoprazole and primaquine (figure 1C). In these cells, Shenin *et al.* (2000) reported transcriptional activation of CYP1B1 by TCDD by use of CYP1B1 promoter-luciferase reporter gene assays. Contrary to these latter results, several authors did not detect any induction of endogenous CYP1B1 in HepG2 cells after 24-h TCDD treatment (Spink *et al.* 1994, Kress and Greenlee 1997, Shenin *et al.* 2000). Although TCDD was not included in our study, the time course of CYP1B1 mRNA induction (figure 2C) provides a possible explanation for this observation. The amount of CYP1B1 mRNA is extensively upregulated after 4 h of treatment with omeprazole, lansoprazole and β -naphthoflavone, but has nearly dropped to control level after 24 h (for omeprazole and lansoprazole). Induction by TCDD might also be transient and therefore not detectable in a 24-h experiment. As a conclusion from these data, expression of hepatic CYP1B1,

though barely detectable in normal liver, can be induced in permanent hepatoma cells and primary hepatocytes, but is unlikely to contribute substantially to the carcinogenic potential caused by the CYP1 enzymes because of the transient nature of the induction.

Although all CYP1 mRNAs were upregulated by the same drugs after 4 h of treatment, there are considerable differences between the enzymes concerning the time dependence of this effect. Similar results have been observed by Shih *et al.* (1999) in primary rabbit hepatocytes, where omeprazole-induced CYP1A1 mRNA was down-regulated earlier than CYP1A2 mRNA. For all CYP1 forms, omeprazole- and lansoprazole-mediated induction was more transient than induction by β -naphthoflavone. This observation is in agreement with a comparison of CYP1A1 induction by omeprazole and 3-methylcholanthrene in rabbit hepatocytes, which demonstrated a decrease in omeprazole-induced CYP1A1 mRNA after 24 h, whereas the same mRNA induced by 3-methylcholanthrene remained unchanged up to 30 h (Shih *et al.* 1999). In HepG2 cells treated with omeprazole and lansoprazole, the duration of mRNA induction was as follows: CYP1B1 was the most short-lived, CYP1A2 was long lasting and CYP1A1 was intermediate. This result seems contradictory to a recent report by Lekas *et al.* (2000) stating that TCDD-induced CYP1A1 mRNA is more rapidly degraded in HepG2 cells than the mRNA for CYP1A2 and CYP1B1. The latter authors used DRB (5,6-dichloro-1- β -D-ribofuranosylbenzimidazole) to inhibit transcription. We were not able to estimate the half-life of CYP1 mRNAs by inhibiting transcription with actinomycin D, although this method worked well for control mRNAs (figure 4D). Whereas pretreatment with actinomycin D completely inhibited subsequent induction of all CYP1 mRNAs by omeprazole (figure 3), this inhibitor prolonged CYP1 induction when added after mRNA synthesis had taken place. This RNA-stabilizing effect of actinomycin D was most prominent for CYP1B1 and CYP1A2, completely inhibiting the decrease of CYP1B1 mRNA observed after 24 h without actinomycin D (figure 4C). As a conclusion, the rapid decay of CYP1A1 reported by Lekas *et al.* (2000) might result from a TCDD-triggered effect that does not occur with benzimidazoles or β -naphthoflavone. This effect might depend on the different mechanisms of CYP1A1 induction, requiring binding to AHR for TCDD, but not for omeprazole (reviewed in Delescluse *et al.* 2000). Nevertheless, CYP1A2 and CYP1B1 mRNAs might be stabilized by DRB as well as by actinomycin D, so that rapid decrease of CYP1B1 would not be observed under the experimental conditions used by Lekas *et al.* (2000).

Stabilization of proteins is a well-known effect caused by the inhibitor of protein synthesis, cycloheximide. The molecular mechanism of this stabilization is assumed to be dependent on cycloheximide-mediated inhibition of the synthesis of proteins with high turnover, which are involved in the degradation process of the stabilized proteins (Ma *et al.* 2000, Ma and Baldwin 2002). The stabilizing effect on CYP1B1 and CYP1A2 mRNAs produced by actinomycin D might be based on a similar mechanism. The data presented in figure 4 are consistent with the hypothesis that one or several labile proteins, whose synthesis is blocked by actinomycin D, participate in active degradation of CYP1B1 and CYP1A2 mRNAs. Omeprazole-mediated induction of CYP1A1, CYP1A2 and CYP1B1 was also increased by cycloheximide (data not shown).

Omeprazole-mediated CYP mRNA induction was completely inhibited by actinomycin D. This result, together with a significant mRNA increase after 1 h of

treatment, confirms *de novo* synthesis of mRNA as the mechanism of induction for all cytochromes tested rather than post-transcriptional mechanisms like RNA stabilization. Induction by *de novo* synthesis of RNA has also been reported for omeprazole-mediated CYP1A1 induction in Caco-2 cells (Daujat *et al.* 1996). Transcriptional regulation of CYP1A1, CYP1A2 and CYP1B1 has been supported by reporter gene assays (Postlind *et al.* 1993, Shenin *et al.* 2000), by correlation of CYP1A1 and CYP1A2 mRNA amounts and enzyme activity in primary human hepatocytes (Rodriguez-Antona *et al.* 2001) and by correlation of CYP1A1 mRNA, protein and enzyme activity in HepG2 cells (Krusekopf *et al.* 1997). Although additional post-translational regulation by primaquine has been reported recently for CYP1A1 in a rat hepatoma cell line, the main mechanism of primaquine-mediated CYP1A1 induction is XRE (xenobiotic response element)-dependent and therefore transcriptional (Werlinder *et al.* 2001).

Despite the low expression of CYP1A2 in untreated HepG2 cells compared with human liver, all xenobiotics that induce CYP1A1, CYP1A2 and CYP1B1 mRNAs in HepG2 cells also do so in primary human hepatocytes. This corresponding regulation in primary and permanent cells is also seen for the only non-inducing drug, pantoprazole. Therefore, HepG2 cells can be used as an *in vitro* test system to predict human CYP1 induction.

Acknowledgements

The authors thank Dr G. Jennings from HepaVec for providing the primary human hepatocytes and Professor K. Ruckpaul for a critical reading of the manuscript. Dr W. H. Schunck and his team are gratefully acknowledged for the opportunity to work in his laboratory. This work was supported by the Federal Institute for Drugs and Medical Devices.

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Induction of hepatic cytochrome P450s responsible for the metabolism of xenobiotics by nicardipine and other calcium channel antagonists in the male rat

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Received 19 June 2002

1. The effects of nicardipine and three other calcium channel antagonists, nifedipine, diltiazem and verapamil, on hepatic gene expression of cytochrome P450s (P450), CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP3A2 in male rats were examined by an RT-PCR method.

2. Treatment of rats with nicardipine resulted in a significant increase in hepatic expression of all the P450 genes examined. Other calcium channel antagonists, nifedipine, diltiazem and verapamil, also enhanced the gene expression of CYP2B1, CYP2B2, CYP3A1 and CYP3A2, although these showed no capacity for activating CYP1A1 and CYP1A2 genes.

3. We have demonstrated for the first time that nicardipine activated not only the genes of CYP2B1, CYP2B2, CYP3A1 and CYP3A2, but also those of CYP1A1 and CYP1A2 in the rat liver and have further suggested that calcium channel antagonists may show a common capacity for activating the genes of CYP2B1, CYP2B2, CYP3A1 and CYP3A2. Furthermore, this increased expression of P450 genes was demonstrated to contribute to increase in the protein level of the corresponding P450s.

Introduction

Calcium channel antagonists are widely used for the treatment of hypertension. However, these drugs have been known to serve as inhibitors of cytochrome P450 (P450) forms in animals and humans (Guengerich *et al.* 1986, 1991, Mäenpää *et al.* 1989, Pichard *et al.* 1990, Kroemer *et al.* 1993, Murray and Butler 1996) and to participate in drug–drug interactions (Bertz and Granneman 1997). On the other hand, administration of calcium channel antagonists such as nifedipine, verapamil and diltiazem to rats has been reported to increase the metabolism of several drugs *in vivo* and shorten the hexobarbital-sleeping time (Koleva and Stoychev 1993, 1995). More recently, calcium channel antagonists such as nifedipine and nicardipine have been demonstrated to increase the level of protein and activity of hepatic P450s, including the CYP2B and CYP3A subfamily enzymes, in rats (Zangar *et al.* 1999). These reports suggest that it is important to determine the mechanism of calcium channel antagonist-mediated induction of hepatic P450s.

In the present study, we have examined the effects of nicardipine and three other calcium channel antagonists on the expression of hepatic P450 genes in male

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rats and demonstrated that the calcium channel antagonists selectively increase several P450 mRNAs.

Materials and methods

Chemicals

Nicardipine hydrochloride (Nic), nifedipine (Nif), diltiazem hydrochloride (Dil) and verapamil hydrochloride (Ver) were purchased from Wako Pure Chemicals, (Osaka, Japan). All chemicals used were the highest grade available.

Animals and treatment

Male F344 rats (6 weeks old) were purchased from Japan SLC Animal (Hamamatsu, Japan), and used at 7 weeks of age. They were kept in plastic cages in an air-conditioned room and given MF diet (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Nic and Nif, and Dil and Ver were dissolved in corn oil and in saline, respectively. Rats were treated orally (p.o.) with the calcium channel antagonists and were killed at the indicated time. In addition, rats were starved for 12 h before treatment with a calcium channel antagonist.

RNA preparation and RT-PCR analysis

Total hepatic RNAs were prepared by the method of Chomczynski and Sacchi (1987) and used for the determination of P450 gene expression, including CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP3A2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an internal control. A portion (4 µg) of total RNA was converted to cDNA by use of poly d(N)₆ primer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) and Moloney murine leukaemia virus reverse transcriptase (GIBCO BRL, Tokyo, Japan) in an RT-reaction mixture (20 µl). PCR was performed in a total reaction mixture (25 µl) containing 0.8 µl of the RT-reaction mixture, 0.5 µl of the corresponding primer sets (table 1), and AmpliTaq gold DNA polymerase (Applied Biosystem, Branchburg, NJ, USA). The primers and amplification conditions used are summarized in table 1. PCR products were separated by electrophoresis on a 2% agarose gel. The amount of the separated PCR product was determined densitometrically with a computer using Kodak 1D Image Analysis Software (Macintosh 1D version 2.02) after visualization by ethidium bromide staining under ultraviolet light, and was normalized to that of the GAPDH gene.

Microsome preparation

Liver was homogenized with 3 vols (w/v) of 1.15% KCl in a Polytron homogenizer (Kinematica, Littau, Switzerland). Microsomes were prepared from the liver homogenates by differential centrifugation as described by Degawa *et al.* (1989), and microsomal protein was assayed by the method of Lowry *et al.* (1951).

Western blot analysis of P450s

Western blot analysis was performed by the method, as described by Degawa *et al.* (1989). Briefly, each microsomal preparation (15 µg protein/lane for CYP1A and CYP3A, 30 µg protein/lane for CYP2B) solubilized with sodium dodecyl sulfate (SDS) was developed by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred from the gel to a nitrocellulose sheet and then immunostained by means of horseradish peroxidase-linked immunosorbent assay using 0.05% 3,3'-diaminobenzidine tetrachloride (Sigma-Aldrich, Tokyo, Japan) and a mouse anti-rat CYP1A1 monoclonal antibody APL-2 (Degawa *et al.*, 1989), a goat anti-rat CYP2B1 polyclonal antibody, or a rabbit anti-rat CYP3A2 polyclonal antibody (Daichi Pure Chemicals, Tokyo, Japan). In addition, APL-2 monoclonal antibody is reactive not only with CYP1A1 but also with CYP1A2 (Degawa *et al.* 1989). Likewise, the goat anti-rat CYP2B1 and rabbit anti-rat CYP3A2 polyclonal antibodies used were reactive with CYP2B1/2 and CYP3A1/2, respectively.

Measurement of microsomal P450 activities

Hepatic microsomal activities for methoxyresorufin *O*-dealkylation (MROD) and pentoxyresorufin *O*-dealkylation (PROD) were determined by the method as described by (Burke *et al.* 1994). Briefly, a reaction medium (495 µl) contained hepatic microsomes (50 µg protein for MROD, 100 µg protein for PROD) and an NADPH generating system (0.8 mM NADP⁺, 8mM glucose 6-phosphate, 2.5mM MgCl₂, 2 unit ml⁻¹ glucose 6-phosphate dehydrogenase) in 100 mM potassium phosphate buffer (pH 7.4). After pre-incubation of the incubation medium for 2 min at 37 °C, the reaction was started by the addition of a

Table 1. PCR primer sets and PCR conditions used in the present experiments.

P450 forms	Primer set	Reaction condition			Product size	Reference
		Denaturation	Annealing	Elongation		
CYP1A1 forward	5'- TGACCTCTTTGGAGCT-3'	95 °C 1 min	50 °C 0.5 min	72 °C 2 min	1050 bp	Kim <i>et al.</i> (1991)
CYP1A1 reverse	5'-TTGAGCCTCAGCAGAT-3'					
CYP1A2 forward	5'-GATGAGAAAGCAGTGGAAGACC-3'	95 °C 1 min	50 °C 0.5 min	72 °C 2 min	328 bp	Gomez-Lechon <i>et al.</i> (1998)
CYP1A2 reverse	5'-AAAAAGAAAGGAGAAACA-3'					
CYP2B1 forward	5'-GCTCAAGTACCCCATGTGC-3'	93 °C 1 min	54 °C 1.5 min	72 °C 1 min	109 bp	Omiecinski <i>et al.</i> (1990)
CYP2B1 reverse	5'-ATCAGTGTATGGCATTTTACTGCGG-3'					
CYP2B2 forward	5'-CTTTGCTGGCAC TGAGACCG-3'	93 °C 1 min	54 °C 1.5 min	72 °C 1 min	163 bp	Omiecinski <i>et al.</i> (1990)
CYP2B2 reverse	5'-ATCAGTGTATGGCATTTTGGTACGA-3'					
CYP3A1 forward	5'-GATGTTGAAATCAATGGTGTGT-3'	95 °C 1 min	60 °C 1 min	72 °C 2 min	289 bp	Oinonen and Lindros (1995)
CYP3A1 reverse	5'-TTCAGAGGTATCTGTGTTTCC-3'					
CYP3A2 forward	5'-AGTAGTGACGATTTCCAACATAT-3'	95 °C 1 min	60 °C 1 min	72 °C 2 min	252 bp	Oinonen and Lindros (1995)
CYP3A2 reverse	5'-TCAGAGGTATCTGTGTTTCCCT-3'					
GAPDH forward	5'-TTCAACGGCACAGTCAAGG-3'	95 °C 1 min	60 °C 1 min	72 °C 2 min	373 bp	Nemoto <i>et al.</i> (2000)
GAPDH reverse	5'-CATGGACTGTGGTCATGAG-3'					

substrate (500 μM methoxyresorufin or 500 μM pentoxyresorufin; 5 μl) dissolved in dimethylsulfoxide, and 5 min later stopped by the addition of cold ethanol (500 μl). The reaction mixture was centrifuged for 10 min at 2500g, and the amount of resorufin formed in the supernatant was measured with a spectrofluorometer (Hitachi F2000) at excitation (550 nm) and emission (575 nm).

Microsomal nifedipine oxidase activity was determined by the method of (Bourrie *et al.* 1996). Briefly, the reaction medium (495 μl) contained liver microsomes (250 μg protein) and an NADPH-generating system (0.8 mM NADP⁺, 8 mM glucose 6-phosphate, 2.5 mM MgCl₂, 2 unit ml⁻¹ glucose 6-phosphate dehydrogenase) in 100 mM potassium phosphate buffer (pH 7.4). After pre-incubation of the reaction medium for 5 min at 37 °C, the reaction was started by the addition of substrate (20 mM nifedipine; 5 μl) dissolved in ethanol and 10 min later stopped by the addition of cold ethanol (500 μl). The reaction mixture was centrifuged for 10 min at 2500g, and the amount of the oxidized metabolite of nifedipine in the supernatant was measured by high-pressure liquid chromatography (ODS-2 column; 5 μm particle size, elution solvent; 60% methanol in water, flow rate; 0.8 ml min⁻¹, detection; 235 nm).

Statistical analysis

Significant differences from the corresponding controls were assessed by the Student's *t*-test.

Results

Enhanced gene expression of P450s

We first examined the dose-effect of Nic on hepatic gene expression of P450s, including CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP3A2, in male rats. Rats were treated with Nic at doses of 50, 100, 200, 400 and 600 $\mu\text{mol kg}^{-1}$ and killed 24 h later. Hepatic RNA preparations obtained were used for the assessment of expression level of each P450 gene by the RT-PCR method. Representative expression patterns of the P450 genes are shown in figure 1. Gene expression levels of the P450s, with the exception of CYP1A1, were significantly increased at a dose of 50 $\mu\text{mol kg}^{-1}$, and significant increase in the CYP1A1 gene was observed at doses greater than 200 $\mu\text{mol kg}^{-1}$ (figure 2). The expression levels of CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and

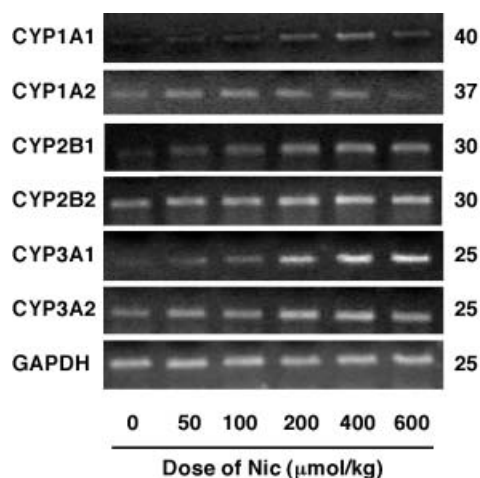


Figure 1. Representative gene expression patterns of several P450s from Nic-treated rat liver. Rats were treated with Nic at doses of 50, 100, 200, 400 and 600 $\mu\text{mol kg}^{-1}$, and were killed 24 h later. Hepatic total RNAs were extracted from the pooled livers from three rats in each experimental group, and were used for RT-PCR analysis. Each number on the right indicates the number of cycles set for PCR.

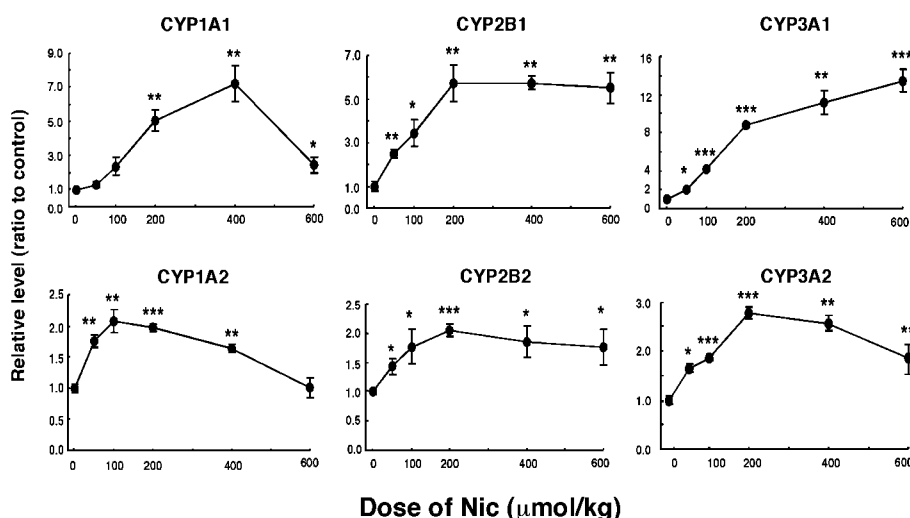


Figure 2. Dose-dependency of Nic-enhanced gene expression of hepatic P450s in the rat. Rats were treated with Nic at doses of 50, 100, 200, 400 and 600 $\mu\text{mol kg}^{-1}$, and were killed 24 h later. Total hepatic RNAs were extracted from three individual rats in each experimental group and were used for RT-PCR analysis with PCR cycles as shown in figure 1. Values represent relative levels to the corresponding controls, and closed circles and bars show means and their standard deviations, respectively. Significant differences from the corresponding controls (assessed by Student's *t*-test); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

CYP3A2 reached their maxima at doses of 400, 100, 200, 200, 600 and 200 $\mu\text{mol kg}^{-1}$, respectively. In addition, no change in the gene expression level of GAPDH, an internal control, was observed in any of the experimental groups.

We next examined the time-dependency of the changes in P450 gene expression after Nic treatment. In these experiments, we selected 200 $\mu\text{mol kg}^{-1}$ as the dose because it produced significant increases in the expression of the all P450 genes examined (figure 2). Both expression levels of the CYP1A1 and CYP1A2 genes were significantly increased at 24 and 36 h, but not at any other periods (figure 3). On the other hand, the expression levels of the CYP2B1 and CYP2B2 genes increased up to 12 h and 24 h, respectively, and were maintained up to 36 h and returned to the corresponding control values at 48 h. As for the CYP3A1 and CYP3A2 genes, expression levels of the genes were increased in a time-dependent fashion up to 24 h, and were maintained up to 36 h and, thereafter, reduced. In addition, no change in the expression level of the GAPDH gene, an internal standard, was observed throughout the experiments.

We selected Nif, Dil and Ver as calcium channel antagonists with different chemical characters and examined whether or not these antagonists were capable of activating P450 genes. Representative expression patterns of the P450 genes from Nif-, Dil- and Ver-treated rats are shown in figure 4. All three calcium channel antagonists enhanced CYP2B1, CYP2B2, CYP3A1 and CYP3A2 gene expression (figure 4). In contrast to Nic, however, these other three antagonists showed no capacity for activating CYP1A1 and CYP1A2 genes. As for the gene activation of CYP2B1 and CYP2B2, Nif and Nic showed higher activity than did the others (figure 5), whereas for CYP3A1 and CYP3A2, the inducing ability of Nif, Dil and Ver were less than that of Nic.

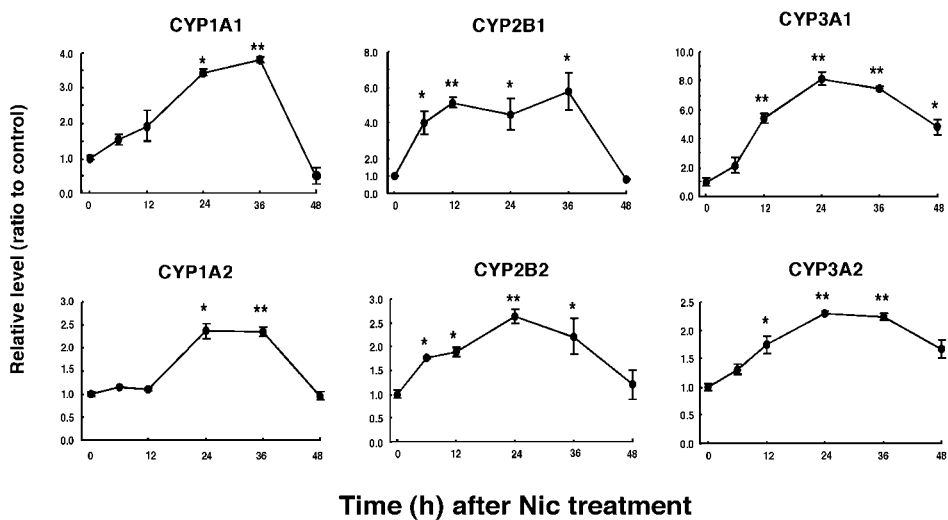


Figure 3. Time dependency of Nic-enhanced gene expression of hepatic P450s in the rat. Rats were treated with Nic ($200\mu\text{mol kg}^{-1}$) or a vehicle alone (control, time 0) and killed 6, 12, 24, 36 or 48 h later. Total hepatic RNAs were extracted from three individual rats in each experimental group and were used for RT-PCR analysis with PCR cycles, as shown in figure 1. Values represent relative levels to the corresponding controls, and closed circles and bars show means and their standard deviations, respectively. Significant differences from the corresponding controls (assessed by Student's *t*-test); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

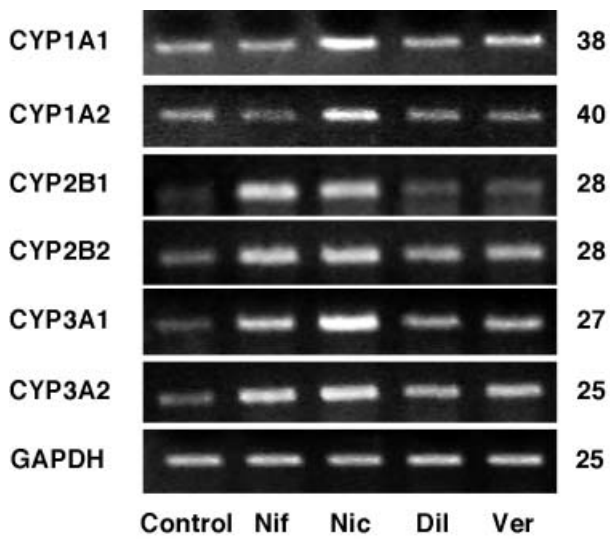


Figure 4. Representative gene expression patterns of hepatic P450s after treatments of rats with calcium channel antagonists. Rats were treated with corn oil (control) or a calcium channel antagonist (Nif, Nic, Dil or Ver; $200\mu\text{mol kg}^{-1}$), and were killed 24 h later. Total hepatic RNAs were extracted from the pooled livers from three rats in each experimental group, and were used for RT-PCR analysis. Each number on the right indicates the number of cycles set for PCR.

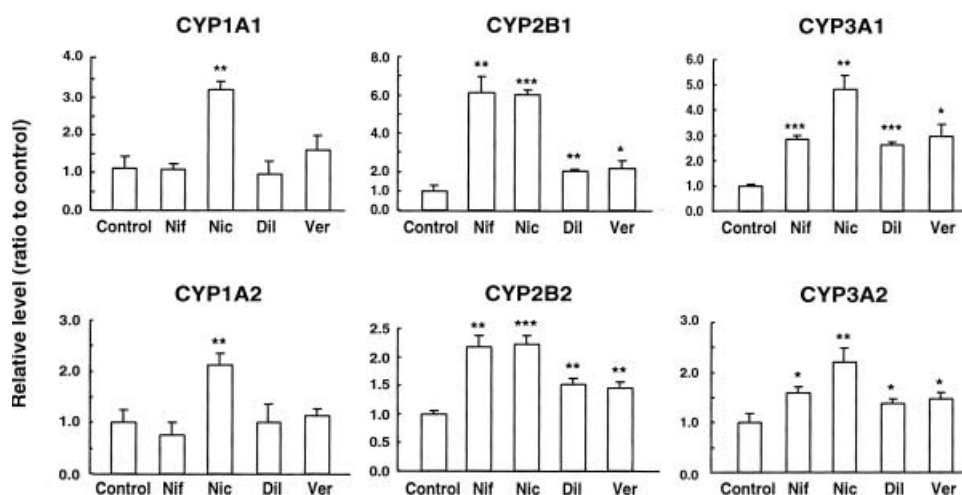


Figure 5. Enhanced gene expression of hepatic P450s by calcium channel antagonists. Rats were treated with corn oil (control) or a calcium channel antagonist (Nif, Nic, Dil or Ver; $200\mu\text{mol kg}^{-1}$), and were killed 24 h later. Total hepatic RNAs were extracted from three individual rats in each experimental group and were used for RT-PCR analysis with PCR cycles, as shown in figure 4. Values represent relative levels to the corresponding controls, and data and bars show means and their standard deviations, respectively. Significant differences from the corresponding controls (assessed by Student's *t*-test); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Effect on P450 protein levels

To determine whether or not increases in the expression levels of P450 genes by the calcium channel antagonists Nic, Nif, Dil and Ver result in increases in the corresponding protein levels, we examined this by Western blot analysis after doses of $200\mu\text{mol kg}^{-1}$ (figure 6). In Nic-treated rats, increases in protein levels of CYP1A2, CYP2B1, CYP2B2 and CYP3A were observed, whereas increased level of CYP1A1 protein was hardly observed. In Nif-, Dil- or Ver-treated rats, protein levels of CYP2B1, CYP2B2 and CYP3A, but not those of CYP1A1 and CYP1A2, were increased. With respect to the CYP2B proteins, Nif showed the highest inductive effect, whereas Nic was the best inducer of CYP3As.

Effect on microsomal P450 activities

Hepatic microsomal activities for MROD, PROD and nifedipine oxidation, which are catalysed mainly by CYP1A2, CYP2B1/2 and CYP3A1/2 enzymes, respectively, were examined in rats treated with Nic, Nif, Dil or Ver ($200\mu\text{mol kg}^{-1}$). MROD activity was significantly increased by Nic treatment but not by any of the other antagonists (figure 7). Microsomal activities for PROD and nifedipine oxidation were significantly increased by treatment with all calcium channel antagonists examined. Nif, which produced the largest increase in CYP2B1 protein (figure 6), showed the highest capacity for increasing microsomal PROD activity (figure 7). Microsomal nifedipine oxidation was greatest in the rats treated with Nic, which also showed the highest capacity for increasing the level of CYP3A protein(s) (figure 6).

Discussion

In the present study, we have demonstrated for the first time that Nic activates not only CYP2B and CYP3A genes, but also those of CYP1A forms. Other calcium channel antagonists (Nif, Dil and Ver) also activated CYP2B and CYP3A genes but not that of CYP1As, suggesting that calcium channel antagonists show a common capacity for activating CYP2B and CYP3A genes but not necessarily for those of CYP1As. Furthermore, increases in the levels of protein and activity of the CYP2B and CYP3A enzymes by calcium channel antagonists corresponded to increases in the level of the corresponding mRNAs, although increases in levels of protein and activity of the CYP2B and CYP3A subfamilies by calcium channel antagonists have been reported (Zangar *et al.* 1999). However, increased levels of protein and activity of each P450 enzyme were not necessarily coincident with that of the corresponding mRNA. For example, Nic treatment resulted in a significant increase in CYP1A1 mRNA, whereas no significant increase in the corresponding protein level was observed. Likewise, increased levels of CYP2B1 mRNA by Nic and Nif were almost the same, whereas the corresponding increased levels of protein were much greater in Nif than that for Nic-treated rats. In addition, increased levels of the P450 proteins closely correlated with those of the corresponding enzyme activities.

Increases in the gene expression of CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A1 and CYP3A2 by Nic were dose- and time-dependent, although these dependencies were different between the P450 genes. The present findings suggest that an increase in the level of each P450 mRNA by Nic occurs through transcriptional activation of the corresponding P450 gene but not through stabilization of the mRNA. This was further supported by the fact that no change in the expression level of the GAPDH gene, an internal control, was observed in any Nic-treated rats. Furthermore, the other calcium channel antagonists, Nif, Dil and Ver, increased the CYP2B and CYP3A mRNA levels but not that of CYP1As, indicating that these also increased the level of CYP2B and CYP3A mRNAs by transcriptional activation.

As for the phenobarbital-mediated induction of CYP2B and CYP3A in mouse and rat primary cultured hepatocytes (Galisteo *et al.* 1999, Marc *et al.* 2000, Joannard *et al.* 2000), the calcium ion and Ca^{2+} /calmodulin-dependent protein kinase II have been reported to act as positive regulators. In the present study, however, calcium channel antagonists, which decrease the level of cellular calcium in rat hepatocytes (Hughes *et al.* 1986, Striggow and Bohnensack 1993), were found to activate CYP2B and CYP3A genes, suggesting that the mechanism for activation of the P450 genes by a calcium channel antagonist is different from that by phenobarbital. Furthermore, different expression patterns of P450 genes were observed by use of different calcium channel antagonists. For example, Nic and Nif showed similar capacities for activating CYP2B genes, whereas for activation of CYP3A genes, Nic was better than Nif. Likewise, Nic, but not any other calcium channel antagonists used, activated CYP1A genes. Thus, differences between the calcium channel antagonists also suggest that the enhanced gene expression of P450s by calcium channel antagonists not only occurs through a change in the concentration of cellular calcium. In addition, it is noteworthy that in spite of the chemical similarity between Nic and Nif, they showed different capacities for activating CYP1A genes: Nic, but not Nif, activated CYP1A genes, although a mechanism for the Nic-mediated gene activation remains unclear.

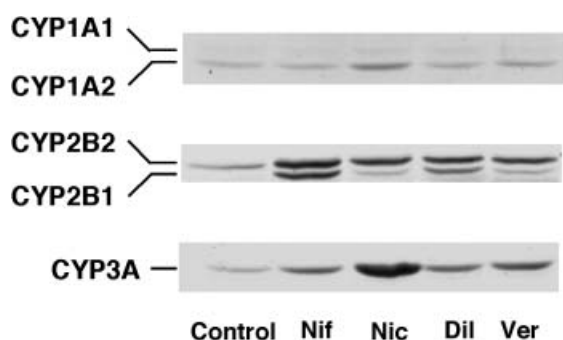


Figure 6. Representative expression patterns of several rat hepatic P450 proteins after calcium channel antagonist treatment. Rats were treated with corn oil (control) or a calcium channel antagonist (Nif, Nic, Dil or Ver; $200\mu\text{mol kg}^{-1}$), and were killed 24 h later. Hepatic microsomes were prepared from the pooled livers from three rats in each experimental group and were used for Western blot analysis.

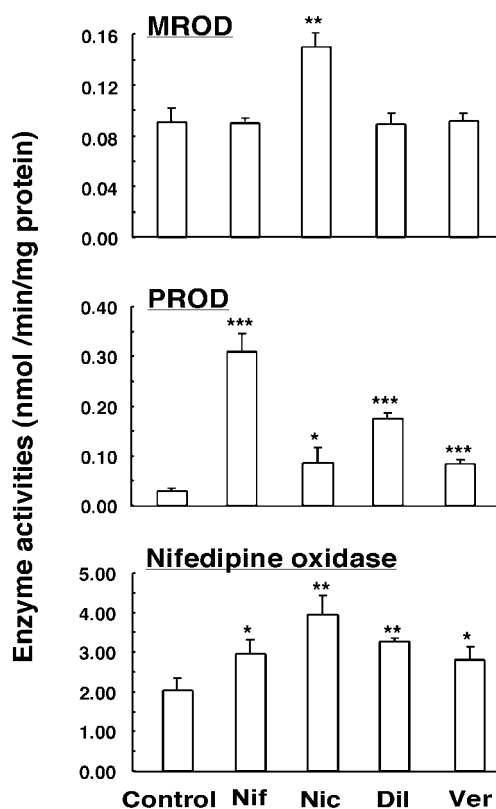


Figure 7. Increased microsomal enzyme activities after treatment with calcium channel antagonists. Rats were treated with corn oil (control) or a calcium channel antagonist (Nif, Nic, Dil or Ver; $200\mu\text{mol kg}^{-1}$), and were killed 24 h later. Hepatic microsomes were prepared from three individual rats in each experimental group and were used for measurement of enzyme activities. Value and bars represent means and their standard deviations, respectively. Significant differences from the corresponding controls (assessed by Student's *t*-test); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Since Nic does not possess the chemical structure required for binding to the Ah receptor, which is thought responsible for activation of the CYP1A genes (Whitlock 1999), Nic-mediated gene activation occurs by an unknown mechanism.

Recently, in human hepatocytes, Nic and Nif have been reported to act as ligands for the pregnane X receptor (PXR), which directly regulates gene expression of CYP2B6 and CYP3A4 (Drocourt *et al.* 2001). Accordingly, enhanced gene expression of CYP2B and CYP3A by Nic and Nif in rat liver likely occurs in part through a PXR-mediated pathway. Such a PXR-mediated pathway, however, is not a sufficient explanation for the difference in altered expression of the CYP2B and CYP3A genes by Nic and Nif. Likewise, since Dil and Ver are substantially different in chemical structure from the dehydropyridine type calcium channel antagonists, Nic and Nif, the former may not serve as PXR ligands.

We have demonstrated herein that calcium channel antagonists induce several P450 enzymes, especially CYP2B and CYP3A, through transcriptional activation of the corresponding P450 genes. In view of the fact that calcium channel antagonists are widely used for treatment of hypertension, our present findings indicate that clinical drug–drug interaction and toxicological drug–xenobiotic interaction may occur through induction of hepatic P450 enzymes by calcium channel antagonists.

Acknowledgements

This work was supported, in part, by a Grant-in-Aid from Scientific Research from the Japan Society for the Promotion of Science.

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Many P-glycoprotein substrates do not inhibit the transport process across cell membranes

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Received 4 July 2002

1. The critical role of P-glycoprotein (P-gp) in the clinical exposure of many pharmaceuticals and toxins has become widely appreciated. The P-gp-mediated influence can often be more significant than that of other well-known xenobiotic defence enzymes in both breadth and impact. The inhibition of P-gp, therefore, has often been examined by testing a compound for its influence on the P-gp-mediated transport of some marker substrate, often the compound is also evaluated for its active efflux mediated by P-gp.

2. Although a substrate for a xenobiotic defence enzyme is logically presumed to be an inhibitor of that enzyme toward an alternate substrate, that is not necessarily the case with a transmembrane active efflux transporter. A substrate that is ejected from the cytosolic side of the membrane bilayer that does not rapidly cross the membrane by passive diffusion back into the cell interior will not occlude the substrate binding site. Hence, some substrates may not significantly affect the overall P-gp function of causing a concentration gradient by efficient net transport. A wide variety of compounds that are documented as substrates of P-gp are characterized here as having no effect on the ability of P-gp to transport several conventional P-gp marker substrates.

3. Transbilayer passive diffusion apparently dictates the ability of a P-gp substrate to be an inhibitor, as described herein based on relative rates of transport (active efflux versus passive re-entry) and the interaction of amphipathic compounds with the cell membrane.

4. The portion of P-gp substrates whose disposition is dependent on P-gp function and which are not also inhibitors is striking. It is therefore important to characterize both the efflux rate parameters and those of inhibition.

5. This report affords a valuable list of known P-gp substrates that are non-inhibitors.

Introduction

Mammalian cells possess a natural battery of defence mechanisms against xenobiotic assault. A particular class of independent proteins actively transports an extensive array of structurally unrelated large lipophilic compounds from the cell, providing what is often known as multiple drug resistance (MDR) (Gottesman and Pastan 1993). Multidrug resistance is characterized by active efflux or pumping of xenobiotics and pharmaceuticals via transmembrane proteins acting as hydrophobic 'vacuum cleaners' (Gottesman and Pastan 1993, Gottesman *et al.* 1996). The protein product of the MDR1 gene encodes a 170 kD integral plasma membrane phosphorylated glycoprotein, P-glycoprotein (P-gp), which is the best known and most extensively studied among these transporters and which thus far appears to have the largest complement of substrates. The gross structural features

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of P-gp appear to be shared by a large family of membrane transporters known as ATP-binding cassette (ABC) transporters, which evidently act as ATP-driven pumps that remove xenobiotics from the interior of cells. Expression of P-gp in normal human tissues — particularly within the cellular membranes of the gastrointestinal tract, liver, blood–brain barrier, adrenals and kidneys — suggests that this protein plays a role in cellular protection as well as in secretion (Ambudkar *et al.* 1999). While the primary function of this protein is unknown, its ability to confer resistance to a wide variety of structurally and chemically unrelated compounds remains impressive. Indeed, the number of substrates list for this transporter reveals that P-gp shares a similar tolerance or acceptance as cytochrome P450 3A4 (CYP3A4), the predominant intestinal and hepatic cytochrome P450 oxygenase enzyme, and may even prove to be more extensive in its substrate recognition and as a route of drug elimination (Fisher *et al.* 1996, Wang *et al.* 2001b).

It is known that some P-gp inhibitors are not themselves actively transported by P-gp (not substrates). However, a xenobiotic defence (multifunctional) enzyme substrate defines competitive inhibition of that site to which it binds and is consequently regarded as a potential inhibitor of activity upon a co-administered substrate (even if slight). Yet, many P-gp substrates do not inhibit P-gp function, causing an effective net transport (or significant concentration gradient) across a cell membrane. Furthermore, we have characterized a remarkable number of substrates that lack detectable inhibition of overall P-gp efficiency. This distinction will be very important when evaluating the potential for drug interactions mediated by P-gp. A P-gp inhibitor can clearly affect the disposition of another P-gp substrate, while the many substrates that are non-inhibitors can still be significantly affected via the P-gp mechanism of drug interactions. Therefore, both characteristics must be quantified to examine rigorously the potential for P-gp-mediated drug interactions. In this paper, we also provide an explanation for the cause of this unique quality of a substrate's failure to inhibit competitively effective transport of another known P-gp substrate.

Materials and methods

Chemicals

DNR (Daunorubicin); Rho (Rhodamine 123); vinblastine, verapamil, EGCG ((-)-epigallocatechin gallate), erythromycin, digosin, lidocaine, cholchicine and cimetidine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tributylmethylammonium bromide, triethylmethylammonium bromide were obtained from Fluka (Milwaukee, WI, USA). Ciprofloxacin was obtained from US Biologicals (Swampscott, MA, USA). LDS (LDS 751) was purchased from Molecular Probes (Eugene, OR, USA). Cyclosporin A was purchased from Alexis Biochemicals (San Diego, CA, USA). Norfloxacin and puromycin were obtained from ICN (Costa Mesa, CA, USA). Hanks' balanced salt solution, Dulbecco's minimum essential medium (DMEM), penicillin/streptomycin, foetal bovine serum (FBS) and trypsin-EDTA were obtained from Life Technologies, Inc. (Rockville, MD, USA). All other reagents were of the highest grade commercially available.

Cell lines

The NIH-3T3-G185 cell line expressing the gene product of human MDR1 was licensed from the NIH and maintained in DMEM as described by Wang *et al.* (2001a).

Membrane microsome preparations

NIH-3T3-G185 cell membranes enriched with the MDR1 gene product transport enzyme were used for preparation of membrane microsomes. Colchicine ($0.5\text{ }\mu\text{g ml}^{-1}$) was added to the growth

culture medium to prevent phenotypic reversion. Cells were washed with complete Hanks' buffer before being resuspended in 10 ml lysis buffer (Tris-HCl 50 mM, mannitol 50 mM, EGTA 2 mM, dithiothreitol 2 mM, pH 7.0 at 25°C) containing protease inhibitors (phenylmethylsulfonyl fluoride 1 mM, aprotinin 10 µg ml⁻¹, leupeptin 10 µg ml⁻¹). All subsequent steps were performed at 4°C. The cells were lysed by nitrogen cavitation (Kontes Glass Co., Vineland, NJ, USA) at 500 psi for 20 min twice with whole cell sedimentation by centrifugation at 1000g for 10 min after each nitrogen cavitation. Nuclei and mitochondria were sedimented by centrifugation at 4000g for 10 min. The microsomal membrane fraction was then sedimented by centrifugation at 100 000g for 60 min. The pellet was resuspended in 0.25 M sucrose buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and homogenized using a Potter-Elvehjem homogenizer. Aliquots of membrane microsomes were rapidly frozen and stored at -80°C until analysis. Protein content was determined by the Lowry method (Lowry, 1951).

ATP hydrolysis and phosphate release

The consumption of ATP was quantified by determining the amount of liberated inorganic orthophosphate that forms a colour complex with molybdate (Wang *et al.* 2000a). The ATP hydrolysis assay based on phosphate-release determination using membrane microsome preparations was carried out in a 96-well microplate. The microsomes were thawed on ice before diluting to 15 µg protein per well in ice-cold ATPase buffer (sodium ATP 3 mM, KCl 50 mM, MgSO₄ 10 mM, dithiothreitol 3 mM, Tris-HCl 50 mM, pH 7.0) containing 0.5 mM EGTA (to inhibit Ca-ATPase), 0.5 mM ouabain (to inhibit the Na/K-ATPase) and 3 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume including the various inhibitors was 100 µl. The incubation reaction was initiated by transferring the plate from ice to 37°C, the plate was incubated for 30 min and the reaction was terminated by the addition of 50 µl 12% SDS solution at room temperature, followed by the addition of 50 µl of a colour development solution (equal volumes) of 18% fresh ascorbic acid in 1 N HCl and 3% ammonium molybdate in 1 N HCl. After 4 min, 100 µl of a solution of 2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid was added to fix the colour formation. After 30-min incubation at room temperature, the fixed released phosphate was quantitated colorimetrically using a microplate reader (Tecan SpectraFluor Plus, Research Triangle Park, NC, USA) at 830 nm. By comparison with a standard curve, the amount of phosphate released — and hence ATP consumed — was quantified. Water-insoluble drugs were dissolved in methanol or DMSO; the maximum methanol or DMSO concentration (2% v/v) was shown not to affect the ATPase activity.

FACS flow cytometry

Fluorescence measurements of individual cells were performed using a Becton-Dickinson FACS-calibur fluorescence-activated cell sorter (San Jose, CA, USA), equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 nm and 570/30 nm band-pass filters). Analysis was gated to include single viable cells on the basis of forward and side light scatter and was based on acquisition of data from 10 000 cells. Log fluorescence data were collected and displayed as single-parameter histograms. A modified inhibition assay for the SPGP efflux pump in viable transfected cells was performed with the flow cytometer (Wang *et al.* 2000c).

Cell viability test

Cell viability was assessed using propidium iodide staining. Dead cells in which propidium iodide was bound to double strands of DNA or RNA were detected in certain regions of the cytometry dot plots and not included in the final data calculations.

Calculation of relative fluorescence

The fluorescence intensity of individual cells was recorded as histograms. The mean fluorescence intensity of 10 000 cells was used for comparison among different conditions. Cyclosporin A was selected as a positive control and used to normalize the measurements because it can significantly inhibit the SPGP-mediated active efflux of fluorescent substrate markers. Relative fluorescence was used for quantitation and comparison among different compounds. The relative fluorescence (% maximal or reference inhibition) represents a ratio obtained through the following formula: the geometric mean fluorescence of a discrete sample divided by the geometric mean fluorescence in the presence of 50 µM cyclosporin A, times 100 or expressed as:

$$\text{Relative fluorescence} = \left(\frac{\text{fluorescence of sample geometric mean}}{\text{fluorescence of reference std. geometric mean}} \right) \times 100.$$

The resulting values represented the per cent inhibition of SPGP normalized for the reference (standard) inhibition and were used to compare the test drugs for inhibition potential.

Results

With the observation that various asserted P-gp substrates were unable to affect the ability of P-gp to transport some fluorescent P-gp substrates out of a viable cell, we characterized several of these P-gp substrates for transport kinetic parameters. As ATP is consumed at a purported rate of about one or two per transport event, the rate of hydrolysis of ATP represents transport rate or activity assay of function (Eytan *et al.* 1996a, Ambudkar *et al.* 1997, Stein 1997, Shapiro and Ling 1998, Sauna and Ambudkar 2000, 2001, Wang *et al.* 2000a). In the absence of exogenous substrate, the enzyme can still hydrolyse ATP to produce a baseline level of activity, a process that is probably due to the transport of endogenous substrates. Therefore, $V_{\max}$ data are presented as a per cent of the basal or control activity, as any change in the rate of ATP hydrolysis from basal activity of the exogenous substrate represents the transport activity of the added substrate. This influence (stimulus or supplanting) can result in a deficit from the baseline activity when the $V_{\max}$ of the test compound is lower than basal activity. The presence of erythromycin causes a concentration-dependent increase in the rate of ATP hydrolysis relative to the baseline rate, which indicates that it is a comparatively rapid substrate for P-gp (figure 1). K_m is about 0.12 mM, and $V_{\max}$ is about 2-fold above baseline (figure 1). Similar quantitative results following evaluation of selected compounds investigated in this study are shown in table 1.

P-glycoprotein inhibition

The IC_{50} (concentration at half maximum inhibition) could be determined from a simple function (figure 2), where the retained fluorescence was measured for samples of viable cells by a flow cytometer at varying concentrations of compound (Wang *et al.* 2000b). The concentration dependency of inhibition displayed a sigmoidal response curve (figure 2), a consequence of cooperativity (Wang *et al.* 2000b), with the Hill equation for allosteric interaction enzymes therefore being the appropriate function for fitting to the data:

$$I = I_{\max} S^n / (IC_{50} + S^n),$$

where I is the inhibition extent, $I_{\max}$ is the maximum inhibition, IC_{50} is the concentration at 50% inhibition, S is the inhibitor (or substrate) and n is the Hill

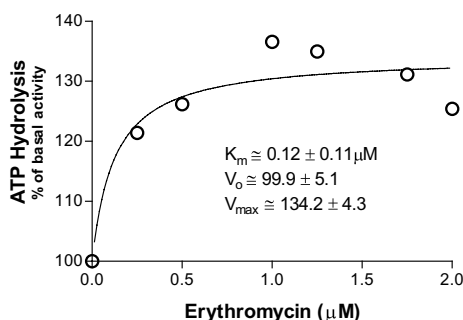


Figure 1. P-gp-mediated ATP hydrolysis rates in the presence of erythromycin. The data are fit to a hyperbola and the $V_{\max}$ = 2-fold above control with a K_m = $0.12 \pm 0.11 \mu M$. Enzyme activity is derived from microsomes of NIH-3T3-G185 cells overexpressing human P-gp and data points are the average of three determinations.

Table 1. Compounds showing activity as a P-gp substrate and no effect on active transport of the marker substrate.

Substrate	K_m (μM)	$V_{\max}$ (%)	IC_{50} (μM) ^a	Reference ^b
Azidoprocainamide	0.71 ± 0.8	67	ne	Smit <i>et al.</i> (1998)
Cimetidine	0.6 ± 2.0	12	ne	Pan <i>et al.</i> (1994); Collett <i>et al.</i> (1999)
Chlorogenic acid	2.0 ± 1.9	63	ne	
Ciprofloxacin	1.6 ± 1.2	58	ne	
Debrisoquin	2.4 ± 4	213	ne	
Dexamethasone	1.8 ± 0.8	63	ne	Schinkel <i>et al.</i> (1996)
Digoxin			ne	Schinkel <i>et al.</i> (1996)
Erythromycin	0.12 ± 0.11	134	ne	Schuetz <i>et al.</i> (1998)
L-Dopa	0.59 ± 0.34	38	ne	Soares-da-Silva and Serrao (2000)
Lidocaine	1.79 ± 1.5	63	ne	
Norfloxacin	1.72 ± 2.4	48	ne	
Puromycin	1.37 ± 0.7	32	ne	Ambudkar <i>et al.</i> (1999)
Tributylmethyl-ammonium	0.53 ± 0.44	170	ne	Smit <i>et al.</i> (1998)
Valinomycin	0.8 ± 0.3	> 200	3.2 ± 0.2	

^ane = No effect.^bOther references with substantive evidence or characterization of compounds as P-gp substrates are included if available.

coefficient. The IC_{50} for valinomycin on DNR transport in the NIH3T3-G185 cell line (which over-expresses the gene product of human MDR1) was about $3.2 \mu\text{M}$, and valinomycin achieved about 86% of maximal (reference) inhibition (figure 2). Other well-characterized inhibitors of P-gp were shown to inhibit the P-gp-mediated transport of DNR efflux and are designated in table 1 with the corresponding IC_{50} . However, some P-gp substrates had no effect on the transport of the marker substrate (DNR, Rho or LDS) over a concentration range of 0–250 μM (figure 2, one example shown) and are given in table 1.

Discussion

Although a substrate typically competes with alternate substrates for the active site of a xenobiotic disposition enzyme, many P-gp substrates are not competitive inhibitors for overall efflux. We have shown that many P-gp substrates have no effect on the ability of P-gp to cause net removal of the marker substrates from a viable cell, and others have indicated a potentially similar distinction for other compounds (Scala *et al.* 1997, Eytan *et al.* 1996b, Polli *et al.* 2001). It is very important to define a compound properly as a substrate, inhibitor or both in the context of evaluating the potential for drug interactions and drug–herb interactions as well as exposure to toxins and drugs. This distinction between a substrate and its ability to inhibit P-gp can be explained by an element of the natural system: P-gp exports its substrates across a lipid bilayer that is intended to preclude permeation of adverse xenobiotics. A requirement for a P-gp substrate also to be an inhibitor is the rapid passive transbilayer movement across the membrane bilayer, a process that allows the substrate to re-enter the cell quickly and hence effectively occupy the P-gp active site (illustrated by the schematic of figure 3).

Many studies indicate the critical role of membrane permeation or passive transmembrane movement rate of a substrate toward the inhibition of P-gp (Sonveaux *et al.* 1999, Wielinga *et al.* 2000). Although P-gp-mediated active rate of compound transfer or efflux is slow (about 900 min^{-1} ; Urbatsch *et al.* 1994), the

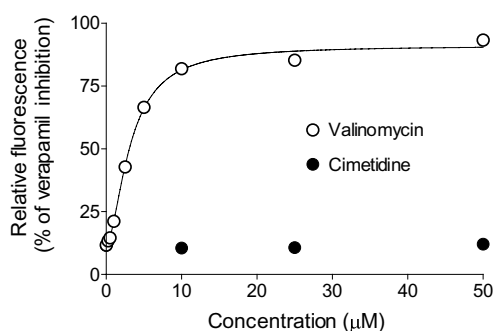


Figure 2. Intracellular retention of daunorubicin in NIH3T3-G185 cells versus competing valinomycin or cimetidine concentration. Fluorescence intensity is expressed as relative fluorescence. The function for the line through the data is the Hill equation: $I = I_{\max} S^n / (IC_{50} + S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown in table 1. Cimetidine has no significant effect on retention of the marker substrate in the MDR1-transfected cell line, as shown by the open symbols. Data points are derived from the 10000 viable cells per assay.

Table 2. Reported P-gp substrates with no effect on active transport of the marker substrate.

Substrate	IC_{50} (μM) ^a	Reference
Actinomycin	ne	Ambudkar <i>et al.</i> (1999)
Cefoperazone	ne	Yusa & Tsuruo (1989)
Corticosterone	ne	Wolf and Horwitz (1992)
Etoposide	ne	Ambudkar <i>et al.</i> (1999)
Fluconazole	ne	Wang <i>et al.</i> (2002)
Loperamide	ne	Schinkel <i>et al.</i> (1996), Wandel <i>et al.</i> (2002)
Mithramycin	ne	Carulli <i>et al.</i> (1990)
Pravastatin	ne	Wang <i>et al.</i> (2001)a
Propantheline	ne	Saitoh and Aungst (1995)
Propranolol	ne	Yang <i>et al.</i> (2000)
Ranitidine	ne	Collett <i>et al.</i> (1999)
Talinolol	ne	Zschiesche <i>et al.</i> (2002)
Trimethoprim	ne	Polli <i>et al.</i> (2001)
Vincristine	ne	Ambudkar <i>et al.</i> (1999)

^a ne, No effect.

'flip-flop' rate of its many substrates is even slower ($t_{1/2} > 2$ min.) against those for which there is 'resistance'. In other words, to create a concentration gradient, the P-gp substrate must have relatively slow transmembrane passive transport rate (figure 3). Conversely, P-gp substrates with relatively rapid permeation will overcome the pace of P-gp to re-enter the binding site and competitively inhibit function (figure 3). The rate of active efflux transport relative to the rate of passive permeation or influx determines the net movement of drug from inside to outside of the cell membrane; a substrate cycling rapidly back into cytosol will compete for P-gp binding site access. MDR-type drugs are amphipathic (hydrophobic and positively charged) and as such bind readily to negatively charged phospholipid head groups of the membrane. Therefore, transmembrane movement often requires a flip-flop through the membrane bilayer and is slow. Indeed, the passive transbilayer diffusion of phosphatidylcholine (PC), the most abundant membrane lipid, is very slow ($t_{1/2}$ of days) in both artificial and natural membranes

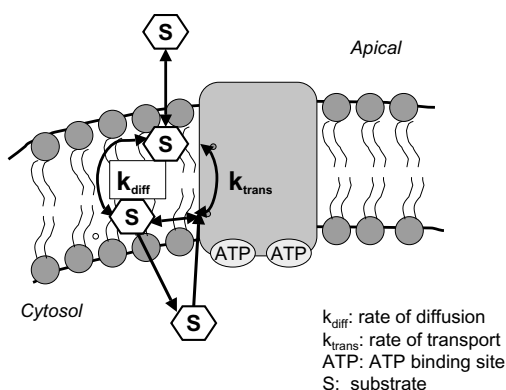


Figure 3. Some of the steps involved in passive and active transport across the cellular membrane bilayer. When the active efflux rate is greater than the passive transbilayer transport rate (diffusion), a concentration gradient is caused. When the diffusion is significantly more rapid than the rate of P-gp mediated efflux, the substrate can effectively re-occlude the transport binding site.

(Zachowski 1993). By measuring the transport rate and passive transbilayer permeation rate of five inhibitors and five substrates, it was previously shown that P-gp inhibitors cross the bilayer membrane faster than the egress rate of P-gp, thereby resulting in rapid equilibration rates (Eytan *et al.* 1996b). At an approximated turnover rate of 900 min^{-1} , P-gp can keep pace with a compound like Rho with a transbilayer movement lifetime of minutes. Conversely, P-gp is inefficient in protecting MDR cells against molecules rapidly permeating through lipid bilayer membranes, for example potent P-gp inhibitors such as the carrier-type ionophore valinomycin, which traverse membranes within microseconds ($k > 25 \times 10^4 \text{ s}^{-1}$). Conversely, gramicidin, effectively excluded by P-gp, has a transmembrane flip-flop rate with a lifetime of minutes. Additionally, the transmembrane movement rate is critical to the overall efficiency of P-gp removal of rhodamine dyes from MDR cells. Indeed, rhodamine B and tetramethylrosamine exhibit high affinity for P-gp, but rhodamine B was the fastest membrane-traversing dye and the least efficiently excluded from the cell. There was a similar corollary for all of the related dyes tested (Eytan *et al.* 1997). Another efficient marker of efflux, doxorubicin exhibits a flip-flop rate with a $t_{1/2}$ of about 1.7 min and 30% cholesterol addition to the vesicles to reduce membrane fluidity decreased the rate 7-fold (Regev and Eytan 1997). The fluidizer benzyl alcohol accelerated the rate, consistent with the role of membrane bilayer permeability dictating the rate of equilibration. Consequently, of compounds ejected by P-gp, those that are relatively slow to cross the lipid bilayer are efficient 'substrates', and those that rapidly permeate the membrane are (also) good 'inhibitors'. This effect has been modelled using the highly permeable compound nifedipine and further supports this conclusion (Doppenschmitt *et al.* 1999, Ito *et al.* 1999), as does ranitidine which exhibits very low passive permeability (Lentz *et al.* 2000) and is not a P-gp inhibitor.

Lipophilicity could sometimes be used to discriminate between P-gp substrates and inhibitors. Among a series of anthracyclines, the less lipophilic derivatives were correlated with slower passive diffusion and resulted in lower intracellular accumulation (Mankhetkorn *et al.* 1996, Mankhetkorn and Garnier-Suillerot 1998,

Sonveaux *et al.* 1999, Wielinga *et al.* 2000). Indeed, lipophilicity is inversely correlated with P-gp efflux efficiency of some vinca alkaloids (Borrel *et al.* 1995), steroids (Barnes *et al.* 1996) and peptides (Borgnia *et al.* 1996); and directly relates to P-gp inhibition for cyclosporins (Tanaka *et al.* 1996), steroids (Barnes *et al.* 1996) and linear hydrophobic peptides (Sarkadi *et al.* 1994). Although more lipophilic compounds appear to be generally better inhibitors (as opposed to efficiently ejected as substrates), this correlation does not apply outside of a particular set of analogues (Chiba *et al.* 1996, Ferte *et al.* 1999). This result is probably because lipophilicity parameters are inadequate for judging the interaction of structurally diverse compounds with the complexities of membranes (Ferte 2000).

We can now appreciate that the number of P-gp substrates that are not also inhibitors is striking, and many substrates could be expected to have no substantial effect on P-gp function in the viable cell. Despite this absence of effect on P-gp overall function by many substrates, these P-gp substrates can still be affected pharmacokinetically by the modulators of P-gp activity. It is therefore important to characterize both the efflux rate parameters and those of inhibition.

Acknowledgements

The authors are very grateful to Professor Adriane L. Stewart for editorial assistance and for the independent comments of Sylvia Johnson.

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CYP2D6-mediated catalysis of tamoxifen aromatic hydroxylation with an NIH shift: similar hydroxylation mechanism in chicken, rat and human liver microsomes

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Received 29 May 2002

1. 4-Tritiated-tamoxifen (4-[³H]-tamoxifen) and 4-deuterated-tamoxifen (4-[²H]-tamoxifen) were synthesized to examine tamoxifen metabolism by human P450 (CYP) forms and also for the possibility of determining tamoxifen-4-hydroxylation in humans *in vivo*.

2. Liver microsomes from several species and cDNA-expressed human P450s were incubated with 4-[³H]-tamoxifen and the reaction monitored by assaying 4-hydroxytamoxifen (4-OH-tam) and ³H₂O formed. However, tamoxifen-4-hydroxylation did not generate stoichiometric amounts of ³H₂O and the expected unlabelled 4-OH-tam but instead yielded radiolabelled 4-OH-tam, apparently from [³H]-migration to the *ortho*-position, referred to as the NIH shift.

3. CYP2D6 was the prime catalyst of tam-4-hydroxylation, whereas CYP2B6, 2C9 and 2C19 yielded only low levels of 4-OH-tam; nevertheless, in all cases the 4-OH-tam was radioactive, apparently resulting from reactions involving an NIH shift.

4. Chicken liver microsomal preparation, being catalytically the most active in tamoxifen-4-hydroxylation, was incubated with deuterated tamoxifen (4-[²H]-tamoxifen) in order to determine whether an NIH shift occurs. Ion-trap mass-spectrometry of the HPLC-purified 4-OH-tam, from that incubation, indicated about 60% of [²H]-retention in 4-OH-tam, signifying an NIH shift. These findings indicate that the aromatic hydroxylation of tamoxifen does not entail hydroxyl insertion with an Sn2-displacement of hydrogen or a hydrogen isotope (²H or ³H), but apparently involves epoxidation followed by migration of the ³H, ²H or ¹H to the *ortho*-position, and dissociation of the ¹H in preference to ³H or ²H, i.e. retention of the hydrogen isotope appears to be related to the bond strengths: C-³H > C-²H > C-¹H.

Introduction

Tamoxifen is currently the therapeutic endocrine agent of choice for all stages of breast cancer and for prophylactic use against the onset of that disease in women at risk (Chlebowski *et al.* 1993, Jordan 1995, Alberg *et al.* 1999). The major paths of tamoxifen metabolism involve N-demethylation, aromatic hydroxylation, side chain α -hydroxylation and N-oxidation (figure 1) (Foster *et al.* 1980, Reunitz *et al.* 1984, Lyman and Jordan 1986, McCague and Seago 1986). The first three reactions are catalysed by P450 enzymes; however, N-oxidation is primarily catalysed by the flavin-containing monooxygenase (Mani *et al.* 1993a, b, Dehal

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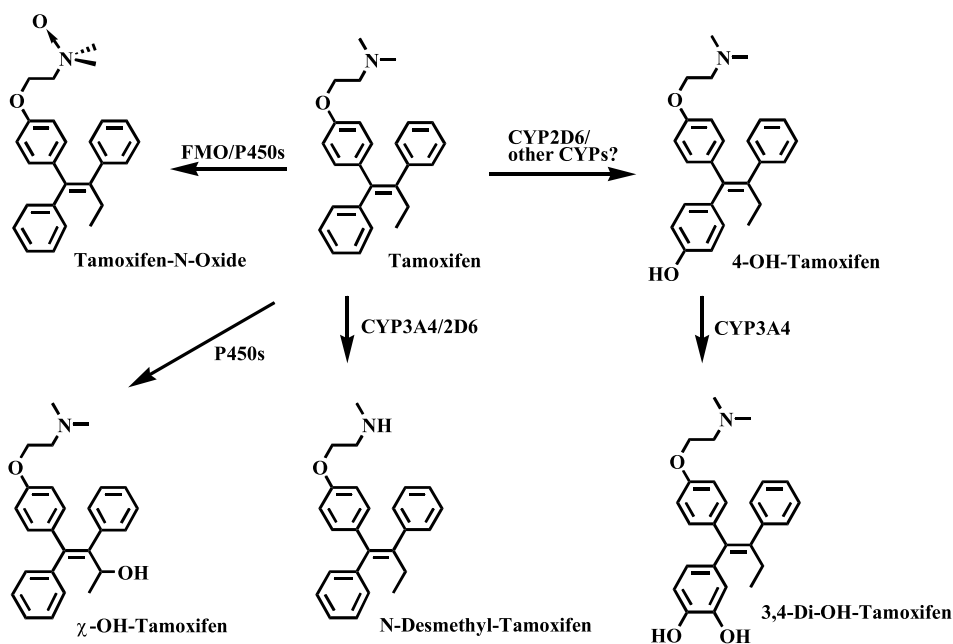


Figure 1. Tamoxifen metabolism as deduced from *in vitro* and *in vivo* studies.

and Kupfer 1997, Boocock *et al.* 1999). The aromatic hydroxylation of tamoxifen yields primarily 4-OH-tamoxifen (4-OH-tam) (Fromson *et al.* 1973a, b, Borgna and Rochefort 1981, Lyman and Jordan 1986), a metabolite that is a 50–100-fold more potent anti-oestrogen than tamoxifen (Borgna and Rochefort 1981, Furr and Jordan 1984). Consequently, it has been speculated that tamoxifen is a prodrug and that 4-OH-tam is the active form of tamoxifen in breast cancer treatment (Borgna and Rochefort 1981, Dehal and Kupfer 1997). Since tam-4-hydroxylation appears to be catalysed primarily by CYP2D6 and to a minor extent by other hepatic P450s (Dehal *et al.* 1996, Crewe *et al.* 1997, Dehal and Kupfer 1997) and that CYP2D6 expression is polymorphic (Marez *et al.* 1997), it is conceivable that a poor response or development of resistance to tamoxifen treatment could be due to inadequate generation of 4-OH-tam.

The original aim of the study was to develop an assay to quantify the rate of the aromatic 4-hydroxylation of tamoxifen in humans *in vivo*, and to determine whether there is a positive correlation between extent of tam-4-hydroxylation and tamoxifen therapeutic efficacy. The assay to be developed relied on the expectation that urinary $^3\text{H}_2\text{O}$ derived from administered 4- $[^3\text{H}]$ -tamoxifen will permit an accurate quantification of the 4-OH-tam formed. However, our current *in vitro* studies demonstrated that the release of $^3\text{H}_2\text{O}$ from $[^3\text{H}]$ -tamoxifen was only minimal and not congruent with the more extensive generation of 4-OH-tam that remained radioactive. These results revealed that the low yield of $^3\text{H}_2\text{O}$ and the residual radiolabel tracer in the 4-OH-tam were a consequence of migration of the ^3H to the *ortho*-position observed in certain aromatic hydroxylations in a mechanism referred to as an NIH shift (Guroff *et al.* 1967, Jerina *et al.* 1971, Koerts *et al.* 1998) but not observed in other aromatic hydroxylations (Reinhold and Bruni 1976, Kupfer *et al.* 1981, Forte *et al.* 1984).

Because 4-hydroxylation represents a substantial activation of tamoxifen into an effective anti-oestrogen (i.e. 50–100-fold more potent than tamoxifen) and possibly serving as the active agent in breast cancer therapy, an understanding of the mechanism of the aromatic hydroxylation of this drug by hepatic P450s is of particular interest. These considerations prompted the investigation of the NIH shift encountered in our current studies.

Materials and methods

Materials

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were purchased from Sigma (St Louis, MO, USA). HPLC-grade acetonitrile was purchased from Mallinckrodt (St Louis, MO, USA). HPLC columns were obtained from Phenomenex-Prodigy 5 C₈ 250 × 4.6 mm (Torrance, CA, USA).

Synthesis of 4-deuterated and 4-tritiated tamoxifen

The synthesis of these compounds was accomplished from 4-iodotamoxifen by unequivocal synthesis involving lithium–halogen exchange chemistry (figure 2). 4-Iodotamoxifen was chemically synthesized using previously published procedures (McCague *et al.* 1989, Hardcastle *et al.* 1995). To a solution of 4-iodotamoxifen (0.250 g, 0.503 mmol) in diethyl ether (10 ml), cooled to -80°C , was added *n*-butyl lithium (2.5 M, 0.4 ml) and stirred for 30 min. D₂O or T₂O were then added (3 equivalents), stirred for 30 min, then warmed to 25°C and stirred for 1 h. The solution was evaporated to dryness under a stream of nitrogen. The 4-deutero-tamoxifen in CDCl₃ was analysed by ¹H- and ²H-NMR analysis, confirming replacement of the iodo with the aryl-deuterium [²H]. An ¹H-NMR of deuterio-tamoxifen (CDCl₃ solvent) relative to TMS [0.0] yielded: 0.93(t, 3H), 2.27(s, 6H), 2.45(q, 2H), 2.61(t, 2H), 3.91(t, 2H), 6.57(d, 2H), 6.77(d, 2H) and 7.08–7.36(9H); tamoxifen has a nearly identical ¹H-NMR spectrum except that the 7.08–7.36 multiplet integrates to 10 protons. The ²H-NMR (CD₂Cl₂ solvent) showed one peak at 7.1 for the aryl-D signal, providing evidence that the deuterium was in the aromatic ring. The incorporation of the deuterium into the 4-position of tamoxifen was 75% (obtained from mass spectrometry). The [N-C³H₃]-tamoxifen (80 Ci mmol⁻¹) was purchased from NEN (Boston, MA, USA). Unlabelled tamoxifen was from Sigma-Aldrich (St Louis, MO, USA). The purity of the synthesized radiolabelled tamoxifen was analysed by TLC. The 4-[³H]-tamoxifen (see the chromatographic conditions below under 'Incubation'), appears to be over 95% radiochemically pure. The synthesized 4-[²H]-tamoxifen, exhibited only 75% deuterium incorporation with the rest being hydrogen incorporation (mass spectroscopic data).

Preparation of liver microsomes. Chicken and rat liver microsomes were prepared by differential ultracentrifugation as previously described (Burstein and Kupfer 1971, Dehal and Kupfer 1996). Human liver microsomes (analysed for the various P450 forms catalytic activities) were purchased from Gentest, Inc. (Woburn, MA, USA).

Incubations

Liver microsomes. Incubations of 4-[³H]-tamoxifen or of [N-C³H₃]-tamoxifen (200 000 dpm, 100 μM final concentration in the incubate was added in 10 μl ethanol) with liver microsomes (2 mg ml⁻¹ protein), from several species, were conducted at 37°C in a Dubnoff incubator while shaking in an atmosphere of air. The incubation medium contained the following constituents: MgCl₂ (10 μM), NADPH (1 mM), Na-phosphate buffer (70 mM, pH 7.4) in a final volume of 1 ml. Usually, after 60 min the reaction was terminated by the addition and mixing of methylene chloride and the products were

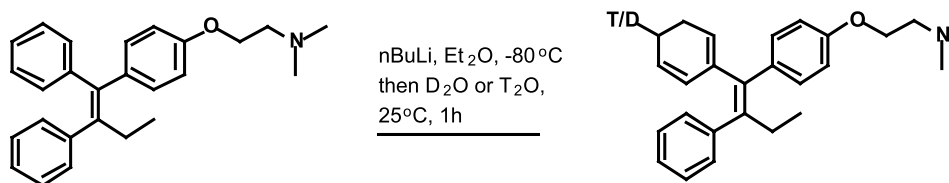


Figure 2. Synthesis of 4-[³H]-tamoxifen and 4-[²H]-tamoxifen.

extracted with 2×3 ml methylene chloride. Analysis of products was achieved by normal-phase silica gel TLC containing a fluorescent indicator (LK6DF silica gel 60Å (Whatman, Inc., Clifton, NJ, USA), developed with chloroform/methanol/ammonium hydroxide (80:20:0.5) and radiometric monitoring with a Bioscan System 200 Imaging Scanner (Washington, DC, USA); R_f values for tamoxifen (0.75) and 4-OH-tam (0.60); the assay sensitivity was about 1000 dpm. Consequently in incubations requiring high sensitivity, i.e. in those yielding low product formation, the specific radioactivity of the substrate was increased to 40 000 dpm nmol⁻¹. Structural assignment of products was obtained using non-labelled authentic compound(s) in adjoining TLC lanes and visualized by UV light. Alternatively, the analysis was conducted by HPLC, using a 5 μ -C₈ reversed-phase 250 $\times$ 4.6 mm 'Prodigy' 5 column (Phenomenex, Torrance, CA, USA) with methanol/water/dimethylamine (80:20:0.1), 1 ml min⁻¹ at room temperature. Detection was by UV (280 nm); the retention time for 4-OH-tam was 3.5 min and for tamoxifen was 10.8 min; the assay sensitivity was 0.25–0.50 nmol. Additionally, at times the generation of ³H₂O was also assayed by treating the incubation mixture (after incubation) with dextran-coated charcoal to eliminate residual 4-[³H]-tamoxifen and metabolites and by determining the radioactivity representing ³H₂O in the supernatant (Kupfer *et al.* 1981).

Human cDNA-expressed P450s. Incubations of [4-³H]-tamoxifen or of [N-C³H₃]-tamoxifen (200 000 dpm, 100 μ M) were conducted with various cDNA-expressed P450s co-expressed with NADPH-P450 reductase in an insect cell line – referred to as supersomes (from Gentest Co., Woburn, MA, USA); these were supplemented with cytochrome b₅ (b₅/CYP2D6 at a ratio of 3.3:1) (100 pmol P450) in a volume of 1 ml. The incubation medium contained the following: MgCl₂ (10 μ M), NADPH (1 mM), Na-phosphate buffer (70 mM, pH 7.4). The incubation of CYP2D6 (50 nM) and [N-C³H₃]-tamoxifen with NADPH (0.5 mM) and its regenerating system (glucose 6-phosphate 10 mM, glucose 6-phosphate dehydrogenase 2 IU) over 30 or 60 min yielded similar rates of 4-hydroxylation as when NADPH (1 mM) alone was used. However, neither conditions yielded linearity. Therefore, in subsequent experiments the regenerating system was deleted. Since incubations with CYP2B6, 2C9 and 2C19 yielded low amounts of the 4-hydroxylation product, being too low for radiometric detection, the substrate concentration was lowered to 5 μ M (increasing the specific radioactivity to about 40 000 dpm nmol⁻¹). In the cDNA-expressed incubations, analysis of products and their quantification was as described above under 'Incubations of liver microsomes'.

Chicken liver microsomes (incubation with deuterated (²H)-tamoxifen). Incubation of [4-²H]-tamoxifen (50 μ M) with chicken liver microsomes was conducted in the presence of NADPH (1 mM) in a volume of 1 ml under an atmosphere of air at 37 °C for 1 h in a Dubnoff incubator with shaking. The 4-OH-tam formed and present in the extract (prepared essentially as described above) was isolated using an HPLC-C₈ column (see above) with methanol/water/dimethylamine (80:20:0.1) and subjected to ion-trap mass spectrometry in a Thermo-Finnigan LCQ Duo. The ESI conditions were: sheath gas flow rate, 20; spray voltage, 4.50 KV; capillary temperature, 200 °C; capillary voltage, -10.0 V; tube lens offset, -0.0 V. Ion optics conditions were: octapole 1 offset, -3.00 V; lens voltage, -16.00 V; octapole 2 offset, -7.00 V; octapole RF amplitude, 400.0 V.

Determination of tritium retention in 4-OH-tam

The relative production of 4-OH-tam in incubations of 4-[³H]-tam with liver microsomes or cDNA-expressed P450 was calculated as the ratio of radioactive counts of 4-OH-tam/unreacted 4-[³H]-tam or [N-C³H₃]-tamoxifen (using radioscanning of the TLC chromatograms). The determination of the percent tritium retention was achieved by comparing the ratio of [³H]-4-OH-tam (derived from 4-[³H]-tam) to unreacted 4-[³H]-tam as compared with the ratio of [N-C³H₃]-4-OH-tam to the unreacted N-[C³H₃]-tam. Both values were obtained from simultaneously performed incubations. Incidentally, the 4-OH-tam generated from [N-C³H₃]-tam retained the specific radioactivity of the parent compound since there was no loss of tritium during the 4-hydroxylation of [N-C³H₃]-tam.

Results and discussion

At the outset it was assumed that the enzymatic hydroxylation of [4-³H]-tamoxifen would yield a stoichiometric release of tritiated water (³H₂O) and non-radiolabelled 4-OH-tam. Consequently, it was anticipated that this procedure would provide a facile and accurate radiometric assay of ³H₂O reflecting the formation of 4-OH-tam. The assay was designed to involve the quantification of the release of ³H₂O employing a method similar to an earlier procedure of enzymatic hydroxylation of 2-[³H]-oestradiol, yielding 2-OH-oestradiol and stoi-

chiometric amounts of $^3\text{H}_2\text{O}$ (Kupfer *et al.* 1981). However, in the current studies involving incubations or $[4\text{-}^3\text{H}]$ -tamoxifen, it soon became evident that there was a lack of congruency (absence of stoichiometry) between the formation of 4-OH-tam and the release of $^3\text{H}_2\text{O}$ (the latter was assayed after removal of substrate and metabolites with dextran-coated charcoal). In fact, incubation of liver microsomes from phenobarbital (PB)-treated rats with $[4\text{-}^3\text{H}]$ -tamoxifen yielded radiolabelled 4-OH-tam and only a minimal amount of tritiated water (data not shown). The radiolabelled metabolites $[^3\text{H}]$ -tam-N-oxide, $[^3\text{H}]$ -N-desmethyl-tam and $[^3\text{H}]$ -4-OH-tam were quantified on TLC, and the $^3\text{H}_2\text{O}$ was determined by scintillation spectrometry (Kupfer *et al.* 1981, Dehal and Kupfer 1997).

The above findings prompted further investigation of the mechanism of the aromatic hydroxylation of tamoxifen that leads to tritium retention.

Metabolism of $[4\text{-}^3\text{H}]$ -tamoxifen by liver microsomes from rats

Incubation of $[4\text{-}^3\text{H}]$ -tamoxifen (100 μM) with untreated-rat liver microsomes in the presence of NADPH yielded radiolabelled N-desmethyl-tamoxifen, 4-OH-tam and tamoxifen-N-oxide as the major metabolites. These incubations yielded 8 nmol/60 min 4-OH-tam. Additionally, this finding indicated that there was a considerable amount of tritium retention in 4-OH-tam (table 1), albeit, as expected, to a lesser extent than found in incubations of $[\text{C}^3\text{H}_3\text{-N}]$ -tamoxifen (^3H is present in the N-methyl moiety). This suggested that 4-hydroxylation of $[4\text{-}^3\text{H}]$ -tamoxifen proceeds largely with an NIH shift, i.e. accompanied with migration of the $[^3\text{H}]$ to the *ortho*-position (figure 3); there was a $52 \pm 6\%$ retention of $[^3\text{H}]$ when compared with incubation of $[\text{C}^3\text{H}_3\text{-N}]$ -tamoxifen. Since $[^{14}\text{C}]$ -tamoxifen (with the label in a metabolically stable position) was not commercially available, we carried out incubations instead with $[\text{C}^3\text{H}_3\text{-N}]$ -tamoxifen.

Table 1. Retention of tritium (T) or deuterium (D) in 4-OH-tam derived from incubation of 4- $[^3\text{H}]$ -tamoxifen or $[^2\text{H}]$ -tamoxifen with animal and human liver microsomes or with c-DNA-expressed DYP2D6.

Enzyme source (microsomes or cDNA-expressed P450)	Retention of T or D (%) ^d	Substrate ^c
Rat liver	52 $\pm$ 6 (T)	4-T-tamoxifen
Chicken liver	99 (T)	4-T-tamoxifen
Chicken liver	60 (D)*	4-D-tamoxifen
Human (H006) liver (CYP2D6 activity undetected) ^a	76 $\pm$ 9 (T)	4-T-tamoxifen
Human (H064) liver (CYP2D6 activity 'normal') ^b	59 $\pm$ 3 (T)	4-T-tamoxifen
CYP2D6 (supersomes)	85 $\pm$ 6 (T)	4-T-tamoxifen

Incubation conditions were: microsomes (1.9–2.0 mg protein ml⁻¹); CYP2D6 (100 pmol ml⁻¹).
T, tritium; D, deuterium.

* Data from mass spectrometry.

^a 4-Hydroxylase activity in the absence of 2D6 was apparently due to other CYPs, possibly CYP 2B6, 2C9 and 2C19 (see table 2).

^b 2D6 activity = bufarolol 1'-hydroxylation (210 pmol mg⁻¹ protein min⁻¹), activity inhibited by 1 μM quinidine.

^c Substrate concentration: 100 μM for 4-T-tamoxifen and 50 μM for 4-D-tamoxifen.

^d Average of duplicates and SD.

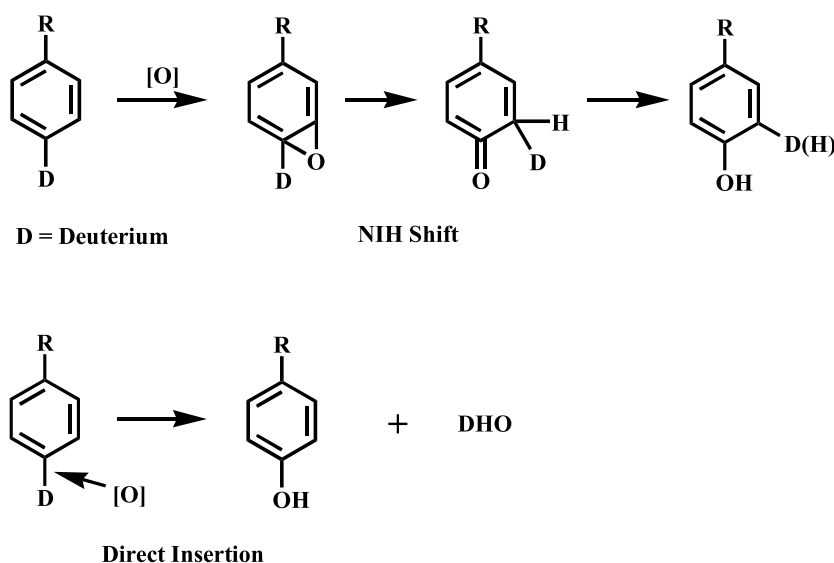


Figure 3. Proposed mechanism for the NIH shift in the 4-hydroxylation of tamoxifen as contrasted with direct oxygen insertion (2-hydroxylation of oestradiol is an example of the latter).

Metabolism of [4-³H]-tamoxifen or [4-²H]-tamoxifen by liver microsomes from untreated chicken

Among liver microsomes of the various species examined, the chicken liver preparation was the most active in tam-4-hydroxylation (about 14 nmol/60 min). Therefore, this liver preparation was used for further studies of the NIH shift. Incubation of [4-³H]-tamoxifen with chicken liver microsomes in the presence of NADPH yielded N-desmethyl-tamoxifen and 4-OH-tam as major products and a minimal amount of tamoxifen-N-oxide. The retention of ³H was about 99% (analysed by radiometric scanning). An incubation of 4-deuterated-tamoxifen ([4-²H]-tamoxifen) yielded 4-OH-tam with 60% retention of deuterium (table 1), as determined by ion-trap mass spectrometry (figure 4) which showed the comparison of MS/MS of unlabelled 4-OH-tam versus the ²H-retained 4-OH-tam. The 60% retention rate was calculated based on the purity of 4-[²H]-tamoxifen and enhancement of the M + 1 peak.

Interestingly, by contrast to human liver where tam 4-hydroxylation is catalysed by CYP2 enzymes (primarily by CYP2D6) (Dehal and Kupfer 1997), in the chicken this catalysis appears to be primarily by CYP1A5 (Arleen Rifkind, personal communication), a P450 inducible by TCDD (Kupfer *et al.* 1994). This finding indicates that with the current knowledge of metabolism, a drug metabolized by one class of P450 isoforms in a given species does not predict with any degree of certainty whether the same P450 class will catalyse that function in another species.

Metabolism of [4-³H]-tamoxifen by CYP2D6 and other human P450s (supersomes)

Incubation of [4-³H]-tamoxifen with CYP2D6 in the presence of NADPH yielded N-desmethyl-tam and 4-OH-tam (4 nmol/60 min). There was a high level of tritium retention in the 4-OH-tam, namely about 85% (table 1). The extent of

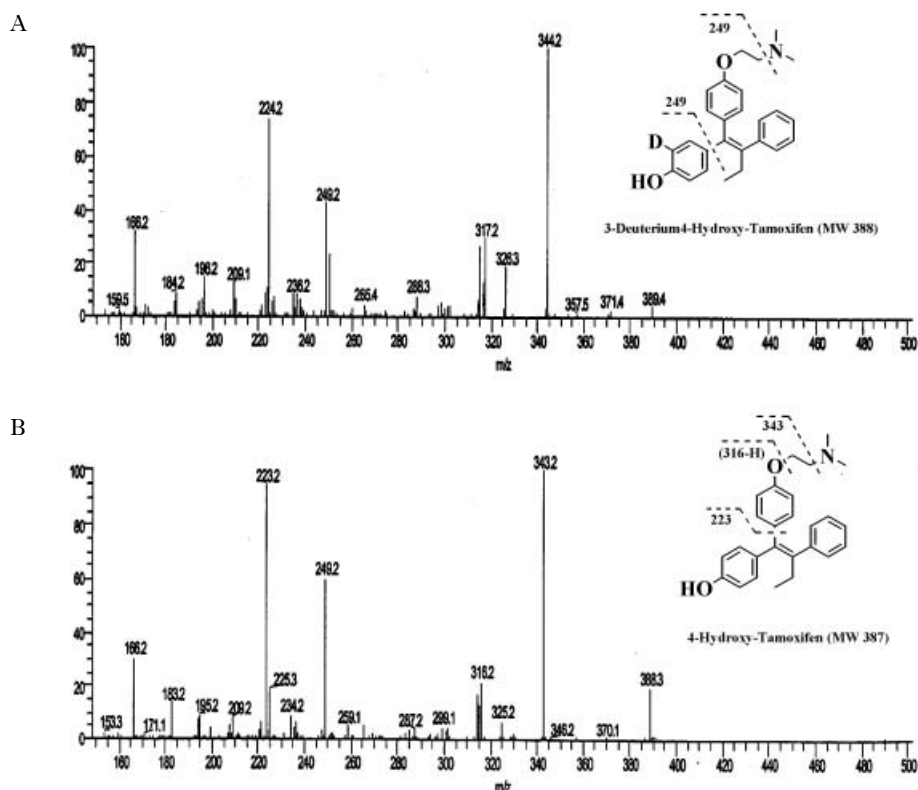


Figure 4. Mass spectrometry of $[^2\text{H}]$ -4-OH-tamoxifen (A) and $[^1\text{H}]$ -4-OH-tamoxifen (B) isolated from the incubation of 4- $[^2\text{H}]$ -tamoxifen and 4- $[^1\text{H}]$ -tamoxifen, respectively, with chicken liver microsomes. The extent of deuterium retention was determined by measuring a select ion of molecular mass (4-OH-tam); $[M]$ and $[M+1]$ and calculating the enhancement of $[M+1]$ (deuterium incorporated versus non-deuterated).

tritium retention in 4-OH-tam from $[4\text{-}^3\text{H}]$ -tamoxifen was calculated by comparison with the $[\text{C}^3\text{H}_3\text{-N}]$ -4-OH-tam formed during incubation of $[\text{C}^3\text{H}_3\text{-N}]$ -tamoxifen (see the Materials and methods). Incubation of $[4\text{-}^3\text{H}]$ -tamoxifen with CYP2B6, 2C9 and 2C19 yielded only about one-tenth to one-twentieth of the amount of 4-OH-tam as compared with 2D6 catalysis (table 2). However, catalysis by the latter P450 forms also exhibited the 4-hydroxylation with an NIH shift.

Metabolism of $[4\text{-}^3\text{H}]$ -tamoxifen by human liver microsomes

Incubation of $[4\text{-}^3\text{H}]$ -tamoxifen with human liver microsomes and NADPH yielded N-desmethyl-tamoxifen, 4-OH-tam (3–4 nmol/60 min) and tamoxifen-N-oxide. Extracts from incubation with liver microsomes from two donors: H006 (without detectable 2D6 activity, i.e. exhibiting no quinidine-mediated inhibition of the bufaralol-1'-hydroxylation), and H064 (exhibiting 'normal' CYP2D6 activity) were analysed (table 1). Both these human liver preparations exhibited tam-4-hydroxylation. Our observation that a general inhibitor of P450s, SKF525A at 1 mM concentration, inhibited tam-4-hydroxylation by human liver microsomes by more than 90% (without affecting the pH of the incubate) indicates

Table 2. Tamoxifen-4-hydroxylation by cDNA-expressed CYP 2B6, 2C9, 2C19 and 2D6.

P450	Incubation time (min)	4-OH-tam (pmol) ^a	Tritium retention (%) ^a
2B6	30	45	47
2C9	30	75	15
2C19	30	75 ^b	46
2C19	60	110 ^b	38
2D6	30	1150	ND

Incubations of 4-[³H]-tamoxifen (5 μ M, 200 000 dpm) with cDNA-expressed human P450s (supersomes) at 50 nM concentration were carried out as described in the Materials and methods.

ND, not done.

^a Because of the low levels of tam-4-hydroxylation by CYP 2B6, 2C9 and 2C19, except by CYP2D6, the values (given as the average of duplicates) represent only approximations.

^b Catalysis of 2C19 mediated 4-hydroxylation did not exhibit linearity with time (30 versus 60 min).

that most, if not all, of tam-4-hydroxylation by human liver is catalysed by P450s (data not shown). Interestingly, retention of tritium in 4-OH-tam generated by liver microsomes H006 and H064 was 76 and 59%, respectively. The finding that in the absence of detectable 2D6 activity (sample H006) there was a significant tam-4-hydroxylation indicated that P450s other than CYP2D6 can also catalyse tam-4-hydroxylation, and that the reaction mediated by these P450s proceeds to a considerable extent by an NIH shift. Although our previous studies revealed that tam 4-hydroxylation was catalysed essentially only by CYP2D6 (Dehal and Kupfer 1997), nevertheless the possibility that other human cDNA-expressed P450s were also able to catalyse that reaction albeit at a much lower rate was examined. To facilitate the detection of an even lower level of catalytic activity expected from these P450s, an attempt was made to raise the sensitivity of the assay by increasing the specific radioactivity of the substrate [4-³H]-tamoxifen by about 20-fold from 2000 dpm nmol⁻¹ (at 100 μ M substrate) to 40 000 dpm nmol⁻¹ (using 5 μ M substrate). (This approach was predicated on the lack of linearity with substrate concentration of tam-4-hydroxylation by CYP2D6 – 5 μ M tamoxifen and 30-min incubation yielded 1.2 nmol 4-OH-tam and 100 μ M tamoxifen yielded 3 nmol 4-OH-tam. The possibilities considered were that at high tamoxifen concentrations there was substrate inhibition of hydroxylation and/or that the K_m values were substantially below 5 μ M – these K_m values, however, were not determined.) Indeed, CYP2B6, 2C9 and 2C19 (at 50 nM P450) demonstrated low but significant 4-hydroxylation activity (45–75 pmol/30 min) as compared with CYP2D6 (1150 pmol/30 min) (table 2). Nevertheless, similar to CYP2D6 and liver microsomes, the former P450s exhibited a significant NIH shift. Thus, it can be concluded from the above findings that the presence or absence of an NIH shift in aromatic hydroxylation does not depend appreciably on the particular P450 isoform in that catalysis, but is primarily the function of the compound, e.g. tamoxifen, undergoing aromatic hydroxylation.

Mechanism of NIH shift during aromatic hydroxylation

A mechanism, based on our results (table 1) and on the bond strengths of C-H versus C-D for the NIH shift was proposed (figure 3). The increase in

bond strength relative to C-H for deuterium was $\Delta G = 1.47 \text{ kcal mol}^{-1}$ and for tritium was $2.05 \text{ kcal mol}^{-1}$; these values for benzene at 298 K were determined for us by Brett Lewis and Vern Schramm. (The bonds strength values for C-T, C-D and C-H were specifically derived for the current study. However, there are additional earlier citations, albeit they bear less distinctively on the current subject; Wiberg 1955, Yankwich 1953). Additionally, Dr William Jones, used the latest thermochemical data from the National Institute of Science and Technology (NIST) to calculate the energy of the C-H bond in benzene and obtained the following: C-H bond = $474.1 \text{ kJ mol}^{-1}$; C-D bond = 479 kJ mol^{-1} . Indeed, the experimental retention of deuterium was substantially lower than of tritium (table 1) suggesting that after migration to the neighbouring carbon the tritium was preferentially retained as compared with deuterium and hydrogen.

Conclusions

To our knowledge this is the first demonstration of CYP2D6 and certain other human and animal P450s in livers of several species catalysing aromatic hydroxylation with an NIH shift, thus supporting a mechanism of ring epoxidation and hydrogen migration to an adjoining site coupled with hydrogen release. Additionally, the findings indicate that because of the prevalence of the NIH shift in the 4-hydroxylation reaction, it would not be feasible to develop an *in vivo* method for tam-4-hydroxylation in humans solely based on the release of the ^3H from 4- $[\text{}^3\text{H}]$ -tamoxifen and on the analysis of urinary $^3\text{H}_2\text{O}$. In turn, it is evident that other methods are needed for the development of an *in vivo* assay for the aromatic hydroxylation of tamoxifen.

Acknowledgements

The project was supported by an NIH Grant, No. ES 00834, from the National Institute of Environmental Health Sciences and the views expressed here are solely those of the authors and do not necessarily represent the official views of the NIEHS. Assistance provided by Dr Zhiyong Cui with the mass spectrometry is greatly appreciated. We are very grateful to Drs Brett Lewis and Vern Schramm (Albert Einstein College of Medicine, Bronx, NY) for calculating the bond strengths of C-T and C-D versus C-H. Thanks also to Dr William Jones (University of Rochester, NY) for analysing the zero-point energy difference between C-H and C-D bonds in benzene. Y. H. and S. S. D. contributed equally to this work.

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Identification of a furazolidone metabolite responsible for the inhibition of amino oxidases

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Received 4 July 2002

1 Furazolidone, a drug widely used in human and veterinary medicine, exhibits inhibition of monoamine oxidase activity, as observed in the tissues of a number of different animal species, including man. The aim of the current study was to determine which of the two possible metabolites, 3-amino-2-oxazolidone (AOZ) or β -hydroxyethylhydrazine (HEH), a well-known carcinogenic compound, is involved in the toxicological effects reported.

2 A new spectrometric method was set up to differentiate intracellular HEH from AOZ inside cells. This method works well at low pH where both AOZ and HEH are free in solution and available to react with the chemical chromophore (DAB).

3 The results confirm that furazolidone has to be metabolized in the intact cell in order to exhibit mitochondrial monoamine oxidase inhibition, whereas AOZ itself is able to exert a reversible monoamine oxidase inhibition. AOZ also inhibits bovine serum amino oxidase. On the contrary, HEH gives irreversible inhibition of both enzymes. However, the reversible nature of the AOZ inhibition with respect to HEH suggests that the two metabolites act by different mechanisms which do not require the biotransformation of AOZ to HEH.

4 Cell lysates, previously incubated with AOZ, were directly analysed and the formation of HEH from AOZ was not detected, supporting the conclusion that the amino oxidase inhibition observed on treatment with furazolidone was attributable to AOZ and not to HEH.

Introduction

5-Nitrofuran derivatives that display antibacterial and antiprotozoal activity have been widely used since the 1940s in human and veterinary medicine and as feed additives (Calla 1983). More recently, the use of these chemicals has been significantly reduced following the observation that many derivatives are mutagenic, clastogenic and cytotoxic in bacteria and in cultured mammalian cells (Kobirska-Szeliga and Czacoth 1994, Ali 1999). In particular, furazolidone [*N*-(5-nitro-2-furfurylidine)-3-amino-2-oxazolidone], is one derivative still in use as a poultry feed additive to reduce the incidence of gastrointestinal infections (Graham and Quresci 2000). Because of its antimicrobial activity, it is also used to treat infectious diseases that have developed multidrug resistance to widely used antibiotics (Graham *et al.* 1994, Graham and Quresci 2000, Gardner and Hill 2001). Furazolidone exhibits differential toxicity towards mammalian cell lines in culture, induces cardiomyopathy in poultry (Lax and Kukolich 1992, Lax *et al.* 1994) and affects enzyme levels in chickens, such as glutathione peroxidase (GSH-

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Px), glutathione reductase (GSH-R), catalase (CAT) and superoxide dismutase (Sas 1993). The potential toxicity as a consequence of exposure to residues observed in sources such as catfish (Rupp *et al.* 1993), poultry muscle tissues and eggs (Kumar *et al.* 1994), and swine liver, kidney and rump muscle (Hoogenboom *et al.* 1992) is of concern but has yet to be demonstrated.

The chemical structure of furazolidone comprises two rings (figure 1), a 5-nitrofuran and a 3-amino-2-oxazolidone ring (AOZ). In bacteria, furazolidone is activated in the first ring by reductive metabolism associated with nitroreductases (Lu *et al.* 1998), leading to stable 5-aminofurans and open-chain nitriles (cyano-metabolite). The AOZ has been found as an intact chemical compound tightly bound to proteins of animals treated with furazolidone. In fact, at least 75–80% of the protein-bound residues in the case of microsomes and cells, and 15–25% in the case of livers of animals treated with furazolidone still harbour intact AOZ (Hoogenboom *et al.* 1991a).

Pig liver microsomes were used to investigate the molecular mechanism by which AOZ binds to proteins and firm evidence has emerged that the binding of metabolites to protein was due to the formation of a reactive site in the nitrofuran moiety as a result of the reduction of the nitro-group, leaving the AOZ side-chain unaltered (Vroomen *et al.* 1986). This is of particular interest since AOZ might be released not only from the parent compound in the animal treated, but also from protein-bound metabolites of the drug which can be present as residues in the

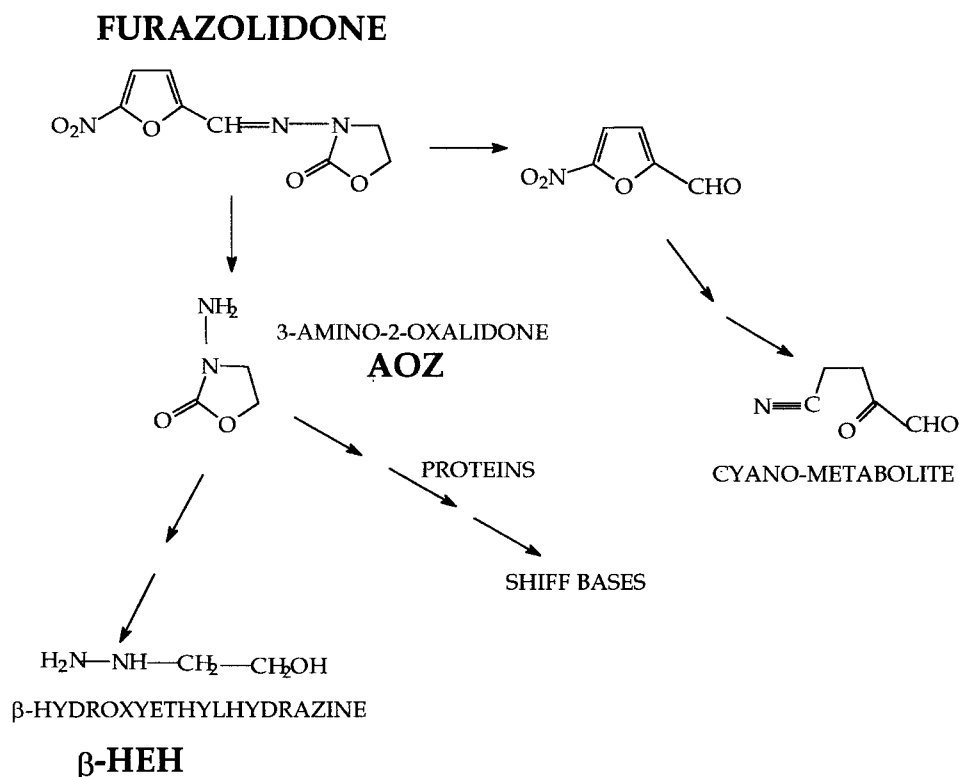


Figure 1. Proposed metabolic pathway for the degradation of furazolidone.

edible tissues of farm animals (Vroomen *et al.* 1988) under conditions like those in the stomach of the consumer (Hoogenboom *et al.* 1991a).

Thus, the open question remains: Can AOZ be biotransformed into a more toxic compound or can it cause adverse effects itself? Inhibition of an amino oxidase (MAO) in animals treated with furazolidone was observed to increase upon repeated exposure, disappearing only gradually after termination of the treatment (Hoogenboom *et al.* 1991a, Hoogenboom *et al.* 1991b). It was concluded that irreversible binding of a metabolite of furazolidone to the MAO enzyme(s) had taken place. Furthermore, the subsequent formation of a reactive metabolite from AOZ appears to underlie this irreversible type of inhibition of the monoamine oxidase (MAO) activity by furazolidone, as observed in tissues of a number of different animal species including man (Stern *et al.* 1967, Ali and Bartlett 1982). Stern *et al.* hypothesized that this metabolite might be β -hydroxyethylhydrazine (HEH) formed by hydrolysis of 3-amino-2-oxazolidinone (AOZ). Based on this hypothesis, it cannot be excluded that AOZ, released from stable protein-bound residues of furazolidone, can be converted to the carcinogenic HEH (Toth 2000) and that these residues should therefore be regarded as carcinogenic.

The aim of the present study was to investigate further the MAO-inhibiting properties of furazolidone using cells, tissue homogenates and purified amino oxidase to clarify which metabolite of furazolidone is involved in this effect. A new spectrophotometric method was developed to detect the biotransformation of AOZ into HEH directly in cell lysates.

Materials and methods

Mitochondrial amino oxidase activity in CaCo-2 cells and pig liver homogenate

CaCo-2 cells (obtained from Dr Stammati, ISS, Rome, Italy) were cultured at 37 °C under a 5% CO₂ atmosphere, essentially according to Pinto *et al.* (1983). The cells were seeded in 75-cm² Falcon flasks (Becton-Dickinson, Franklin Lakes, NJ, USA) and grown in monolayers as Dulbecco's modified Eagle's medium (GIBCO, Paisley, UK) supplemented with 10% inactivated foetal calf serum, 4 mM glutamine, 1% (w/v) non-essential amino acids, 3.7 g l⁻¹ sodium bicarbonate, 100 µg ml⁻¹ streptomycin and 100 IU ml⁻¹ penicillin. After 21 days (differentiated cells) of culture, the cells were detached by trypsin/EDTA treatment (0.2% trypsin, 2 mM EDTA), washed twice in Chance buffer (6.2 mM KCl, 145 mM NaCl, 11 mM sodium phosphate, pH 7.4) and counted. Cells used for the determination of enzyme activities were detached mechanically and washed twice in 140 mM NaCl and 20 mM sodium phosphate, pH 7.4. The final cell pellet was resuspended in the same buffer.

Whole liver was obtained from a slaughterhouse (in Italy), 120 kg, male pig, and transported on ice to the laboratory. Lobes were perfused with 0.9% NaCl and homogenized in a Potter homogenizer with 9 vols ice-cold medium consisting of 220 mM mannitol, 70 mM sucrose, 0.1 mM EDTA and 5 mM hepes buffer (pH 7.5).

In the case of CaCo-2, cells (10⁶ cells ml⁻¹, corresponding to 200 µg protein) were incubated in RPMI 1640 containing furazolidone, AOZ and HEH (RIKILT Wageningen, The Netherlands) at a different concentration, whereas for pig liver aliquots of 10 µl of homogenate containing 200 µg protein were incubated with the three chemicals as above.

Mitochondria from both lysates, CaCo-2 (Stammati *et al.* 1988) cells and pig liver homogenate were isolated by sequential centrifugations. The homogenate was centrifuged twice at 800g for 10 min at 4 °C; the resulting supernatant was then centrifuged at 12 000g for 20 min. The mitochondrial pellet was suspended (1:5 w/v) in 100 mM phosphate buffer (pH 7.4), fractionated in 0.5-ml samples and stored at -70 °C. The protein content of mitochondrial preparations was determined according to the method of Lowry *et al.* (1951) in the presence of 0.01% sodium dodecyl sulfate, using bovine serum albumin as standard.

Determination of mitochondrial amino oxidase activity

Mitochondrial MAO activity was determined by spectrophotometric and fluorimetric assay. The spectrophotometric method consists of incubating mitochondria (an aliquot of 10 µl mitochondrial

suspension 60 µg protein) in a buffer containing benzylamine 10 mM at pH 7.4 for 50 min at 37 °C. The reaction was blocked by addition of TCA (15% w/v) and the sample centrifuged at 7600g. Then, 500 µl of supernatant were dissolved in 2.5 ml cyclohexane and the benzaldehyde absorption read at 250 nm at 25 °C.

The fluorimetric method consisted of incubating 20 µl homogenized mitochondria with kynuramine 0.1 mM in Hepes/KOH 50 mM, pH 7.4, for 30 min at 37 °C. The reaction was blocked by TCA 15% w/v, the sample centrifuged at 7600g for 15 min and 200 µl of the supernatant were dissolved in 3 ml NaOH (1 N) and the fluorescence read at 390 nm upon excitation at 315 nm (Morinan and Garratt 1985).

In order to obtain an accurate determination of the amino oxidase activity, both spectrophotometric and fluorimetric methods were used.

Preparation of bovine serum amino oxidase (BSAO) and determination of its activity

BSAO was purified by the method of Turini *et al.* (1982) with the addition of two more purification steps on Q-Sepharose columns, pHs 8.0 and 6.0, as suggested by Janes *et al.* (1990). A single band was obtained on sodium dodecyl sulphate (SDS) gel electrophoresis. The enzymatic activity was measured spectrophotometrically at 25 °C by monitoring the oxidation of benzylamine to benzaldehyde at 250 nm ($\epsilon = 12500 \text{ M}^{-1} \text{ cm}^{-1}$) (Tabor *et al.* 1954). The specific activity of the samples used was 0.3–0.4 IU mg⁻¹, with IU (international unit) defined as micromoles of oxidized product per minute. The protein concentration was measured from the 280-nm absorbance using an absorption coefficient of $1.741 \text{ g}^{-1} \text{ cm}^{-1}$ (Suzuki *et al.* 1983).

Preparation of S9-fraction and microsomes from pig liver

Whole liver was obtained from a pig and transported on ice to the laboratory. Lobes were perfused with 0.9% NaCl and homogenized in the presence of liquid nitrogen in twice the volume of a 1.15% KCl solution using a Braun blender mixer. Part of the homogenate was centrifuged for 20 min at 9000g (4 °C), yielding an S9 supernatant fraction. Another aliquot of the homogenate was dissolved in sucrose (0.4 M) containing 1 mM EDTA and 0.02% heparin at pH 7. Following centrifugation for 20 min at 1200g and subsequent centrifugation of the supernatant for 20 min at 105 000g, the pellet-containing microsomes were resuspended in Hepes/KOH 50 mM at pH 7.4.

Spectrophotometric method for metabolite analysis

Reactions of dimethylaminobenzaldehyde (DAB) with HEH or AOZ were followed spectrophotometrically using a Varian 4 UV-Vis spectrophotometer equipped with a thermostat cell holder. Solutions of DAB (Merck, Milan) (10 mM) were prepared in 20-mM aqueous SDS (Sigma, Milan). A constant pH was maintained by using 10 mM acetate-phosphate buffer in a pH interval of 2–6. In more acidic media, the desired pHs were obtained by addition of aliquots of standard 1.0 M HCl solution. Since addition of SDS to acid solutions increases the pH even in the presence of buffers, the pHs of surfactant solutions were controlled with a glass electrode initially calibrated with HCl solutions of known concentrations and were adjusted to desired values with 1.0 or 0.10 M HCl. Kinetic and equilibrium measurements normally used 10 and 300 µM solutions of HEH and AOZ, respectively. In a typical experiment, 3.0 or 2.5 ml DAB solution containing the desired concentration of SDS at the desired pH in a 1.0 cm cuvette was placed in the spectrophotometer and equilibrated thermally for 5 min. The incubated solution containing AOZ was then added (100 µl) with a micropipette into the cuvette to initiate the reaction. All experiments were carried out at 20 °C. After 30 min of reaction the solution in the cuvette was read at 465 nm.

Biotransformation of AOZ to HEH in pig liver and CaCo-2 cells

Homogenized pig liver, aliquots of 10 µl, containing 200 µg protein, and CaCo-2 cells, $10^6 \text{ cells ml}^{-1}$, containing 200 µg protein, were incubated at 37 °C with AOZ ($5 \times 10^{-4} \text{ M}$; $5 \times 10^{-3} \text{ M}$). At different times, samples were taken and the amount of HEH formed was determined by the DAB method.

Results

Inhibition of amino oxidases

Both CaCo-2 cells and fresh pig liver homogenate were incubated with 400 µM furazolidone, or AOZ or HEH at different times, and mitochondria were subsequently isolated in order to compare the MAO activity with that of untreated samples. It was observed that the inhibition of MAO by furazolidone was time-

dependent reaching a maximum effect after 8 h, whereas AOZ showed a significant effect by 1–2 h and HEH in an even shorter time (data not shown). Moreover, both intact CaCo cells and fresh pig liver homogenate showed comparable results, suggesting that, first, the three drugs pass through the membrane and, second, that fresh homogenates remain metabolically functional. Table 1 shows the values of MAO inhibition assayed on mitochondria from CaCo cells and fresh pig liver homogenate which were recorded over 8 h following treatment with the substrate and two metabolites at different concentrations. It was observed that although the inhibition time-course was different, after these long incubation times the enzyme inhibition of furazolidone was comparable with that observed for AOZ, whereas HEH was more effective. This is consistent with the hypothesis that furazolidone must be metabolized in order to exert an inhibitory effect, whereas AOZ and HEH themselves possess inhibitory potential. On the other hand, after addition of furazolidone to isolated mitochondria (figure 2), independently from CaCo cells or pig liver homogenate, MAO activity remained unaffected whereas a concentration-dependent inhibition was observed for both AOZ and HEH, although to different extents. This experiment indicates that mitochondria cannot metabolize furazolidone into AOZ and/or HEH and that intact cells or fresh homogenate are required to transform the substrate into an active inhibitor. With regard to the recovery of enzyme activity, upon prolonged dialysis (2 days) of mitochondria treated with the three compounds, the MAO activity, previously reduced to 22–35% during incubation, was almost completely restored (about 95%) in furazolidone and AOZ-treated organelles, but not in those treated with HEH, suggesting that the latter causes irreversible damage to amino oxidases. The reversible nature of the AOZ inhibition with respect to HEH suggests that the two metabolites act by different mechanisms.

To explore this inhibition further, we performed *in vitro* studies on the isolated enzyme. Since it is well documented that both MAO-A and MAO-B enzymes lose their activity once extracted from mitochondria (Abell and Kwan 2000), we used purified BSAO for our investigation. This enzyme has a similar amino acid

Table 1. Effect of furazolidone and its derivatives on the mitochondrial monoamine oxidase activity of CaCo-2 cells and pig liver homogenate.

Drug	Chemicals concentration (μM)	MAO activity	
		CaCo-2 cells % of control $\pm$ SD	Pig liver homogenate % of control $\pm$ SD
Furazolidone	400	29 $\pm$ 1.2	33 $\pm$ 1.3
	200	63 $\pm$ 1.9	68 $\pm$ 1.7
AOZ	400	24 $\pm$ 1.2	25 $\pm$ 2.3
	200	59 $\pm$ 2.0	56 $\pm$ 1.8
HEH	400	13 $\pm$ 1.5	14 $\pm$ 1.5
	200	35 $\pm$ 1.7	32 $\pm$ 1.9
	1	50 $\pm$ 1.6	54 $\pm$ 1.4

Cells were incubated for 8 h with the three chemicals at different concentrations. MAO activity was determined in mitochondria prepared by centrifugation using the method reported in the Materials and methods.

Absolute value of 100% control activity was 0.25 IU mg^{-1} .

Data were derived from three replicate determinations.

Samples for MAO activity were collected each hour. Both fluorimetric and spectrophotometric methods were used.

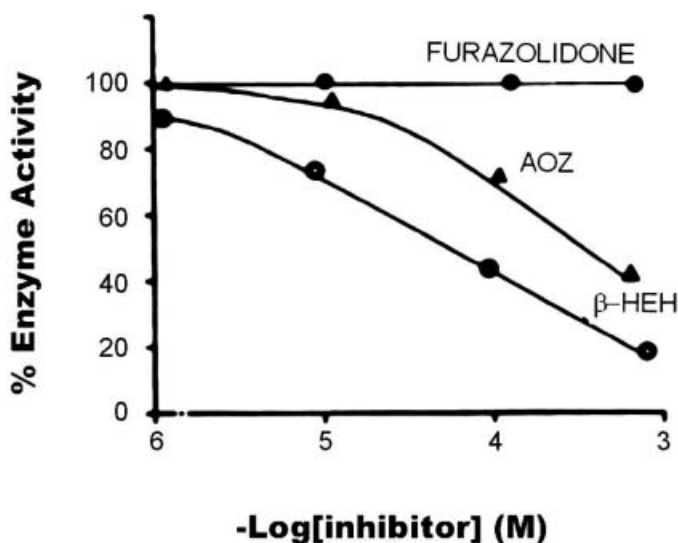


Figure 2. Mitochondrial monoamine oxidase (MAO) inhibition in pig liver mitochondria upon the addition of different concentrations of: furazolidone (●), AOZ (▲) and β -HEH (○). Aliquots of 10 μ l of mitochondrial suspension (60 μ g protein), in a buffer containing benzylamine 10 mM at pH 7.4, were incubated with one of the three chemicals for 30 min at 37 °C. Enzymatic activity was measured spectrophotometrically at 37 °C by monitoring the oxidation of benzylamine (10^{-3} M) to benzaldehyde at 225 nm. Data points were derived from three determinations. Absolute values of 100% control activity was 0.23 IU mg^{-1} .

sequence to the MAO enzyme and a similar molecular mechanism of catalysis but contains a copper atom, although it is not clear if this contributes to the enzyme's activity (Abell and Kwan 2000). The inhibitory profiles of the two metabolites (figure 3) are similar to that observed for mitochondrial MAO inhibition, except that it occurred at lower concentrations: the BSAO enzyme activity decreases as a function of increasing AOZ and HEH concentrations whereas furazolidone does not affect the enzyme activity. Furthermore, when HEH binds to the BSAO protein, it causes a linear concentration-dependent optical absorption at 400 nm (see below) which is not observed upon the addition of AOZ. BSAO activity is irreversibly lost upon HEH treatment and as with the mitochondrial sample, this was not restored by prolonged dialysis (data not shown). In contrast, enzyme activity was observed to recover following several hours' incubation with AOZ, but is irreversibly lost with a longer incubation time (2 or 3 days). Finally, the Michaelis–Menten plot obtained on the addition of AOZ to BSAO saturated with benzylamine, a chemical compound known to bind to the active site of the enzyme, revealed that the inhibitory effect of AOZ was non-competitive (figure 3). In contrast, HEH caused irreversible enzyme inhibition in benzylamine-saturated BSAO, suggesting that despite binding of the amino oxidase active site, further reactions may occur. All the evidence seems to support the idea that HEH and AOZ may recognize different binding sites on the protein or produce different effects at the active site. Thus, the BSAO enzyme is also inhibited by AOZ and HEH, but through different mechanisms, as presumed for the MAO enzyme. At this point, it was therefore crucial to clarify if HEH was a metabolite of AOZ.

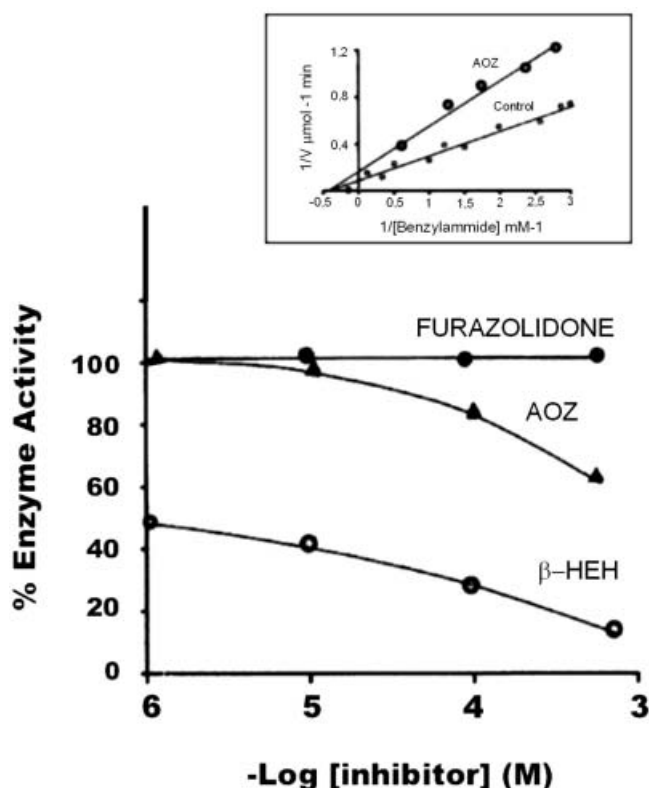


Figure 3. Bovine serum amino oxidase (BSAO) inhibition upon the addition of different concentrations of furazolidone (●), AOZ (▲) and β -HEH (○). Enzyme (1 μ M), dissolved in a buffer containing benzylamide 10 μ M, at pH 7.4, was incubated with each chemical for 30 min at 37 °C. The enzyme activity was measured as above. Data were derived from three determinations. Inset panel B shows the Michaelis–Menten plot obtained upon the addition of benzylamide to BSAO (1 μ M) saturated with AOZ (5 mM)

Biotransformation of AOZ to HEH

To determine if AOZ is transformed to HEH inside the cell, we used the method for the determination of hydrazine and phenylhydrazine previously performed with DAB *p*-(dimethylaminobenzaldehyde) (Gutierrez *et al.* 1989). The reaction of DAB with hydrazine involved a consecutive reversible addition of two aldehyde molecules, contrary to phenylhydrazine which binds only one aldehyde molecule and where the reaction is also slower owing to the presence of a hydrophobic phenyl group. Since AOZ is a cyclic hydrazine and more hydrophobic than HEH, we have tried to adapt this method of determination to discriminate between the two derivatives. At room temperature DAB binds to HEH to give a distinctive optical absorption at 465 nm which increases over time, reaching a maximum after 15 min, remaining constant until 30 min then falls thereafter (figure 4). Addition of 10 mM SDS increases the stability of the complex for longer. The signal is strongly pH-dependent, being high at low pH (below pH 3) (figure 4). This peculiar behaviour allows both hydrazines to be determined, even in the presence of macromolecules. In fact, it has been reported that AOZ at neutral pH is tightly bound to the proteins and thus undetectable, but working at

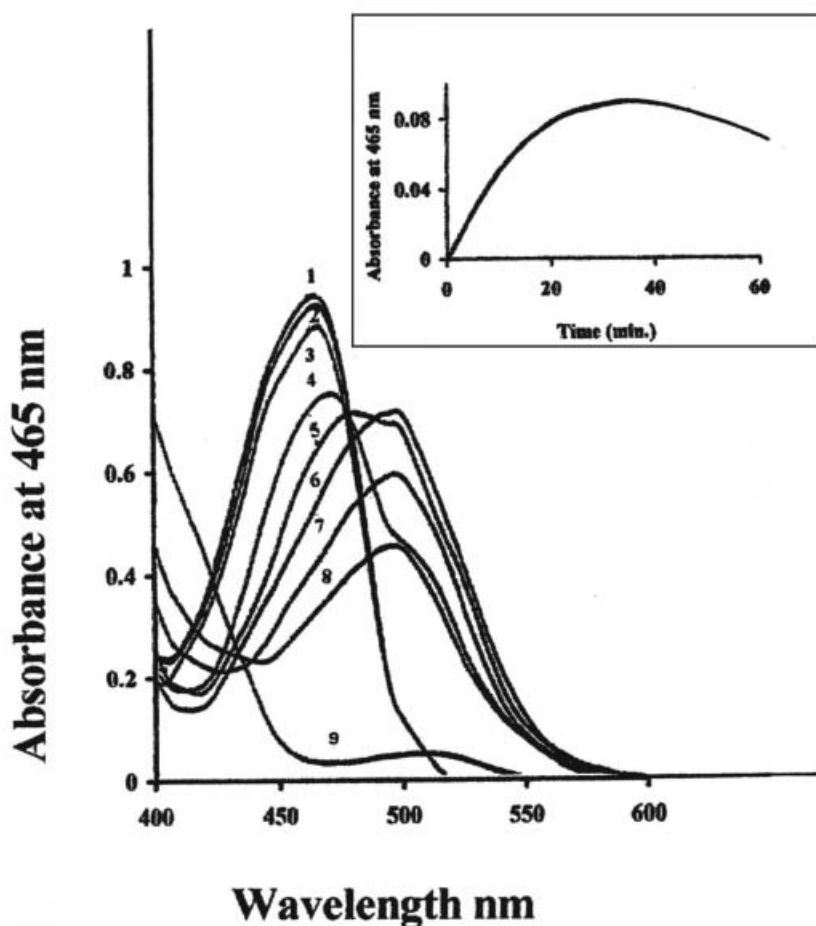


Figure 4. Absorption spectra of $10\text{ }\mu\text{M}$ HEH in 20 mM SDS at different pHs: 1.0 (1), 2.0 (2), 3.0 (3), 3.5 (4), 4.5 (5), 5.5 (6), 6.5 (8) and 8.0 (9). Insert: time-course of the DAB reaction with HEH at room temperature.

low pH (below 2) means this method can be used to detect the presence of hydrazine, in water or in biological solution, in the presence of proteins.

Figure 5A shows the increase in optical absorption when DAB is equilibrated at different concentrations with HEH $10\text{ }\mu\text{M}$ or AOZ 1 mM . It was observed that the two compounds show different extinction coefficients upon binding to DAB and that HEH is more reactive than AOZ. Interestingly, at low concentrations of DAB the formation of azine takes place. Therefore, it is theoretically possible, operating at low DAB concentration (below 1 mM), to determine the presence of low amounts of HEH before AOZ starts to react. Figure 5B shows that small amounts of HEH (below $1\text{ }\mu\text{M}$) may be easily detected even in the presence of large amounts of AOZ (above $100\text{ }\mu\text{M}$). This means that if AOZ is added to a cell at concentrations around $100\text{ }\mu\text{M}$, and only 1–5% is metabolized to HEH, this can be detected spectrophotometrically. However, a reaction in a biological solution may differ from that observed in aqueous solution, since both HEH and AOZ can bind tightly to the macromolecules present. We investigated this possibility by comparing the optical signal recorded when the same concentration of HEH ($1\text{ }\mu\text{M}$) was

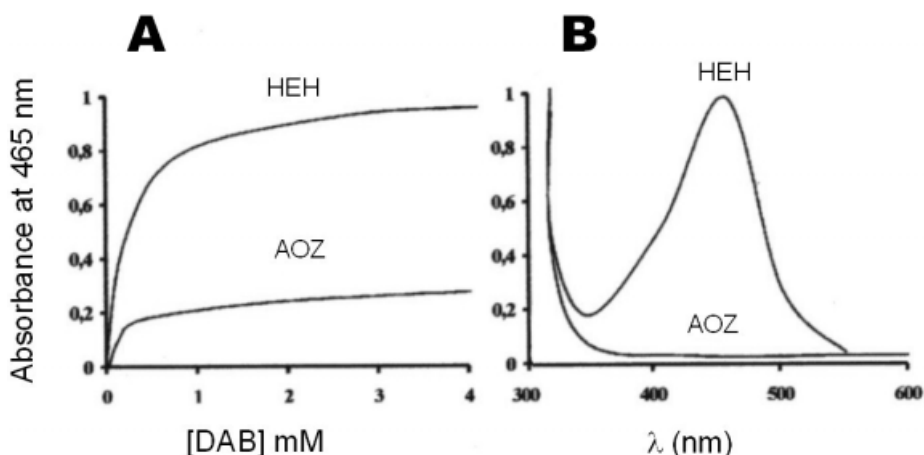


Figure 5. (A) Absorbances of equilibrated solutions of HEH and AOZ versus DAB concentration. DAB 1 mM dissolved in 20 mM and 10 mM SDS was added to 10 μ M HEH at pH 1.0 or to 170 μ M AOZ at pH 2.0. The signal was recorded at $\lambda = 468$ nm for HEH and at 465 nm for AOZ, respectively. Data were derived from three determinations. (B) Absorption spectra of DAB 0.5 mM in the presence of AOZ 100 μ M and of HEH 1 μ M. If about 1–5% of AOZ was metabolized to HEH, then the two spectra should be observed.

added to the same final volume of buffered solution or lysed cells. The optical absorption recorded was similar in both aqueous solution or cell lysate, indicating that under the experimental conditions established (SDS 10 mM, DAB 1 mM, at pH < 2, at 37 °C and after 20 min of incubation) DAB detection of HEH in cell lysates provides a reliable measurement (data not shown).

To detect the possible formation of HEH from AOZ *in vivo*, pig liver microsomes, which are supposed to contain the enzyme(s) responsible for the conversion of AOZ (Klee *et al.* 1999), and liver homogenate were incubated at 37 °C with AOZ (500 μ M) in the presence of NADP⁺ and/or NAD⁺ (1 mM), Mg²⁺ (3 mM) and low oxygen tension for 3 h. At different times the incubation was stopped by lowering the pH to 1.7 by addition of buffered DAB in SDS. After 40 min of incubation the supernatant was placed in a cuvette and read at 465 nm. No optical absorption was recorded. When HEH (10^{−6} M), enough to give 50% of MAO inhibition (table 1), was added to the solution the characteristic HEH spectrum was observed as expected, indicating that the method works and can reveal low concentrations of HEH. Similar experiments were performed with CaCo cells and the same results were observed (data not shown).

Discussion

Oral treatment of man and animals with furazolidone has been shown to result in the inhibition of MAO activity (Hoogenboom *et al.* 1991a, b). There are two distinct isoforms of mitochondrial MAO (termed A and B) (Abell and Kwan 2000) that differ by their substrate and inhibitor specificity, tissue distribution and immunological characteristics (Patel *et al.* 1984), but for the purpose of this study the overall activity was assayed in isolated mitochondria or in lysates cells. Moreover, enzyme inhibition was estimated in cells of two particular types: liver cells, where MAO functions as a detoxifying enzyme against foreign amine

compounds that enter the body (Srolin-Benedetti and Tipton 1998), and the CaCo-2 cells, a derived mucosal cell type that closely mimics *in vivo* events that take place in the first cells which come into contact with AOZ present in foods. Freshly homogenized pig liver instead of cultured pig liver cells were used because of the latter short lifetimes. However, it has been demonstrated that homogenized tissue is capable of metabolizing furazolidone (Hoogenboom *et al.* 1991a). Since it is well documented that in the central and peripheral nervous system MAO catalyses the metabolic transformation of amine neurotransmitters, and in the liver it functions as a detoxifying enzyme against foreign amine compounds that enter the body (Srolin-Benedetti and Tipton 1998), an analytical study to explore the possible molecular mechanism of this inhibition may be of general interest.

We started by comparing the effects of furazolidone and two of its derivatives, AOZ and HEH, on two amino oxidases: mitochondrial MAO and purified BSAO. The results reported herein confirm that the amino oxidase inhibition observed in animals treated with furazolidone (Hoogenboom *et al.* 1991b) is not due to the drug itself but to its metabolite(s). In fact, inhibition was not observed in isolated mitochondria incubated with furazolidone, whereas inhibition was clearly apparent when the drug was added to fresh pig liver homogenate or CaCo-2 cells, supporting the idea that the native molecule has to be metabolized further. Moreover, the toxic effects were observed in different cell types, making it less likely that the inhibition was tissue-specific. Furthermore, the oxazolidone moiety of furazolidone (AOZ) was itself able to produce the observed effects without being metabolized to HEH, which, because of its chemical reactivity, was previously considered to be the final biometabolite of furazolidone and solely responsible for the observed MAO inhibition (Stern *et al.* 1967). Similar results were obtained by addition of the three drugs to purified BSAO.

Two chemical rings are present in the furazolidone molecule: the nitrofuran ring, which undergoes reductive metabolism in the exposed animal, eventually to be metabolized to a biologically inactive product with open-chained nitriles (Lu *et al.* 1998), and an unchanged AOZ, which was present in protein-bound residues in tissues of animals treated with furazolidone (Hoogenboom *et al.* 1992, 1994). Clearly, the probability that AOZ may cause enzyme toxicity either directly or by subsequent metabolism is of concern. Our data show that AOZ itself causes MAO inhibition in isolated mitochondria as well as with the purified protein (BSAO) in contrast to previous studies that reported that MAO inhibition in cultured pig liver cells treated with AOZ becomes more evident after prolonged incubation time while it was not observed at all in isolated mitochondria (Hoogenboom *et al.* 1991b). This discrepancy is probably due to the enzyme assay used to determine the activity, since the tyramine method used in previous work was more cumbersome and also ineffective in respect of the two methods used in our current investigation. The reversibility of AOZ inhibition of MAO observed in isolated mitochondria may not be ascribed to *de novo* synthesis of the enzymes (Hoogenboom *et al.* 1991b) but simply due to removal of the metabolite from the active site since the activity is also recovered in isolated mitochondria. This hypothesis is supported by the observation that intact AOZ can be released from protein binding sites on hepatocytes by addition of HCl to the incubation medium since it is bound as a Schiff base (Horne *et al.* 1996, Rossi *et al.* 1996). In contrast, HEH inhibition is always irreversible. When AOZ is incubated with purified BSAO for a relatively short time, inhibition is reversible. This is not true for long incubation times,

suggesting that at least in interactions with this enzyme the AOZ molecule may undergo further chemical changes. However, the absence of characteristic spectra in optical absorption at 400 nm, which would indicate the peculiar of binding of HEH to protein, confirms that AOZ is not being metabolized to HEH. These data are also consistent with the Michelis–Menten plot analysis that revealed that AOZ acts in a non-competitive manner in contrast to HEH which seems to affect directly the active site of the enzyme causing irreversible inhibition and the previously mentioned characteristic optical absorption at 400 nm. The non-competitive inhibitory effect observed for AOZ agrees with previous studies performed to investigate the relationship between chemical structure and inhibitory activity of some hydrazine-thiazole derivatives on rat liver mitochondria MAO (Tipton 1994, Raciti *et al.* 1995). Raciti *et al.* demonstrated that numerous compounds belonging to structurally diverse substituted hydrazines behave as non-competitive MAO inhibitors. A common structural feature of substrates and inhibitors is an amino or imino group, which is assumed to play an essential role in the orientation and complex formation at the active site of the enzyme (Belleau 1965). However, the presence of alkyl or aryl substituents influence the potency of inhibitors, but the reaction is always reversible, contrary to that observed for HEH, which irreversibly inactivates amine oxidases. Thus, it may be reasonable to conclude that HEH and AOZ act via two different mechanisms that do not require the biotransformation of AOZ to HEH.

Clearly, it was important to determine if AOZ was metabolized to HEH inside the cell to eliminate or confirm its role in the inhibition of amino oxidase following AOZ treatment. To address this question, we developed a new spectrophotometric method to discriminate between AOZ and HEH when both are present in a cell. The method is based on the chemical consideration that HEH contains bifunctional hydrazine groups, while AOZ contains one hydrazone with a monofunctional hydrazine group, allowing discrimination between the two chemical substances by using a chemical chromophore which can bind them differentially. Thus, the condensation of DAB proceeds according to stoichiometric equations given in figure 6. The reaction with HEH involves the consecutive reversible addition of two aldehyde molecules. The reaction with AOZ is slower. In this regard, since AOZ is less polar than HEH, addition of SDS to the solution enhanced considerably the binding of this reactant to micelles, in agreement with that observed for phenylhydrazine when compared with unsubstituted hydrazine (Yatsimirsky *et al.* 1992).

The current method is highly sensitive ($> 1 \mu\text{M}$) and can discriminate between HEH and AOZ because it is based on the strong absorption at 460 nm that occurs when DAB reacts with HEH in contrast to the weak absorption with AOZ. Moreover, the evidence that DAB reacts better with hydrazine groups at low pH, giving a higher optical signal around pH 1.7, allows one to perform the determination at low pH where both AOZ and HEH are free in solution and available to react with the added chromophore. This is extremely important for the determination of AOZ, since it has been reported that at neutral pH it is tightly bound to the proteins and is therefore undetectable. Thus, the current method also works well in the presence of macromolecules, indicating the possibility that it may be suitable for detecting the presence of HEH in any environment. The data presented reveal in an unequivocal way that the metabolism of AOZ to HEH occurs neither inside the cell nor in the presence of pig liver microsomes, which

reported herein suggests that treatment of animals with furazolidone should be stopped in time to allow for the gradual disappearance of AOZ after termination of the treatment.

Finally, the key to the molecular mechanisms by which HEH irreversibly inhibits amino oxidase is in progress in our laboratory. Preliminary electron spin resonance (ESR) measurements have revealed that HEH, unlike AOZ, is itself able to produce OH radicals, with a concomitant consumption of oxygen. Thus, irreversible effects of HEH treatment on both amino oxidase enzymes may be caused by oxygen radicals being released from the metabolite. In this regard, preliminary experiments have revealed that HEH is also highly reactive towards DNA causing fragmentation of the oligonucleotide backbone via the production of oxygen radicals. However, more experiments are in progress in our laboratory to understand the molecular mechanism by which furazolidone is mutagenic without the formation of HEH.

Acknowledgements

The authors are grateful to Dr Hoogenboom for assistance in the preparation of the manuscript and to Dr Jaqueline Scarpa for its revision.

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Serotonin (5-hydroxytryptamine) glucuronidation *in vitro*: assay development, human liver microsome activities and species differences

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Received 8 May 2002

1. The main purpose was to develop a high-performance liquid chromatography (HPLC)-based method to assay serotonin glucuronidation activity using liver microsomal fractions. Application of this method was then demonstrated by determining serotonin UDP-glucuronosyltransferase (UGT) enzyme kinetics using human liver microsomes and recombinant human UGT1A6. Interspecies differences were also evaluated using liver microsomes from 10 different mammalian species.

2. Incubation of liver microsomes with serotonin, UDP-glucuronic acid and magnesium resulted in the formation of a single product peak using HPLC with fluorescence and ultraviolet absorbance detection. This peak was confirmed as serotonin glucuronide based on sensitivity to β -glucuronidase and by obtaining the expected mass of 352 with positive-ion mass spectrometry.

3. Following a preparative HPLC isolation, the structure of this metabolite was established as serotonin-5-*O*-glucuronide by ¹H-NMR spectroscopy.

4. Enzyme kinetic studies showed apparent K_m and V_{max} of 8.8 ± 0.3 mM and 43.4 ± 0.4 nmoles min⁻¹ mg⁻¹ protein, respectively, for human liver microsomes, and 5.9 ± 0.2 mM and 15.8 ± 0.2 nmoles min⁻¹ mg⁻¹, respectively, for recombinant UGT1A6.

5. The order of serotonin-UGT activities in animal liver microsomes was rat > mouse > human > cow > pig > horse > dog > rabbit > monkey > ferret. Cat livers showed no serotonin-UGT activity. Heterozygous and homozygous mutant Gunn rat livers had 40 and 13%, respectively, of the activity of the normal Wistar rat, indicating a significant contribution by a rat UGT1A isoform to serotonin glucuronidation.

6. This assay provides a novel sensitive and specific technique for the measurement of serotonin-UGT activity *in vitro*.

Introduction

UDP-glucuronosyltransferase (UGT) 1A6 is an important xenobiotic-metabolizing enzyme and is found in a wide range of tissues including liver, intestine, lung, kidney and brain (Harding *et al.* 1988, Sutherland *et al.* 1993, King *et al.* 1999, Tukey and Strassburg 2001). It catalyses the glucuronidation of clinically used important drugs and environmental chemicals, facilitating their removal from the body and significantly affecting their therapeutic efficacy and toxicity, respectively (Liston *et al.* 2001). Consequently, interindividual variability in the function-

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ing of this enzyme through differential regulation or genetic polymorphism is likely to have important health consequences (Ciotti *et al.* 1997, Liston *et al.* 2001). Accurate and specific determination of the glucuronidation activity of UGT1A6 will be an important initial step in understanding the role of this UGT isoform in human health.

Isoform-specific substrate probes have proved very useful in quantifying drug-metabolizing enzymes (Venkatakrishnan *et al.* 2001), however specific probe substrates for most of the UGT isoforms still need to be identified and validated. Until now, UGT1A6 activity has been quantified using α -naphthol, 4-nitrophenol, 4-methylumbelliferone and acetaminophen. Unfortunately, all these substrates have not proven to be UGT isoform specific. Alternative substrates must therefore be identified for use as probes for more accurate estimation of UGT1A6 activity. Serotonin has been identified recently as a substrate of human UGT1A6 (King *et al.* 1999). Since serotonin glucuronidation is also absent in cats, which lack a functional UGT1A6 gene (Court and Greenblatt 2000), we suspected that serotonin might be glucuronidated only by the UGT1A6 isoform.

To date, serotonin glucuronidation activities have been measured using a colorimetric technique following solvent extraction of the metabolite (Leakey 1978, Abe *et al.* 1992). However, this method tends to lack sensitivity and specificity and is not amenable to high sample throughput. Consequently, the primary aim of the present study was to develop a sensitive and specific high-performance liquid chromatography (HPLC) based method for the *in vitro* measurement of serotonin-UGT activities in human and animal tissues. Mass and nuclear magnetic resonance spectroscopy analyses were used to establish unequivocally the structure of the glucuronide metabolite formed in these reactions. Application of this serotonin-UGT assay was then demonstrated by determining enzyme kinetics using human liver microsomes and recombinant UGT1A6. Interspecies differences in enzyme activities were also evaluated using liver microsomes from 10 different mammalian species.

Materials and methods

Reagents

Unless otherwise indicated, reagents including alamethicin, UDP-glucuronic acid (UDPGA; sodium salt), serotonin (5-hydroxytryptamine) hydrochloride, β -glucuronidase (containing sulphatase activity), type H-3AF from *Helix pomatia* were purchased from Sigma Chemical Co. (St Louis, MO, USA). Microsomes of lymphoblast cells containing cDNA expressed human UGT1A6 were obtained from BD-Gentest (Woburn, MA, USA).

Liver microsomes

Human liver tissue from six donors were randomly selected from frozen banks maintained at the Division of Clinical Pharmacology, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston. Donors were all Caucasian and included five males of 5, 68, 36, 43 and 21 years of age and a 65-year-old female. Liver from the female (LV35) was used for enzyme kinetic analysis. For animal species comparison, livers were obtained from five dogs, eight ferrets, four mice, a cat, a cow, a horse, a monkey, a pig, a rabbit and three rats (one each of homozygous Gunn mutant—J/J, heterozygous Gunn mutant—J/+, and normal Wistar strain—+/+). Relevant details of these animals are given in table 1. The Animal Research Committee and the Human Investigation Review Committee of Tufts University approved collection and use of these tissues. Liver microsomes were prepared individually (except mouse livers) from frozen liver by differential centrifugation as described by Court *et al.* (1997). The resultant pellet was reconstituted in 20% glycerol/phosphate buffer, aliquoted and stored at -80°C . Frozen microsomes were thawed immediately before use. Microsomal protein concentrations were determined using the bicinchoninic acid assay

Table 1. Derivation of liver tissue used in this study.

Liver i.d.	No.	Genus/species	Race/breed/strain	Age	Gender
Ferret	8	<i>Mustela putorius furo</i>	Domestic	1–2.5 years	four males; four females
Cat	4	<i>Felis domesticus</i>	Domestic short hair	1–2 years	four males
Dog	10	<i>Canis familiaris</i>	Mixed breed	1–3 years	five males; five females
Cow	1	<i>Bos taurus</i>	Unknown	6 months	unknown
Horse	1	<i>Equus caballus</i>	Quarter horse	13 years	male
Monkey	1	<i>Macaca fascicularis</i>	Crab-eating macaque	9–10 years	female
Mouse	4*	<i>Mus musculus</i>	CD-1	12 weeks	four males
Pig	1	<i>Sus scrofa</i>	Yorkshire	1 year	female
Rabbit	1	<i>Oryctolagus cuniculus</i>	New Zealand white	9 months	female
Rat	3	<i>Rattus norvegicus</i>	Gunn (1 each J/J, J/+ +/+; with Wistar background)	6 months	male

* Microsomes prepared from pooled livers of four littermates.

(Pierce, Rockford, IL, USA). The quality of the liver samples used was ascertained by reference to other UGT and CYP enzyme activities measured in this laboratory using the same set of liver samples. Livers that consistently showed low activity values (more than twofold lower for all measured activities) relative to the median activity for the entire liver set were excluded from the study.

Serotonin glucuronidation assay

Microsomal serotonin glucuronidation activities were measured by HPLC. Solutions of serotonin were prepared in methanol and stored at -20°C . After adding the required amount of serotonin to incubation tubes, the solvent was evaporated to dryness in a 45°C vacuum oven before the addition of cofactors. Samples were incubated in duplicate. Incubation mixtures contained 25 mM phosphate buffer (pH 7.5), 2.5 mM MgCl_2 and UDPGA (20 mM for kinetic studies, otherwise 5 mM). The samples were pre-incubated in a water bath at 37°C for 2–3 min. Reactions were initiated by the addition of microsomal protein, and the final volume was 100 μl , unless otherwise indicated. Protein concentrations of 0.25–0.8 mg ml^{-1} of microsomes were used. Incubations were performed in a water bath for 30–60 min at 37°C and terminated by addition of 100 μl acetonitrile containing acetaminophen as the internal standard. The mixture was vortex mixed and spun at 16 000g for 10 min and the supernatants were transferred to HPLC tubes, dried down in a vacuum oven at 45°C and reconstituted with 150 μl water containing 5% acetonitrile.

The HPLC apparatus (Model 1100, Agilent, Palo Alto, CA, USA) consisted of a dual-head pump with autoinjector serially connected to a 250×4.6 mm C_{18} column (Aqua, Phenomenex, Torrance, CA, USA), a UV absorbance detector (set at 270 nm) and fluorescence detector (excitation wavelength 225 nm, emission wavelength 330 nm) connected in series to monitor column effluent. The UV detector was used to quantitate acetaminophen (internal standard) concentrations, while the fluorescence detector determined serotonin glucuronide concentrations. Acetaminophen was used as the internal standard since no fluorescent compound with an appropriate retention time could be identified. The HPLC mobile phase consisted of 20 mM, pH 4.5, phosphate buffer in water (solution A) and acetonitrile (B). Run condition consisted of 5% solution B (and 95% A) for the first 8 min and was raised to 50% solution B using a linear gradient over the next 9 min. A flow rate of 1 ml min^{-1} was maintained throughout. Analyte retention times were typically 7.5 min for serotonin glucuronide, 17.5 min for serotonin and 20 min for the internal standard (acetaminophen). Standard curves were generated by preparing known amounts of purified serotonin glucuronide (10–100 nmoles) in incubation buffer and the peak areas showed linearity in this range with both UV and fluorescence detectors without any interfering peaks. Chromatograms were analysed by measuring serotonin glucuronide peak area normalized to the internal standard peak area. Initial rate conditions with respect to time (up to 60 min) and protein concentration (up to 0.8 mg ml^{-1}) for the formation of serotonin glucuronide were established in preliminary studies at 1 mM serotonin concentration. Further studies at 0.1 mM serotonin concentration showed linearity with protein concentrations up to 0.25 mg ml^{-1} and incubation time to 30 min. Alamethicin dissolved in methanol (0.0025% w/v final alamethicin concentration) was also added to incubations in amounts determined in preliminary studies to cause maximal activation (approximately two fold increase). Enzyme activity was normalized to incubation time and microsomal protein content and given as $\text{nmoles min}^{-1} \text{ mg}^{-1}$ protein. The lower limit of quantitation of the assay under these conditions was $3 \text{ pmoles min}^{-1} \text{ mg}^{-1}$ protein, based on a signal-to-noise ratio of 5:1 using purified glucuronide against blank. The reproducibility of the assay was determined by replicate analysis of the activity of 20 human liver microsomal samples (data not shown). Intra- and interassay variations were found to average less than 5%.

β -Glucuronidase assay

Glucuronidase treatment was used tentatively to identify the metabolite peak as a glucuronide. A reaction mixture (250 μ l) containing the glucuronide metabolite was generated by incubating 1 mM serotonin and 0.8 mg ml⁻¹ human liver microsomes for 1 h at 37 °C. After adding 250 μ l of 1 M acetate buffer (pH 4.5) and 10 μ l (10 000 units) of *Helix pomatia* β -glucuronidase (Sigma, St Louis, MO, USA), it was incubated for a further 16 h at 37 °C and stopped by addition of 250 μ l acetonitrile/acetic acid. Control incubations were identical except that β -glucuronidase was added after the stop solution. The reaction products were analysed by HPLC as described above.

Purification of serotonin glucuronide

To facilitate further metabolite identification and allow calibration, a small amount (approximately 10 mg) of serotonin glucuronide was biologically synthesized and purified as follows. Incubation buffer (2.5 ml) containing 20 mg serotonin, 65 mg UDPGA, 2.5 mM MgCl₂ and 125 μ g alamethicin was incubated with 3.6 mg mouse liver microsomes overnight at 37 °C. After centrifuging at 15 000g for 10 min, the supernatant was filtered using a 5000 kDa cut-off centrifugal filter (Ultrafree, Millipore, Milford, MA, USA) to remove remaining proteins and then concentrated in a vacuum oven for 6 h at 40 °C. Preparative HPLC was used to isolate the glucuronide peak using the method described above except that a shallow linear gradient from 0.2 to 4% of solution B was used over the first 50 min of the run. The eluate was collected and concentrated by vacuum drying in an oven at 40 °C. The residual moisture contained in the pellet was removed by precipitating in 10 ml 100% methanol followed by evaporation of the remaining methanol from the precipitate.

HPLC-mass spectrometry

Mass spectrometry (MS) was used to confirm the molecular weight of the glucuronide peak identified by HPLC. HPLC conditions were similar to those described previously, except that 20 mM (pH 6.0) ammonium acetate buffer was used instead of phosphate buffer. Apparatus consisted of an ion-trap MS detector (Agilent 1100, Palo Alto, CA, USA) operated in positive-ion electrospray mode. The detector was initially tuned by direct injection of purified serotonin glucuronide dissolved in ammonium acetate buffer.

¹H-NMR spectroscopy

¹H-NMR spectroscopy was used to confirm the structural identity of the HPLC-purified glucuronide peak. Proton NMR spectra of serotonin glucuronide and pure serotonin in deuterated dimethylsulfoxide (d₆-dmsO) were obtained using Bruker 300 MHz and 500 MHz NMR spectrometers (DPX300 and AMX500, Bruker, Billerica, MA, USA). Two-dimensional double quantum filtered COSY (correlated spectroscopy) and NOESY (nuclear overhauser effect spectroscopy) spectra of the HPLC-purified serotonin glucuronide were obtained, the latter employing a mixing time of 800 ms.

Enzyme kinetics

For enzyme kinetic analysis activities were measured with a fixed 20 mM UDPGA concentration and serotonin concentrations varying from 0.1 to 40 mM (serotonin kinetics), and with a fixed 40 mM serotonin concentration and UDPGA concentrations varying from 0.05 to 20 mM (UDPGA kinetics). Substrate concentration (*S*) and velocity (*V*) data were fitted to the Michaelis-Menten model by non-linear least-squares regression analysis (GraphPad Prism, GraphPad Software, Inc., San Diego, CA, USA) to derive the enzyme kinetic parameters *V*_{max} (maximal velocity) and *K*_m (substrate concentration at half maximal velocity) (Court *et al.* 2001).

Statistics

Statistical analyses were performed using the Sigmapstat software package (SPSS, Chicago, IL, USA). All activities were determined in duplicate and averaged. *p* ≤ 0.05 was considered as statistically significant. Potential differences in serotonin glucuronidation activities related to gender and species were evaluated by the Mann-Whitney rank sum test.

Results

Serotonin metabolite identification

Incubation of serotonin with UDPGA, magnesium and liver microsomes resulted in the appearance of a novel chromatographic peak that was visible using UV absorbance and fluorescence detectors previously optimized for the

Table 2. NMR parameters of serotonin glucuronide adduct in d_6 -dmsO

Resonance	$^1H(ppm)$
1(N-H)	10.76
4	7.35
7	7.22
2	7.11
6	6.82
1'	4.76
3'	3.47
2'	3.29
4' or 5'	3.24
5' or 4'	3.22
CH2- α	2.96
CH2- β	2.87

detection of serotonin (figure 1A). This peak had a shorter retention time (7.5 min) compared with serotonin (17.5 min), and was dependent on inclusion of UDPGA in the incubation mixture. Treatment of the incubate with β -glucuronidase resulted in disappearance of the metabolite peak (figure 1B). Following HPLC purification, fluorescence and UV absorbance scans of this compound showed optimal excitation and emission wavelengths of 225 and 330 nm, respectively, and UV absorbance maxima at both 225 and 270 nm. These were identical to values obtained for serotonin.

HPLC-MS analysis of both human liver microsome incubate and HPLC-purified metabolite determined the m/z (+1) of this compound to be 353.15 (figure 1C), which is consistent with the predicted molecular weight of a glucuronide conjugate of serotonin (352 mass units). NMR spectroscopy was then used to identify the position of the glucuronide linkage on the serotonin molecule. Proton resonances (table 2) for the purified serotonin glucuronide (figure 2A) were assigned using a combination of the 1D serotonin and serotonin glucuronide (figure 2B) spectra and the COSY spectrum of serotonin glucuronide. No evidence was found in the NOESY spectrum of serotonin glucuronide (figure 2C) for cross peaks between H1' and H2 or between H1' and CH2 β excluding the presence of any N-linked glucuronide. Furthermore, based on strong cross peaks between the glucuronic acid H1' and the indole H4 and H6 resonances in the 2D spectra, it was concluded that adduct formation could only have occurred through an ether linkage at the indole C5 position.

Serotonin glucuronidation kinetics

Enzyme kinetic analyses were performed using representative human liver (LV35) microsomes and recombinant UGT1A6. Michaelis-Menten and Eadie-Hofstee plots of these data were consistent with one enzyme Michaelis-Menten kinetics (figure 3). The highest concentration of serotonin used was 40 mM since higher concentrations resulted in lower activities (presumably from substrate inhibition).

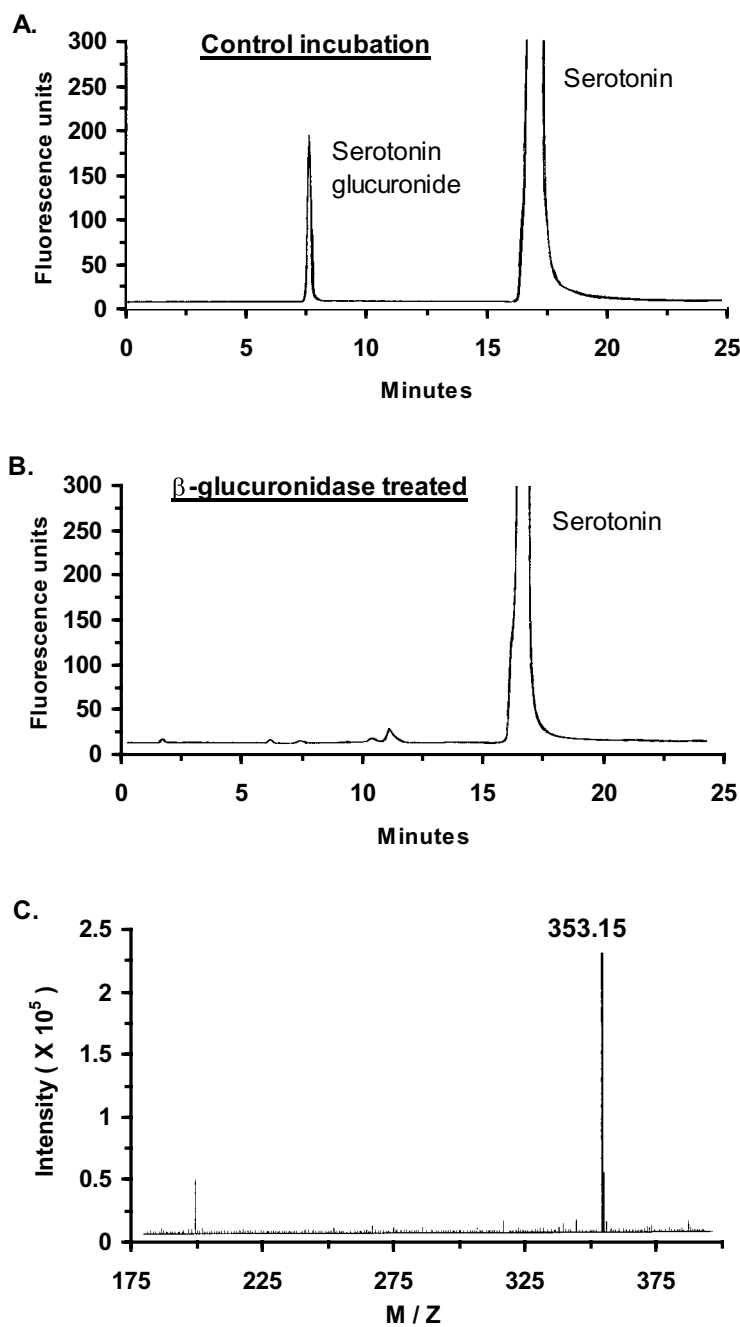


Figure 1. (A) Representative HPLC chromatogram of *in vitro* glucuronidation of serotonin. Injectate was the centrifuged supernatant of a mixture of human liver microsomes, 4 mM serotonin, 5 mM UDPGA and 5 mM MgCl₂ in 25 mM, pH 7.5, phosphate buffer incubated at 37 °C. Shown is the output from the fluorescence detector (225 nm excitation, 330 nm emission wavelengths). (B) HPLC chromatogram of the same incubate shown in (A), following treatment with β-glucuronidase as described in the Materials and methods. (C) Positive-ion mass spectrogram of HPLC-purified serotonin glucuronide. The *m/z* of the only significant molecular ion present was 353.15, which is consistent with the calculated molecular mass of 352 for serotonin glucuronide.

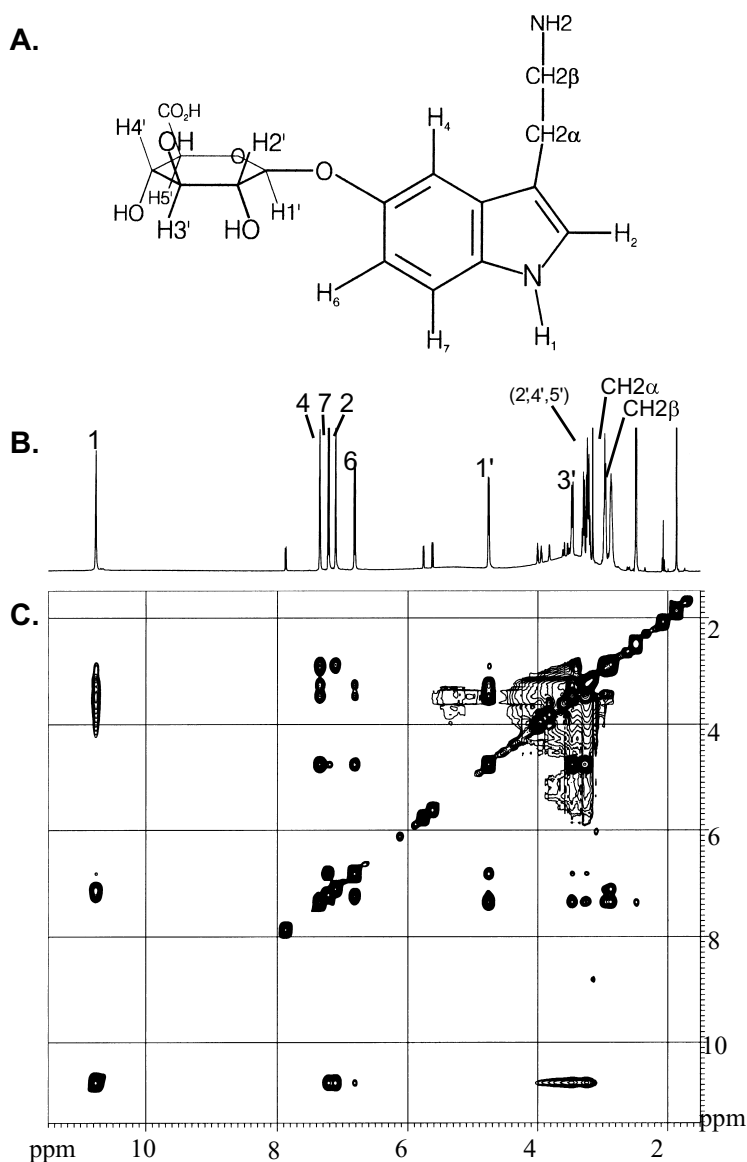


Figure 2. (A) Structure of serotonin 5-*O*-glucuronide. (B) 1D ¹H-NMR spectrogram of HPLC-purified serotonin glucuronide. Shown are resonances corresponding to protons labelled in (A). (C) 2D NOESY NMR plot of HPLC-purified serotonin glucuronide showing proton interactions consistent with the structure shown in (A).

Fit of liver microsome data to the Michaelis–Menten kinetic model resulted in an apparent $K_m = 0.49 \pm 0.04$ (estimate $\pm$ SEE) mM and a $V_{max} = 33 \pm 0.7$ nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein for UDPGA and an apparent $K_m = 8.8 \pm 0.3$ mM and a $V_{max} = 43.4 \pm 0.4$ nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein for serotonin. Recombinant UGT1A6 had an apparent $K_m = 5.9 \pm 0.2$ mM and a $V_{max} = 15.8 \pm 0.2$ nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein for serotonin. Similar apparent K_m for serotonin were also obtained with 10 times lower protein concentrations (0.05 mg ml^{-1} for human liver

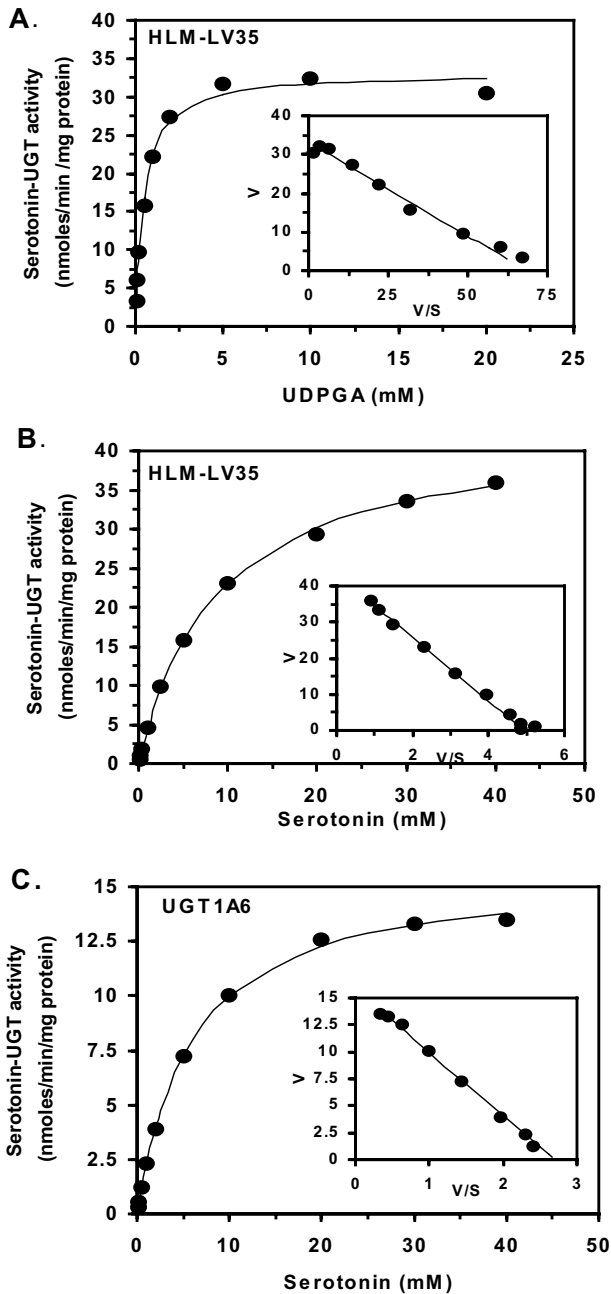


Figure 3. Enzyme kinetics of serotonin glucuronidation by human liver microsomes (LV35) and lymphoblast-expressed UGT1A6. Activities were measured using 40mM serotonin and UDPGA concentrations between 0.05 and 20mM (A) and 20mM UDPGA and serotonin concentrations between 0.1 and 40mM (B, C). Shown for each set of data are Michaelis–Menten and Eadie–Hofstee (inset) plots, as well as curves representing best-fit estimates determined by non-linear curve fit to the Michaelis–Menten equation. Estimates for apparent K_m and V_{max} were 0.49 ± 0.04 mM and 33 ± 0.7 nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein, respectively, for UDPGA (A) and 8.8 ± 0.3 mM and 43.4 ± 0.4 nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein, respectively, for serotonin (B) by human liver microsomes, and 5.9 ± 0.2 mM and 15.8 ± 0.2 nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein, respectively, by expressed UGT1A6 (C). The data points represent the average of two determinations.

microsomes; 0.01 mg ml⁻¹ for recombinant UGT1A6) and when serotonin was freshly dissolved in incubation buffer rather than dried down from a methanol solution (data not shown).

Species differences in serotonin glucuronidation

As shown in figure 4, serotonin-UGT activities in animal liver microsomes at 4 mM serotonin concentration varied widely between species with no detectable activity in all cat livers ($n = 4$; lower limit of sensitivity 3 pmoles min⁻¹ mg⁻¹ protein) to as high as 15.5 nmoles min⁻¹ mg⁻¹ in normal rat liver. Comparison of liver microsomal serotonin-UGT activities between species showed higher values in human (8.0 ± 2.1 nmoles min⁻¹ mg⁻¹ protein) compared with dog (1.3 ± 0.21), ferret (0.2 ± 0.02) and cat (0). Serotonin-UGT activities in homozygous mutant (J/J) and heterozygous (J/+) Gunn rats were 13 and 60%, respectively, of activities measured in the congenic normal Wistar rat. No significant differences in serotonin glucuronidation activities related to gender were found for dog ($p = 0.856$; $n = 5$ of each gender) or ferret ($p = 0.959$; $n = 4$ of each gender) liver microsomes.

Discussion

We have developed an *in vitro* HPLC-based assay that can be used to quantitate serotonin glucuronidation activities using mammalian liver microsomes. Positive identification of the metabolite peak as serotonin glucuronide was based on a requirement for UDPGA in the incubation mixture, sensitivity to treatment with β -glucuronidase and spectral similarities (UV absorbance and fluorescence) to serotonin. HPLC purification of this glucuronide metabolite enabled confirmation of this by MS analysis, which yielded a molecular mass of 352.1, identical to the calculated mass of serotonin glucuronide. Since there are three possible sites of conjugation on serotonin, including the ring nitrogen, the side chain amine and or the phenolic ether, NMR was used to determine the structure of the glucuronide. For this purpose, 2D NMR (particularly NOESY) was most informative in that strong interactions between the H_{1'} on glucuronic acid and the H₄ and H₆ serotonin protons were observed, supporting O-linked glucuronidation. On the other hand, interactions between H_{1'} and H₂ indicating aromatic N-linked glucuronidation, or between H_{1'} and the side chain CH₂ β indicating primary amine glucuronidation, were not observed.

The HPLC method described above has proven to be simple and rapid with a higher sensitivity (3 pmoles min⁻¹ mg⁻¹ protein) compared with the previously reported colorimetric method (about 17 pmoles min⁻¹ mg⁻¹ protein) (Leakey 1978). This increased sensitivity may prove useful in measuring serotonin-UGT activities in animal and human tissues with very low levels of this enzyme.

Preliminary enzyme kinetic studies were performed primarily to establish appropriate serotonin and UDPGA concentrations for more extensive activity studies. The shape of the enzyme kinetic curve can also indicate whether a single or multiple UGT isoforms are likely to catalyse serotonin glucuronidation. Serotonin and UDPGA kinetic curves of both human liver microsomal (LV35) and recombinant UGT1A6 showed a simple Michaelis-Menten kinetic pattern and had very similar apparent K_m 's for serotonin glucuronidation activity, which

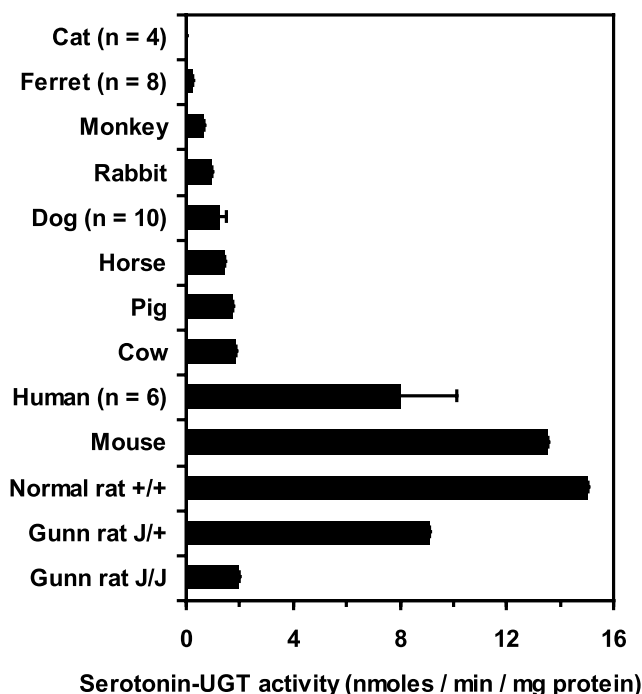


Figure 4. Species comparative serotonin glucuronidation by liver microsomes. For cat ($n = 4$), ferret (8), dog (10) and human (6) liver microsomes, bars are mean activity values, while the error bars indicate the standard error of estimates. For other species, the bar is the average of duplicate determinations using liver microsomes from one animal, except for mouse liver microsomes, which were pooled from four animals.

is indicative of catalysis by either a single enzyme or possibly multiple enzymes with the same K_m .

The apparent K_m for UDPGA in this study with human liver microsomes (0.48 mM) was similar to the value previously obtained using rat liver microsomes (0.6 mM) (Abe *et al.* 1992). In contrast the apparent K_m 's for serotonin determined with human liver microsomes and expressed UGT1A6 in this study were over 10 times higher compared with values reported for expressed human UGT1A6 (0.165 mM) (King *et al.* 1999) and for rat liver microsomes (0.1 mM) (Abe *et al.* 1992). One possible explanation for this difference is non-specific microsomal binding of substrate, which results in higher apparent K_m 's (Venkatakrishnan *et al.* 2001). However, use of very low hepatic microsomal (0.05 mg ml⁻¹) or recombinant UGT1A6 (0.01 mg ml⁻¹) protein concentrations had no effect on apparent K_m 's. Similarly, the apparent K_m did not change when a freshly dissolved buffered aqueous solution of serotonin was used instead of drying down serotonin dissolved in methanol, thereby excluding the possibility of inadequate substrate dissolution. Finally, the difference may relate to the assay technique used to measure serotonin glucuronide concentrations. All previous reports have used a technique that involves differential precipitation of the glucuronide and colorimetric detection without the use of a serotonin glucuronide standard. Although we have not made a direct comparison, the current HPLC technique we have developed is likely to be much more specific.

Gender differences in serotonin glucuronidation were not found for ferret or for dog liver microsomes. Previous studies of acetaminophen glucuronidation in human (Court *et al.* 2001) and ferret (Court 2001) liver microsomes showed higher activities in males compared with females. As previously indicated, acetaminophen is glucuronidated by multiple UGT isoforms in addition to UGT1A6 in humans (Court *et al.* 2001). Consequently, the gender difference in acetaminophen-UGT activity may be due to a UGT isoform other than that responsible for glucuronidating serotonin.

Complete absence of serotonin glucuronidation by liver homogenate from one cat has been reported previously (Leakey 1978) and was confirmed in this study using liver microsomal fractions from four different cats. Since cats have been shown recently to lack a functional UGT1A6 gene (Court and Greenblatt 2000), this observation would support a role for UGT1A6 in the glucuronidation of serotonin. However, in addition to UGT1A6, other UGT isoforms are also lacking in cat liver, including an isoform mediating glucuronidation of morphine (Yeh *et al.* 1971). Of the remaining species, ferrets had the lowest serotonin-UGT activities. Low acetaminophen glucuronidation activities have also been shown for ferret liver, with values close to those of cat liver (Court 2001). However, unlike cat, ferrets have been shown to possess an intact UGT1A6 gene (Court 2001).

Gunn rats (JJ) were studied since they carry a premature stop codon mutation in one of the shared exons of the UGT1A gene locus that results in truncated UGT1A isoforms but normal UGT2 family expression (Iyanagi 1991). Previous work has shown reduced serotonin glucuronidation in homozygous Gunn rat liver homogenates to about 20% of the activity in normal Wistar rat liver homogenates (Leakey 1978). We found a somewhat lower activity in the homozygous mutant Gunn rat liver microsomes (13% of control) and as expected found intermediate activities (60% of control) in the heterozygous mutant liver microsomes. Together, these data indicate that serotonin is glucuronidated primarily by one or more of the UGT1A subfamily isoforms (possibly UGT1A6) in rat liver microsomes. The low but significant activity in the homozygous mutant rat liver indicates that UGT2 isoforms may also contribute to this activity.

In conclusion, we have developed a novel sensitive and specific technique for the *in vitro* measurement of serotonin glucuronidation activity. Based on preliminary results with human and animal liver microsomes, and expressed UGT1A6, further validation of serotonin as a specific probe substrate for UGT1A6 is warranted.

Acknowledgement

This work was supported by Grants RR-00104, GM-61834, DA-05258, MH-58435, DA-13209, DK-58496 and RR-00054 from the National Institutes of Health (Bethesda, MD).

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***l*-Deprenyl metabolism by the cytochrome P450 system in monkey (*Cercopithecus aethiops*) liver microsomes**

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Received 1 August 2002

1. The aim was to clarify the kinetic and cytochrome P450 (CYP) enzymes involved in *l*-deprenyl metabolism by liver microsomal preparations from African green monkeys, an animal model extensively used in the study of Parkinson's disease.

2. CYP levels and monooxygenase activities were similar to those observed in microsomes from other monkey strains. The enzyme kinetics of both *l*-methamphetamine and *l*-nordeprenyl formation were characterized by a high- and low-affinity component. For *l*-methamphetamine, the apparent K_{m1} and K_{m2} were 1.07 ± 0.01 and $350 \pm 2.7 \mu\text{M}$, and V_{max1} and V_{max2} were 4.70 ± 0.01 and $8.9 \pm 0.02 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$, respectively. For *l*-nordeprenyl, K_{m1} and K_{m2} were 0.96 ± 0.05 and $168 \pm 15 \mu\text{M}$, and V_{max1} and V_{max2} were 3.34 ± 0.02 and $3.91 \pm 0.02 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$, respectively. The ratio V_{max}/K_m for both metabolites was 2 orders of magnitude higher for the low K_m component than for the high K_m , suggesting that the former component is the major determinant of *l*-deprenyl N-dealkylation. At $15 \mu\text{M}$ *l*-deprenyl, both ketoconazole and 8-methoxypsoralen significantly inhibited *l*-methamphetamine and *l*-nordeprenyl formation, indicating that CYP3A and CYP2A enzymes were involved in both reactions. At $500 \mu\text{M}$ *l*-deprenyl, however, inhibition studies suggest the involvement of CYP1A and 2D enzymes.

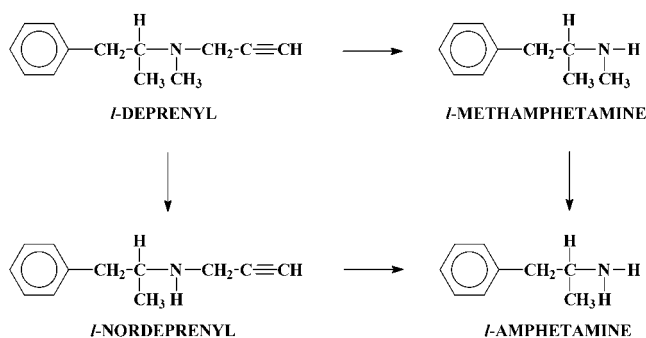
3. The metabolism of *l*-deprenyl by monkey liver microsomes is very efficient, indicating that CYP-dependent metabolism is relevant and could contribute to neuroprotection in primate models of Parkinson's disease.

Introduction

l-Deprenyl [(–)-deprenyl, selegiline] (figure 1) is a selective inhibitor of monoamine oxidase-B (MAO-B). Because of its capacity to increase striatal dopamine levels, it has been used for treating patients with Parkinson's disease (Gerlach *et al.* 1996). As an adjunct to levodopa therapy, *l*-deprenyl has been reported to improve function and reduce motor fluctuations in patients with advanced Parkinson's disease (Birkmayer *et al.* 1985). In patients with early, untreated Parkinson's disease, controlled clinical trials have demonstrated that *l*-deprenyl delays the disability and slows the progression of signs and symptoms of this condition (The Parkinson Study Group 1993).

The observation that pretreating animals with *l*-deprenyl protects them against the neurotoxic effects of compounds such as 1-methyl-4-phenyl-1,2,3,6-

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Figure 1. Major metabolic pathways of *l*-deprenyl.

tetrahydropyridine (MPTP), 6-hydroxydopamine and *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) has stimulated studies aimed at clarifying the pharmacological and biochemical properties of this drug (Tipton 1994). Although it was initially assumed that the benefit from *l*-deprenyl in Parkinson's patients was a result of MAO-B inhibition, more recent studies, both *in vivo* and *in vitro*, have suggested that *l*-deprenyl prevents neurodegeneration by mechanisms that are independent of the inhibition of MAO-B activity (Tatton and Greenwood 1991, Olanow and Tatton 1999). These latter studies also suggested that *l*-deprenyl metabolites contribute to its effect. In humans, as well as in experimental animals, *l*-deprenyl is rapidly metabolized by the liver cytochrome P450 (CYP) system, forming mainly *l*-nordeprenyl (*l*-demethyldeprenyl) and *l*-methamphetamine (*l*-depropargyldeprenyl) (Baker *et al.* 1999). These two compounds are further metabolized to *l*-amphetamine (figure 1).

l-Nordeprenyl, which is a less potent inhibitor of MAO-B than the parent drug both *in vitro* and *in vivo*, is more efficacious in protecting dopamine neurones against oxidative stress damage (Olanow and Tatton 1999). The other metabolite, *l*-methamphetamine, is a more potent inhibitor of presynaptic noradrenaline and dopamine uptake than the parent drug and it has been suggested that this effect contributes to neuroprotection (Sziráki *et al.* 1994). Although the CYP-dependent metabolism of *l*-deprenyl is therefore of clinical importance, few studies have examined this in detail. Grace *et al.* (1994) and Bach *et al.* (2000) have reported that human recombinant CYP2D6 catalyses the formation of both *l*-nordeprenyl and *l*-methamphetamine. However, neither quinidine nor quinine, two competitive inhibitors of CYP2D subfamily enzymes in man and rat, reduce the formation rate of these two metabolites in either control or phenobarbital-induced rat liver microsomes (Grace *et al.* 1994). Furthermore, Scheinin *et al.* (1998) have reported that CYP2D6 is not important in the primary metabolic disposition of *l*-deprenyl and that its biological effects are comparable either in poor or in efficient debrisoquine metabolizers. A recent study in humans has demonstrated that CYP2B6, 2C19 and, though to a lesser extent, 3A4 and 1A2 may be involved in the metabolism of *l*-deprenyl (Hidestrand *et al.* 2001).

The determination of CYP expression patterns and *l*-deprenyl metabolism in African green monkey liver should help to underline the similarities and differences with respect to humans, thus contributing to a more appropriate use of this animal model in Parkinson's disease studies. In fact, the MPTP-treated African green monkey is of great help in the study of Parkinson's disease (Jan *et al.* 2000)

because it shows neuropathological and neurochemical alterations very similar to those found in patients suffering from the disease and particularly in young people who unintentionally were exposed to the toxic effects of MPTP (Taylor *et al.* 1997).

The aim of the present study was to investigate the N-dealkylation pathway of *l*-deprenyl in African green monkey liver microsomes and to clarify which CYP forms are involved. In addition, the characterization of the CYP system in this monkey strain has been carried out.

Materials and methods

l-(-)-*N*, α -dimethyl-*N*-2-propynylphenethyl-amine hydrochloride (*l*-deprenyl-HCl) and *l*-(-)-*N*, α -methyl-*N*-2-propynylphenethyl-amine (*l*-nordeprenyl) were gifts from Chino Chemical Works (Budapest, Hungary), ketoconazole was from Janssen (Rome, Italy); NADPH, NADP and glucose 6-phosphate dehydrogenase were provided by Boehringer (Mannheim, Germany). The human recombinant CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4 were expressed in *E. coli* (Guengerich *et al.* 1996) using plasmids containing cDNA of the major human CYP isoforms kindly supplied by Dr F. P. Guengerich (Nashville, TN, USA). Rabbit polyclonal antibodies against rat CYP1A1, 2B1 and 2E1 were obtained in our laboratory as described (Menicagli *et al.* 1994). Anti-rat CYP1A1 recognized only the human CYP1A1 and 1A2. Anti-rat CYP2B1 cross-reacts with human 2B6 and lightly with 2A6 but not the human recombinants 1A1, 1A2, 2C9, 2D6, 2E1 and 3A4. Rabbit polyclonal antibodies against mouse CYP2a5 were kindly donated by Dr X. Ding (Albany, NY, USA) and recognized, among the recombinant human CYPs, only the CYP2A6. Goat polyclonal antibodies against rat CYP2C11 were kindly supplied by Dr J. B. Schenkman (Farmington, CT, USA) and recognized among the above-mentioned human CYPs only the CYP2C9. Goat polyclonal antibodies against rat CYP3A2 and monoclonal antibody human CYP2D6 were purchased from Gentest Co. (Woburn, MA, USA). Anti-rat CYP3A2 recognized quickly the CYP3A4 but not the other human CYP recombinant isoforms. The specificity of the anti-human CYP2D6 toward 2D6 has been reported by Gentest. Cynomolgus monkey (male; 2–3 years old) liver microsomes were from XENOTECH (Kansas City, KS, USA). Reagents for development by the alkaline phosphatase method were obtained from ICN (Costa Mesa, CA, USA). Nylon membranes were purchased from Sartorius (Göttingen, Germany). All other chemicals and solvents were of the highest grade available and obtained from common commercial sources.

Animals and preparation of microsomes

Female African green monkey (*Cercopithecus aethiops*) (mean age 23 months, range 17–32 months, 2.5 ± 0.6 kg body weight) livers (52.6 ± 4.3 g) were obtained from Chiron Biocine (Siena, Italy), which used kidneys from the same animals for preparing anti-polio vaccine. After dissection, the liver was perfused through the vena cava with ice-cold normal saline solution and weighed. Each liver was suspended in 4 vols 0.154 M potassium chloride and homogenized by four 10-s bursts in an ultraturrax, followed by four passes of a glass-Teflon pestle Potter-Elvehjem homogenizer. The homogenate was centrifuged at 15 000*g* for 20 min and the resulting supernatant was recentrifuged at 100 000*g* for 1 h. The resulting microsomal pellet was suspended in a 0.01 M Tris-HCl buffer, pH 7.6, containing 0.15 M KCl, 0.1 mM EDTA and 20% (v/v) glycerol and stored frozen at -80°C until use. Protein content was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Western blot analysis

Microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) using the method of Laemmli (1970) in a Bio-Rad Protean Mini II apparatus. Western blot analysis was performed according to Towbin *et al.* (1979) using a 5% (w/v) milk/water as a blocking agent. The staining of antigen-antibody complex was carried out by horseradish peroxidase or alkaline phosphatase method.

Enzyme assays

CYP and cytochrome b_5 contents were measured by the method of Omura and Sato (1964). NADPH-cytochrome P450 reductase activity was measured according to Masters *et al.* (1967). Dextromethorphan *O*-demethylase activity was measured by HPLC using the method of Ducharme *et al.* (1996). Testosterone hydroxylase activity was determined by HPLC (Amato *et al.* 1998). Erythromycin demethylase was assayed by measuring the formation of formaldehyde (Tu and Yang

1983). Methoxyresorufin *O*-demethylase, ethoxyresorufin *O*-deethylase, pentoxyresorufin *O*-depentylase and benzoxyresorufin *O*-debenzoylase activities were determined by measuring the formation of resorufin in a spectrofluorimeter (Krijghsheld and Gram 1984). Coumarin and *p*-nitrophenol hydroxylases were assayed according to Ko *et al.* (1987) and Reinke and Moyer (1985), respectively.

In vitro l-deprenyl metabolism

The basic incubation mixture consisted of 0.4 mg ml⁻¹ monkey liver microsomal protein, a variable concentration of *l*-deprenyl (3–500 µM) and phosphate buffer 100 mM, pH 7.4, in a final volume of 1 ml. The reaction was started by adding 100 µl of a solution (NADPH-generating system) containing 1 mM NADP⁺, 4 mM glucose 6-phosphate and 1 U ml⁻¹ glucose 6-phosphate dehydrogenase in 48 mM MgCl₂.

After a 15-min incubation in a shaking water bath thermostatted at 37°C, the reaction was terminated by the addition of 0.2 ml 5 M NaOH and after cooling in an ice bath. Blanks containing boiled microsomes were incubated under the same conditions as the drug incubations.

In vitro inhibition of l-deprenyl metabolism

Inhibition experiments were carried out by incubating microsomal preparations with different concentrations of inhibitors (α -naphthoflavone, 8-methoxypsoralen, sulfaphenazole, quinidine, 4-methylpyrazole and ketoconazole) relatively specific for CYP enzymes (1A, 2A6, 2C9, 2D6, 2E1 and 3A4, respectively). The inhibition rates were assessed at 15 and 500 µM *l*-deprenyl concentrations. The reaction mixture contained 0.4 mg microsomal protein, variable concentrations of inhibitors (1–100 µM) and 100 mM phosphate buffer, pH 7.8, in a final volume of 1 ml. The inhibitor was added first to microsomes followed by 100 µl NADPH-GS and pre-incubated for 10 min in a shaking water bath thermostatted at 37°C. The reaction was started by adding *l*-deprenyl and stopped after 15 min incubation by the addition of 0.2 ml 5 M NaOH.

Extraction procedure and gas liquid chromatography quantification of l-deprenyl and its metabolites

The reaction mixtures were spiked with 100 nmol clorgyline as internal standard and extracted once with 6 ml ethylacetate. The recovery of both parent drug and metabolites was subsequently determined by a modification of the gas-liquid chromatography method described by Grace *et al.* (1994). After derivatization for 30 min at 90°C with pentafluoropropionic anhydride, samples were dried under a nitrogen stream, resuspended in 50 µl ethylacetate and injected in a Perkin-Elmer 3B series gas-chromatograph equipped with a DB-17 column (30 m × 0.32 mm i.d.) and a nitrogen-phosphorus detector. A temperature-programmed separation procedure was used, starting from an initial 100°C and increasing with a linear ramp rate of 7°C min⁻¹ to a final 230°C. Injector and detector temperatures were set at 230 and 270°C, respectively, and the carrier-gas flow rate was 25 ml min⁻¹. Calibration curves were obtained by adding different amounts of the analytes to heat-inactivated microsomes.

Statistical analysis

Kinetic parameters were calculated according to the Michaelis–Menten equation for one or two enzymes by non-linear regression analysis (Prism 3.02 Graphpad Software, Inc., San Diego, CA, USA). Values are presented as means ± SEM and one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons were performed.

Results

Cytochrome P450 activities

CYPs and cytochrome b₅, NADPH-cytochrome P450 reductase and several monooxygenase activities in microsomes from five African green monkey livers are given in table 1.

Testosterone was also oxidized by African green monkey liver microsomes (figure 2) leading to the formation of the same major metabolites (1 α / β -OH-, 6 β -OH-, 16 α -OH-, 16 β -OH-, 2 β -OH-testosterone and androst-4-ene-3,17-dione), though to a lesser extent as compared with cynomolgus monkey liver microsomes (Bullock *et al.* 1995).

Table 1. Mixed function oxidase activities in liver microsomes from African green monkeys.

Enzyme	Content/activity
Cytochrome P450 (nmol mg ⁻¹ protein)	0.88 ± 0.16
Cytochrome b ₅ (nmol mg ⁻¹ protein)	0.41 ± 0.12
NADPH P450 reductase (μmol min ⁻¹ mg ⁻¹ protein)	148 ± 30
pNPH (nmol min ⁻¹ mg ⁻¹ protein)	2.28 ± 0.54
CoH (nmol min ⁻¹ mg ⁻¹ protein)	0.160 ± 0.053
EROD (nmol min ⁻¹ mg ⁻¹ protein)	0.074 ± 0.03
MEROD (nmol min ⁻¹ mg ⁻¹ protein)	0.023 ± 0.005
PROD (nmol min ⁻¹ mg ⁻¹ protein)	0.008 ± 0.003
BROD (nmol min ⁻¹ mg ⁻¹ protein)	0.011 ± 0.003
ErD (nmol min ⁻¹ mg ⁻¹ protein)	1.85 ± 0.22
DEXOD (nmol min ⁻¹ mg ⁻¹ protein)	0.074 ± 0.025

Data are the means ± SEM from experiments performed at least in duplicate using microsomes prepared from five different animals. pNPH, *p*-nitrophenol hydroxylase; CoH, coumarin hydroxylase; EROD, ethoxyresorufin *O*-deethylase; MEROD, methoxyresorufin *O*-demethylase; PROD, pentoxyresorufin *O*-depentylase; BROD, benzyloxyresorufin *O*-debenzoylase; ErD, erythromycin demethylase; DEXOD, dextromethorphan *O*-demethylase activity.

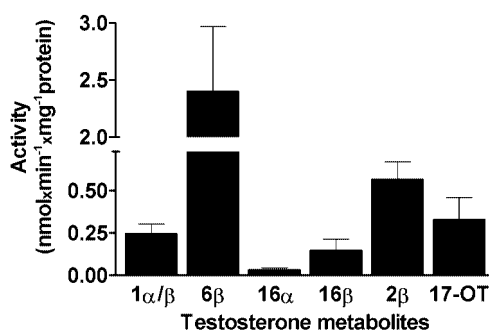


Figure 2. Testosterone hydroxylase activity by African green monkey liver microsomes. Data are means ± SEM (bars) of five liver microsomal preparations. Incubations were carried out at 37°C for 30 min with 1 mg ml⁻¹ microsomal protein. 1α/β, 1α/β-hydroxytestosterone; 6β, 6β-hydroxytestosterone; 16β, 16β-hydroxytestosterone; 16β, 16β-hydroxytestosterone; 2β, 2β-hydroxytestosterone; and 17-OT, androst-4-ene-3,17-dione, were detected.

Immunoblotting of cytochrome P450 isoforms

The main drug-metabolizing CYP isoforms in microsomes from two African green monkey livers were identified by immunoblotting with polyclonal antibodies raised against mouse CYP2a5, rat CYP1A1, 2E1, 2C11 and 3A2, and monoclonal antibody human 2D6. For the sake of comparison, the immunoblot analysis was also determined for a sample of cynomolgus liver microsomes.

Immunoblot evidence for the presence of several proteins immunorelated to CYP1A, 2A, 2B, 2C, 2E and 3A subfamilies in African green monkey liver is shown in figure 3. The protein recognized by monoclonal antibody anti-human CYP2D6 was barely detectable (data not shown), in keeping with Otton *et al.* (1992). Anti-rat CYP1A1 antibody revealed two protein bands in both monkey strains, one a weak signal with a higher molecular weight (mw) and a second more intense signal with a molecular weight about 54 kDa. With the anti-rat 2C11

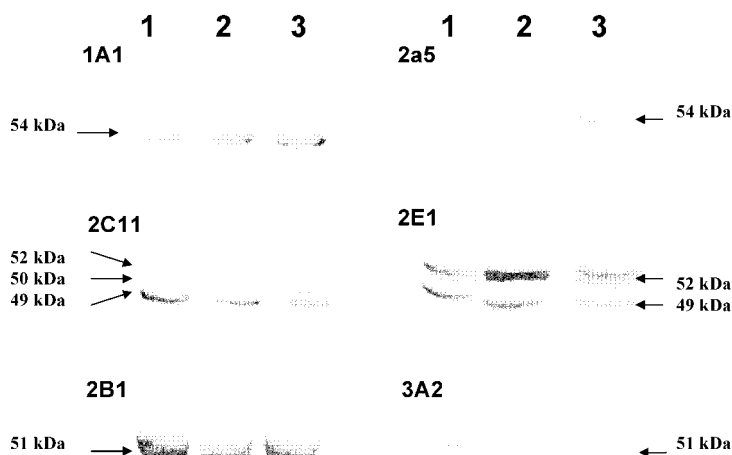


Figure 3. Immunoblots of liver microsomes from two monkey strains. Microsomal proteins (30 μ g) were subjected to SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane and probed with polyclonal antibodies against rat CYP1A1, 2C11, 2E1, 2B1, 3A2 and anti-mouse 2a5. Lane 1 contained a sample from cynomolgus monkey, whereas lanes 2 and 3 contained samples from two African green monkeys. The molecular weight of each band was calculated using human recombinant CYPs as markers.

antibody, three protein bands were revealed (52, 50, 49 kDa mw). These proteins showed similar mw in both strains, that of the lowest molecular weight having the highest intensity. The anti-rat 2E1 antibody revealed two similar protein bands in both monkey strains at molecular weight of 52 and 49, as previously observed in cynomolgus monkey liver microsomes (Amato *et al.* 1998). In the latter strain, single protein bands were also recognized by anti-CYP3A2 and anti-CYP2A5 antibodies at mw of 51 and 52 kDa, respectively. The same antibodies also revealed single protein bands in African green monkey liver microsomes. These protein bands were characterized by molecular weights similar to those recognized in cynomolgus liver microsomes (figure 3).

Kinetics of the formation of l-deprenyl N-dealkylated metabolites

The analytical method employed in this study given a detection limit, calculated as ratio peak/noise > 10 , corresponding to 3 ng ml $^{-1}$ for parent drug and metabolites. The day-to-day coefficient of variation in the slope of calibration curve was 9.2%.

Preliminary assays of *l*-deprenyl N-dealkylation, giving rise to the primary metabolites *l*-methamphetamine and *l*-nordeprenyl, showed that the amount of products formed increased linearly with time up to 30–45 min and that the initial rates were proportional to the amount of microsomal protein added up to 1 mg ml $^{-1}$ (data not shown). The formation of low amounts of the secondary metabolite, *l*-amphetamine, was also detected. Kinetic analysis of *l*-deprenyl N-dealkylation activities was performed in liver microsomal fractions prepared from five individual animals. At all the concentrations of *l*-deprenyl tested, *l*-methamphetamine turned out to be the principal metabolite, its concentration in the assay

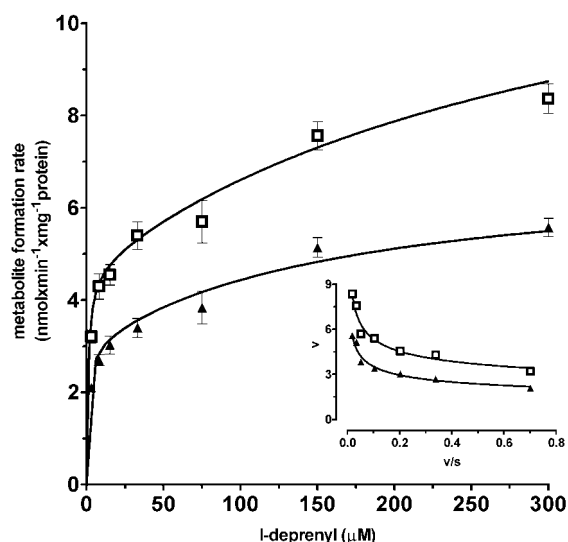


Figure 4. Kinetic analysis of *l*-methamphetamine ($\square$) and *l*-nordeprenyl ($\blacktriangle$) formation. Data fitting was performed according to the Michaelis-Menten equation, while the inset represents the Eadie-Hofstee plot of the same data. V is expressed as $\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$; V/S as $\text{nmol min}^{-1} \text{mg}^{-1} \text{protein mM}^{-1}$. Data are expressed as the mean $\pm$ SEM devised from five animals.

mixture being about 1.5–1.3-fold greater than *l*-nordeprenyl (figure 4). The non-linear Michaelis-Menten analysis of *l*-deprenyl metabolism suggested that at least two components were involved in both N-dealkylation reactions (correlation coefficient 0.9809 and 0.9831 for methamphetamine and nordeprenyl kinetic curves, respectively). In accordance with these observations (figure 4, inset), biphasic Eadie-Hofstee plots were obtained.

As reported in table 2, the high-affinity components for both *l*-nordeprenyl and *l*-methamphetamine formation had similar K_m 's. Also, the low-affinity components had similar apparent K_m 's, but of about 2 orders of magnitude higher than K_m 's of the high-affinity component. The apparent $V_{\max}$'s were quite similar for both components in the two N-dealkylation reactions investigated. As to the low-affinity component, however, the $V_{\max}$ for *l*-methamphetamine formation was about twofold higher than that for the formation of *l*-nordeprenyl. The ratio $V_{\max}/K_m$, as an index of intrinsic clearance, for both metabolites was 2 orders of magnitude higher for the low K_m component as compared with that for the high K_m component. This suggests that the high-affinity component is the major determinant of *l*-deprenyl N-dealkylation pathway by African green monkey liver microsomes.

Inhibition of l-deprenyl metabolism

In order to define the CYPs involved in the N-dealkylation reactions of *l*-deprenyl, an inhibition study was performed by incubating the drug at 15 and 500 μM concentration to account for the low- and high-affinity component, respectively, in the presence of a CYP selective inhibitor. α -Naphthoflavone, 8-methoxypsoralen, quinidine, sulfaphenazole, 4-methylpyrazole and ketoconazole

Table 2. Kinetic constants for the N-dealkylation reaction of *l*-deprenyl by African green monkey liver microsomes.

<i>l</i> -Deprenyl N-dealkylation reactions	K_{m1} (μ M)	K_{m2} (μ M)	V_{max1} ($nmol\ min^{-1}\ mg^{-1}\ protein$)	V_{max2} ($nmol\ min^{-1}\ mg^{-1}\ protein$)	V_{max1}/K_{m1} (min^{-1})	V_{max2}/K_{m2} (min^{-1})
N-depropargylation	1.07 ± 0.01	350 ± 2.7	4.7 ± 0.010	8.9 ± 0.02	4394	25
N-demethylation	0.96 ± 0.05	168 ± 15	3.34 ± 0.02	3.9 ± 0.02	3465	23

Data are means $\pm$ SEM ($n = 5$) from experiments performed at least in duplicate using microsomes prepared from single animals.

were used to inhibit CYP1A, 2A6, 2D6, 2C9, 2E1 and 3A4, respectively (Parkinson 1996, Koenigs *et al.* 1997, Sai *et al.* 2000). When 15 μM *l*-deprenyl was used, only ketoconazole and 8-methoxypsoralen produced a significant inhibition of both *l*-deprenyl N-dealkylation reactions (figure 5). In the presence of 100 μM ketoconazole or 20 μM 8-methoxypsoralen, the *l*-methamphetamine formation rate was

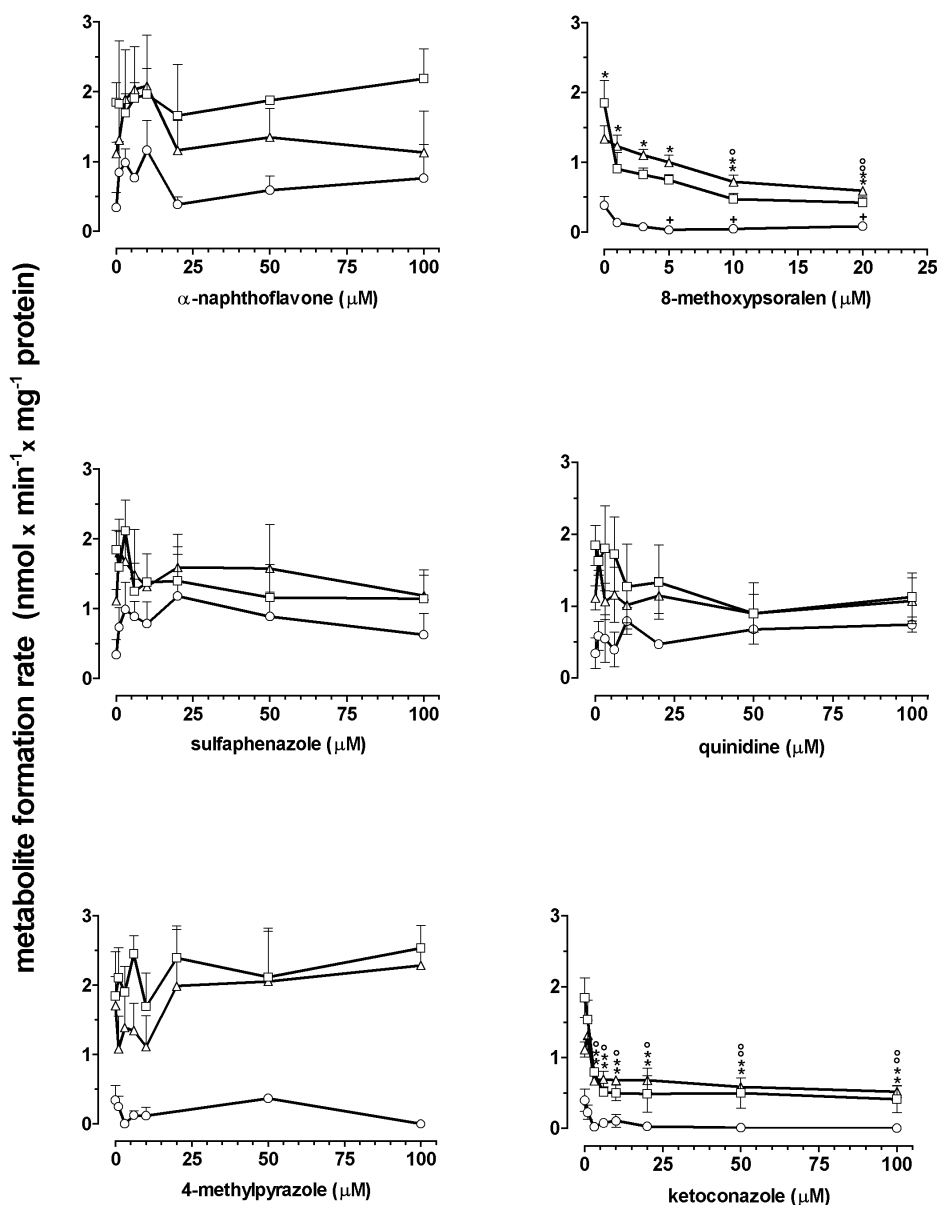


Figure 5. Effects of CYP inhibitors on the formation rate of *l*-methamphetamine (□), *l*-nordeprenyl (△) and *l*-amphetamine (○). *l*-Deprenyl (15 μM) was incubated with African green monkey liver microsomes at 37°C for 15 min in the presence of various concentrations of inhibitor. The comparison between metabolite formation in the absence (control) and presence of inhibitor was performed by using ANOVA test followed by Dunnet post-test; * $p < 0.05$, ** $p < 0.01$ for *l*-methamphetamine; ° $p < 0.05$, °° $p < 0.01$ for *l*-nordeprenyl; + $p < 0.05$ for *l*-amphetamine. Data are the mean $\pm$ SEM from five animals.

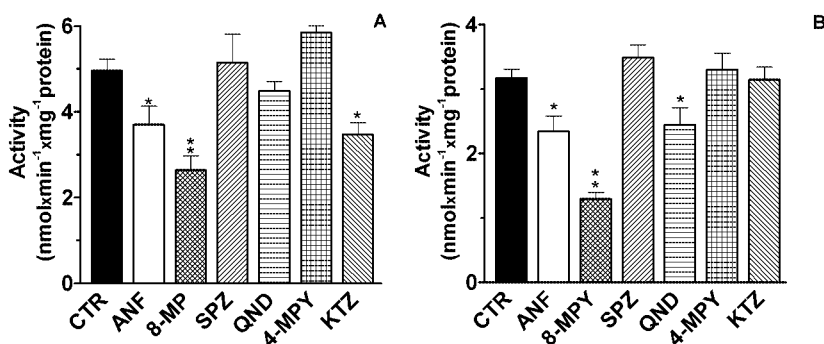


Figure 6. Effects of α -naphthoflavone (ANF), 8-methoxypsoralen (8-MP), sulfaphenazole (SPZ), quinidine (QND), 4-methylpyrazole (4-MPY) and ketoconazole (KTZ) on *l*-methamphetamine (A) and *l*-nordeprenyl (B) formation rates. *l*-Deprenyl (500 μ M) was incubated with African green monkey liver microsomes ($n = 5$) at 37°C for 15 min in presence of 50 μ M α -naphthoflavone, sulfaphenazole, quinidine, 4-methylpyrazole or ketoconazole, and 20 μ M 8-methoxypsoralen. Data are the means $\pm$ SEM (bars) of microsomes from five monkey livers. The comparison between metabolite formation in the absence (control) and presence of inhibitor was performed by using ANOVA test followed by Dunnet post-test; * $p < 0.05$, ** $p < 0.01$.

inhibited by 90 and 78%, whereas the *l*-nordeprenyl formation rate was inhibited by 54 and 56%, respectively. Ketoconazole IC₅₀'s for the formation rate of *l*-methamphetamine and *l*-nordeprenyl were 5.1 and 8.1 μ M, respectively, and 8-methoxypsoralen IC₅₀'s were 3.3 and 38.8 μ M, respectively. To verify the CYP2A selective inhibition by 8-methoxypsoralen in monkey microsomes, testosterone hydroxylase activities were assayed in the presence of this inhibitor at 2 and 5 μ M concentrations to probe the diversity of CYPs. 8-Methoxypsoralen inhibited the formation of both 16b-OH-testosterone and androst-4-ene-3,17-dione by about 10–15 and 25–30%, respectively. Since these testosterone metabolites are relatively specific markers of rat CYP2B enzymes, these data suggest that in monkey 8-methoxypsoralen is not a such specific inhibitor of CYP2As, as has been observed in man (Mäenpää *et al.* 1993), but additionally it inhibits CYP2B-like enzymes.

When 500 μ M *l*-deprenyl was incubated in the presence of 50 μ M α -naphthoflavone, ketoconazole, 4-methylpyrazole, quinidine, sulfaphenazole or 20 μ M 8-methoxypsoralen (figure 6), the formation rate of both *l*-methamphetamine or *l*-nordeprenyl were substantially inhibited (about 50%) only by 8-methoxypsoralen and moderately inhibited (about 30%) by α -naphthoflavone. By contrast, ketoconazole and quinidine significantly inhibited the formation rate of only *l*-methamphetamine and *l*-nordeprenyl, respectively.

Discussion

There is very little information in the literature on the CYP system of African green monkey liver and the present study addresses this gap in the knowledge. Since a gender difference in CYP activities of the different monkey strains tested so far, as well as humans (Stevens *et al.* 1993, 1995, Weaver *et al.* 1994, Bullock *et al.* 1995), has not been observed, it is likely that the values found in female monkeys herein may be also valid for the male counterparts. Total hepatic CYP and b₅ contents, in addition to NADPH-cytochrome P450 reductase and several mono-oxygenase activities, including testosterone hydroxylases, are similar to those

previously described for rhesus and/or cynomolgus monkey liver (Souhaili *et al.* 1986, Stevens *et al.* 1993, Bullock *et al.* 1995).

Ethoxyresorufin-, methoxyresorufin-, pentoxyresorufin-, benzoiloxymresorufin-*O*-dealkylase and erythromycin-*N*-demethylase activities are also similar to those reported for rhesus (Stevens *et al.* 1993) and cynomolgus monkey liver microsomes (Longo *et al.* 1992, Bullock *et al.* 1995). Coumarin hydroxylase activity, however, was lower than that of cynomolgus monkey (Bullock *et al.* 1995) and even lower than that previously reported for African green monkey liver microsomes (Li *et al.* 1997). Dextrometorphan *O*-demethylase activity was lower in green monkey than in cynomolgus monkey (Weaver *et al.* 1999). Furthermore, in African green monkey liver, erythromycin demethylase activity, a relatively specific probe for human CYP3A4/5 (Parkinson 1996), is as high as in cynomolgus monkey, but much higher than in humans. This may reflect a difference in the catalytic activity and substrate specificity between man and cynomolgus/green monkey CYP3A forms.

Assuming the immunorecognition specificity of antibodies is maintained across species, Western blot analysis by anti-mouse CYP2a5, anti-rat CYP1A1, 2E1, 2C11, 3A2 and monoclonal antibody anti-human CYP2D6 has revealed a similarity between African green and cynomolgus monkeys. Green monkey, in fact, like cynomolgus monkey, constitutively expresses two proteins immunorelated to CYP2E1 (Amato *et al.* 1998) and one or two proteins immunorelated to the CYP1A subfamily. The strong protein band recognized by anti-rat CYP1A1 in green monkey may be in fact a mixture of CYP1A1 and 1A2. Furthermore, it was also found that green monkey liver microsomes express a protein immunoreactive with the monoclonal antibody anti-human 2D6 and exhibit dextromethorphan *O*-demethylase activity, a marker activity for this enzyme, in agreement with a previous report on the same monkey strain (Otton *et al.* 1992).

A CYP2A enzyme that catalyses the hydroxylation of coumarin has been purified (Ohmori *et al.* 1993) from cynomolgus monkey liver, and a homologous enzyme might be constitutively expressed in African green monkey since an immunoreactive microsomal protein was recognized by anti-mouse 2a5 antibodies. This confirms a previous observation that microsomes from the same monkey have a coumarin hydroxylase activity susceptible to inhibition by monoclonal antibody anti-human 2A6 and by 8-methoxypsoralen (Li *et al.* 1997), a relatively specific inhibitor of human CYP2A6 (Mäenpää *et al.* 1993).

As previously thought (Weaver *et al.* 1994), cynomolgus liver microsomes have a constitutively high expression of a CYP2B protein, immunoreactive with anti-rat 2B1. A high expression of a same immunoreactive protein with the same molecular weight was also found in African green monkey liver microsomes. In agreement with these data, both strains exhibit 16 β -testosterone hydroxylase activity catalysed by a CYP2B6-like enzyme, which has been purified from cynomolgus monkey (Ohmori *et al.* 1993). As to the presence of the CYP3A subfamily in the microsomes of the two monkey strains, a single, intense protein band was recognized in each strain by anti-rat CYP3A2. In cynomolgus monkey liver this protein has been reported to correspond to CYP3A8, at a high level of expression (about 20% of total CYP), and this supports 6 β -testosterone hydroxylase activity (Ohta *et al.* 1989, Ohmori *et al.* 1993). Thus, it is likely that a very similar CYP3A is present in African green monkey liver.

The CYP2C subfamily is known to be complex in rat, man (Parkinson 1996) and cynomolgus monkey (Weaver *et al.* 1994). In liver microsomes of the latter strain, with use of anti-human CYP2C9 antibodies, three protein bands have been identified but only two enzymes belonging to CYP2C subfamily have been isolated, one more similar to 2C9 and the other (2C20) more related to human CYP2C8 (Ohta *et al.* 1989).

Immunoblotting assays with anti-rat CYP2C11 antibodies carried out in both monkey strains have revealed the presence of at least three constitutive CYP2C immunorelated proteins in cynomolgus monkey and three proteins in African green monkey. The two proteins revealed in both strains have the same molecular weights. Thus, the orthology of the enzymes in the CYP2C subfamily appears not to be maintained in these two monkey strains.

The *in vitro* *l*-deprenyl metabolism by liver microsomes prepared from five African green monkeys was also analysed herein. *l*-Deprenyl N-dealkylation reactions, i.e. N-demethylation and N-depropargylation, exhibited biphasic Eadie-Hofstee plots indicating that at least two CYP enzymes catalyse each of these reactions. It is of interest that the high-affinity component of both *l*-deprenyl oxidation reactions was characterized by substantial rates and similar very low K_m 's (about 1 μ M), are two orders of magnitude lower than those reported for human liver microsomes and two order of magnitude higher than *l*-deprenyl plasma concentrations observed in man after a single therapeutic oral dose of 10 mg (Taavitsainen *et al.* 2000). The K_m 's observed in human microsomes were comparable with values associated with the low-affinity component found in African green monkey. Even K_m 's reported by Hiderstrand *et al.* (2001) as good CYP catalysts of *l*-deprenyl N-dealkylation reactions (human recombinant CYPs expressed in yeast) were higher than those found here for the high-affinity component. Taken together, these findings suggest that in African green monkey liver *l*-deprenyl has high clearance.

In order to identify the CYP enzymes involved in the N-demethylation and N-depropargylation pathway of *l*-deprenyl, inhibition experiments were performed with specific human CYP inhibitors at two concentrations of *l*-deprenyl to account for both the high and low-affinity components. It should be emphasized that the inhibitors used in the present study are relatively selective for human CYP forms, namely α -naphthoflavone, 8-methoxypsoralen, 4-methylpyrazole, sulfaphenazole, quinidine and ketoconazole that inhibit CYP1A1/2, 2A6, 2E1, 2C9, 2D6 and 3A4, respectively. Therefore, an effective and informative inhibition may be expected to take place if homologous enzymes with inhibitor susceptibility similar to human enzymes are also present in African green monkey liver. The clarification of this point awaits the cloning and expression of monkey CYPs.

At a low *l*-deprenyl concentration, the major role seemed to be played by two CYP isoforms susceptible to inhibition by ketoconazole and 8-methoxypsoralen, respectively, thus suggesting the involvement of members of the CYP2A and 3A subfamilies. 16 β -Testosterone hydroxylase activity, which is thought to be linked to a CYP2B6-like enzyme in monkey liver microsomes (Li *et al.* 1997), is inhibited by low concentrations of 8-methoxypsoralen. Thus, it is possible this enzyme, rather than a CYP2A6-like enzyme, is responsible for the observed *l*-deprenyl metabolism which is inhibited by 8-methoxypsoralen. However, the fact that even at the highest concentrations used ketoconazole and 8-methoxypsoralen were unable to suppress the formation rate of both *l*-methamphetamine and

l-nordeprenyl suggests that other CYPs or phase I enzymes, in addition to those discussed above, could be involved in *l*-deprenyl metabolism.

In man, CYP2B6 and CYP2C19 forms have been reported to play a major role in the two N-dealkylation reactions of *l*-deprenyl (Hidestrand *et al.* 2001), although the contribution of both CYP3A and 1A2 forms has been reported (Taavitsainen *et al.* 2000).

The inhibition studies with ketoconazole showed IC₅₀'s for *l*-methamphetamine and *l*-nordeprenyl formation (Taavitsainen *et al.* 2000) one order of magnitude lower than those found in human liver microsomes; a similar species' difference was also observed in the inhibition pattern by 8-methoxypsoralen. This further supports the involvement of CYP3A (ketoconazole-inhibitable) and 2A/2B enzymes (8-methoxypsoralen-inhibitable) in African green monkey *l*-deprenyl metabolism, unlike that observed in human liver microsomes (Taavitsainen *et al.* 2000) or human recombinant CYP enzymes (Hidestrand *et al.* 2001). In addition, the evidence that CYP2E1 is not involved in monkey liver *l*-deprenyl metabolism, as previously reported for the C57BL mouse (Valoti *et al.* 2000), underlines the differences between species in CYP-dependent metabolism of *l*-deprenyl and the dangers of attempting to extrapolate results across species.

When a relatively high concentration of *l*-deprenyl was used, both α -naphthoflavone and quinidine inhibited *l*-deprenyl metabolism, further indicating a catalytic role of other CYPs, possibly members of the CYP1A and 2D subfamilies. However, the latter enzymes may contribute to *l*-deprenyl metabolism only at very high drug concentrations, which are probably not attainable *in vivo*.

In conclusion, the main contribution to *l*-deprenyl metabolism by African green monkey appears to be provided by CYP3A4- and 2A6-like enzymes, although 2B and, at high *l*-deprenyl concentrations, 1A1/2 and 2D can not be excluded. Furthermore, *l*-deprenyl has a very high hepatic clearance similar to that observed in man, the formation of *l*-methamphetamine being more efficient than that of *l*-nordeprenyl. These data support the view that CYP-dependent metabolism is very likely relevant to the neuroprotection afforded in primate models of Parkinson's disease.

Acknowledgements

The authors are grateful to Dr Savina Sapienza and Dr Francesca Benedetti for invaluable technical assistance, and to Dr Giacomo Matteucci (Chiron Biocine S.p.A., Siena, Italy) for providing the monkey tissues. This work was supported by the University of Siena, Fondazione Monte dei Paschi Siena and COST working group: D13/0018/01.

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Pharmacokinetics and tissue distribution of the nonadecapeptide Moli1901 in rats and mice

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Received May 29 2002

1. Administration of aerosolized, radiolabelled Moli1901 (duramycin, 2622U90), a 19 amino acid polycyclic peptide, to rats resulted in the deposition of high amounts of radiolabel in the respiratory tract, with deposited radiolabel persisting almost unchanged through 7 days after dosing. Little to no radiolabel was present in the bloodstream of these rats.

2. Rats absorbed little radiolabel after p.o. administration, with nearly all of the dose excreted in the faeces by 2 days after dosing.

3. At 7 days following an intravenous dose, rats excreted 54% of the radiolabel in faeces and 5.4% in the urine, with 44% remaining in the carcass, primarily in the liver (33%).

4. Following an intratracheal instillation dose to rats, radiolabel was eliminated from the pulmonary system with a half-life of 64 days. Excretion was almost exclusively via faeces, with an elimination half-life of 52 days. Plasma and blood concentrations in these animals were uniformly $< 1 \text{ ng eq. ml}^{-1}$ at all sampling times.

5. Results in mice given intravenous and oral doses were consistent with those observed in rats.

6. Prolonged retention of Moli1901 in pulmonary tissue supports its use in the treatment of respiratory diseases.

Introduction

Cystic fibrosis (CF) is a genetic disease characterized by abnormal chloride ion transport that alters the water content of the airway mucus, impairing its clearance and leading to infections, inflammation, lung damage and death. Moli1901 (commonly named duramycin) is a 19 amino acid polycyclic peptide antibiotic (Shotwell *et al.* 1958, Hayashi *et al.* 1990) that increases chloride transport (Cloutier *et al.* 1990, 1993, Molina y Vedia *et al.* 1996, Henke *et al.* 1998) and water mobilization (Molina y Vedia *et al.* 1996, Henke *et al.* 1998) when applied to the apical surface of airway epithelium. (Moli1901 is used throughout to denote the compound studied. It has also been known as duramycin and 2622U90 and appears as such in the titles of some of the cited material. A preliminary report of

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this work appeared in McNulty *et al.* 1997.) The therapeutic goal in CF and other pulmonary diseases in which the water content of the mucus is altered, e.g. chronic bronchitis and asthma, is to remove retained secretions from the lungs. Retention of viscous mucous secretions frequently complicates many chronic respiratory diseases including chronic bronchitis and asthma. Although not of primary aetiological significance, thickened mucus can severely compromise the function of lungs that already have low reserve (Task Group on Mucoactive Drugs 1994). The ability of Moli1901 to increase chloride permeability in CF and normal epithelium (Cloutier *et al.* 1990, Molina y Vedia *et al.* 1996) and to increase the volume of bronchial secretion in normal healthy dogs (Molina y Vedia *et al.* 1996, Henke *et al.* 1998) suggests that it may have a role in managing these respiratory diseases as well.

The compound is currently in clinical trials. Because the intended route of administration in the clinic is by aerosol inhalation, preclinical studies were undertaken to determine the tissue distribution and elimination of radiolabel in experimental animals exposed to aerosolized ^{35}S -Moli1901. The initial inhalation study indicated significant retention of radiolabel in pulmonary tissue at 1 week after dosing, prompting additional studies in mice and rats to examine the tissue distribution and elimination of radiolabelled Moli1901 after intravenous (i.v.), oral (p.o.) and intratracheal dosing. The results indicate that Moli1901 is minimally absorbed after inhalation, intratracheal or p.o. exposure. Furthermore, the results indicate that the compound has a long half-life in the lungs after intratracheal exposure.

Materials and methods

Chemicals

Moli1901 (MW=2013; figure 1), ^{35}S - and ^3H -Moli1901 were prepared by fermentation from a *Streptovorticilium cinnamoneous* culture grown in the presence of ^{35}S -sulfate or ^3H -phenylalanine, respectively. The peptide was purified by column chromatography before use in animal studies. Radiolabel purity was uniformly $\geq 95\%$; specific activities for tritium labelled batches ranged from approximately 15 MBq (400 μCi) to 0.93 TBq (25 Ci) mmol^{-1} , whereas specific activities for the ^{35}S batches were approximately 3 GBq (80 mCi) mmol^{-1} .

Moli1901 was also purchased (as duramycin) from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade solvents (acetonitrile, methanol, water) were obtained from EM Science (Gibbstown, NJ, USA). Ethyl alcohol was purchased from McCormick Distilling Co., Inc. (Pekin, IL, USA). Trifluoroacetic acid was purchased from Mallinckrodt Chemical Co. (Paris, KY, USA). BondElut C-18 cartridges were obtained from Varian (Harbor City, CA, USA). Flo-Scint III scintillation cocktail and Soluene 350 (tissue solubilizer) were purchased from Packard Instrument Co. (Meriden, CT, USA). Ready Safe liquid scintillation cocktail was purchased from Beckman Instruments, Inc. (Fullerton, CA, USA). Vetalar (ketamine) was purchased from Fort Dodge Laboratories, Inc. (Fort Dodge,

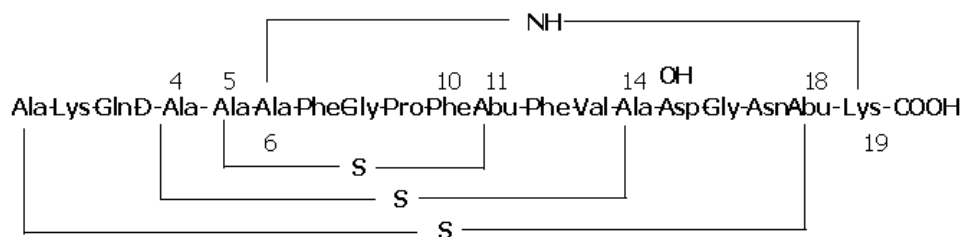


Figure 1. Structure of Moli1901.

IA, USA); Rompun (xylazine) was purchased from Phoenix Pharmaceutical, Inc. (St Joseph, MO, USA); and diazepam was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ, USA).

Animal studies

In separate experiments, male rats were exposed to radiolabelled Moli1901 by aerosol inhalation, and i.v., p.o. and intratracheal dosing. Dosing methodologies were as follows.

Aerosol administration

To determine the disposition of inhaled Moli1901, rats were exposed to an aerosol of ^{35}S -Moli1901. Nine male Sprague-Dawley rats (Charles River Canada, Inc., St Constant, Quebec, Canada), 349 ± 11 g weight range, were exposed (nose only) for 6 min to an aerosol of ^{35}S -Moli1901 ($3.3 \text{ GBq mmol}^{-1}$ or $88.4 \text{ mCi mmol}^{-1}$) in saline, at a chamber concentration of $8.253 \mu\text{g l}^{-1}$. The test atmosphere was generated using a single Restec nebulizer supplied with predried compressed air. The mass median aerodynamic particle diameter and geometric standard deviation of the test atmosphere were $2.4 \pm 1.8 \mu\text{m}$. The estimated achieved dose was $12.0 \pm 0.1 \mu\text{g kg}^{-1}$ calculated using:

$$\text{Achieved dose } (\mu\text{g kg}^{-1}) = \text{RMV} \times \text{AC} \times T \times D/\text{BW},$$

where RMV (l min^{-1}) is the respiratory minute volume calculated as $0.0021 \times (\text{body weight, g})^{0.75}$ (it was assumed that the RMV parameter was unaffected by exposure to the test article), AC ($\mu\text{g l}^{-1}$) is the chamber concentration of active ingredient (^{35}S -Moli1901) determined from the radioactivity content of the gravimetric filter, T (min) is the treatment time (6 min), D is the total aerosol deposition fraction (0.5) according to the particle size determined gravimetrically, and BW (kg) is the body weights of the animals measured on the day of dosing.

The animals were euthanized in groups of three rats per time point immediately following the exposure and at 24 and 168 h after exposure. Blood and selected tissues were collected from each animal, and excreta were collected from those euthanized at 24 and 168 h.

Oral administration

To determine the disposition of Moli1901 after p.o. ingestion, three male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh NC, USA), 300–350 g weight range, were given single $231 \mu\text{g kg}^{-1}$ p.o. doses of ^3H -Moli1901 (equivalent to approximately 0.6 MBq kg^{-1} or $15 \mu\text{Ci kg}^{-1}$). The dosing material was dissolved in water and administered in a dose volume of 1 ml kg^{-1} . Immediately after dosing, the rats were placed in metabolism cages for 7 days for collection of excreta. At the end of this period, the animals were euthanized and the tissue distribution of radioactivity examined.

Intravenous administration

To determine the disposition of Moli1901 in the systemic circulation, three male Sprague-Dawley rats (Harlan (UK) Ltd, Oxon, UK), 166–171 g weight range, received $150 \mu\text{g kg}^{-1}$ i.v. bolus doses of ^3H -Moli1901 (approximately 0.5 MBq or $12 \mu\text{Ci}$ per animal) via the tail vein. The dosing material was dissolved in a vehicle of 10% propylene glycol in normal saline and administered in a dose volume of 1 ml kg^{-1} . Immediately after dosing, the rats were placed in metabolism cages for 7 days for collection of excreta. At the end of this period, the animals were euthanized and the tissue distribution of radioactivity examined. In a separate study, rats were given $150 \mu\text{g kg}^{-1}$ i.v. bolus doses of ^{35}S -Moli1901 (approximately 0.25 MBq ; $6 \mu\text{Ci kg}^{-1}$) by tail vein injection. They were euthanized in groups of up to three rats at 0.25, 4, 8, 24, 72 and 168 h. Radiolabel concentrations in blood and plasma samples were determined. In addition, the metabolic profiles in samples of faeces and liver extract were determined by HPLC with radiochemical detection.

Intratracheal instillation

To determine the half-life of radiolabelled material in the pulmonary system, rats were given a known amount of ^3H -Moli1901 by intratracheal instillation. The radiolabelled peptide was reconstituted in normal saline to a concentration of $2.3 \mu\text{g}/200 \mu\text{l}$. Male Sprague-Dawley rats (Charles River Breeding Laboratories), 150–250 g weight range, were anesthetized with intraperitoneal injections of ketamine, xylazine and diazepam (initial doses of 40, 2 and 1 mg kg^{-1} , i.p., respectively, supplemented as needed during the dosing procedure). When the larynx of each animal was sufficiently relaxed, a 14- or 18-gauge Angiocath catheter was inserted into the rat's trachea and the $2.3\text{-}\mu\text{g}$ dose of ^3H -Moli1901 (approximately 0.02 MBq or $0.5 \mu\text{Ci}$) was instilled in a $200\text{-}\mu\text{l}$ dose volume by syringe. The rat was held in a partially upright position during the dosing procedure so that the solution would flow into the lungs. Following instillation of the dose, the catheter was removed and the rat placed on a warming pad until recovery of consciousness. The animals were housed in stainless steel, wire mesh cages to prevent coprophagy. The dosed rats were euthanized in groups of one to seven animals at various times over the next 120 days. Blood and tissues were analysed for concentrations of radioactivity. In addition, three of

the rats were housed in metabolism cages during an 89-day post-dosing period for collection of urine and faeces.

Radiolabel concentrations in biological samples

In experiments in which the tritium label was used, volumes of plasma, blood and urine were pipetted directly into paper combustion cones. Trachea and bronchi were weighed and then transferred to combustion cones intact. Other tissue samples and faeces samples were mixed with water and homogenized, and weighed amounts of these homogenates were then transferred to combustion cones. In experiments in which the concentration of radiolabel in faeces was low, the faeces were lyophilized and ground into fine powders with a mortar and pestle. Weighed amounts of these powders were transferred to combustion cones. All samples containing tritium were analysed by combustion of the cones in a Packard Oximate 80 Model 307 biological sample oxidizer followed by counting on a scintillation counter (Model 2000CA, Packard Instrument Co., Downer's Grove, IL, USA). All biological samples except trachea and bronchi were analysed at least in duplicate.

Samples containing ^{35}S were homogenized in water and portions were then transferred to scintillation vials. Soluene 350 was added to each vial and the vials were incubated overnight in a 50°C water bath shaker. The solubilized samples were decolourized by addition of 30% hydrogen peroxide (blood, 1 ml; faeces, tissues and femur, 200 µl). These samples were left at room temperature for 30 min or until the bubbling stopped. Finally, 10 ml scintillation cocktail were added into each vial. Samples were stored for several days in the dark before counting to eliminate the effect of chemiluminescence on the count rate. Final values were recorded only after repeated counts indicated a stable count rate had been achieved. DPM (disintegrations per minute) values were corrected for radioisotope decay and the results were converted to µg equivalents of Moli1901, based on the specific activity of the dosing solution. Appropriate sample blanks were run with each analysis; the limit of detection was set at twice the blank count rate.

Preparation of samples for HPLC

Portions of each faeces homogenate were weighed into 50-ml centrifuge tubes, followed by the addition of 3 parts (v/w) 10% trichloroacetic acid (TCA) in water. The mixture was homogenized for 2 min, then mixed on a shaker for 30 min, followed by centrifugation for 30 min at 2000 RPM at room temperature (Sorvall H1000B rotor in a RT6000B centrifuge, Du Pont Co., Wilmington, DE, USA). After centrifugation, the supernatant was collected while the pellet was subjected one more time to the same TCA extraction procedure described. At the end of the second TCA extraction, the supernatants from both TCA extractions were pooled. The pellet from the TCA extraction was then extracted with 3 parts (v/w) absolute ethanol. The mixture was homogenized for 2 min, heated in a boiling water bath for 30 min, mixed in a shaker for 30 min and centrifuged for 30 min at 3000 RPM. The supernatant from this absolute ethanol extraction was added to the pooled TCA extraction supernatants described above. The total volume of the pooled supernatant was measured, and duplicate volumes of the pooled supernatant (200–500 µl) were added to scintillation vials containing 10 ml scintillation fluid and counted. The pooled supernatants were then dried at 50°C on the N-Evap evaporator (Organomation Associates, Shrewsbury, MA, USA). The dried samples were reconstituted in 60% of mobile phase A and in 40% of mobile phase B by slowly adding small volumes of mobile phases until all the dried extract was solubilized. The reconstituted sample was then further purified by a solid-phase extraction procedure (described below) before HPLC analysis. The recovery of ^{35}S using this procedure varied from 50 to 90% of the starting material.

Homogenized tissues were extracted by the same procedure described for the faeces, with a few modifications. For the homogenized tissue, 10% TCA extraction was done once, followed twice by ethanol extractions and twice by 70% ethanol extraction. All of the supernatants from the different extractions were pooled, measured and dried as described in the faeces extractions procedure. The recovery of ^{35}S by this procedure varied from 70 to 90% of the original starting material.

HPLC analysis

The profile of metabolites was determined in extracts of tissues and excreta by HPLC. Separations were achieved on a VYDAC protein C-4, 15 cm × 4 mm analytical column, fitted with a VYDAC protein and peptide C-18 precolumn. Mobile phase A (0.1% trifluoroacetic acid in water) and mobile phase B (0.1% trifluoroacetic acid in acetonitrile) were mixed at a combined flow rate of 1 ml min⁻¹. A mobile phase gradient of 100% A to 100% B over 20 min was used to analyse urine and tissue extracts. Faeces samples were analysed with a 3:1 mixture of A:B, isocratically.

Mass spectrometry

Fractions from HPLC runs of selected extracts were analysed by FAB-MS to determine the identity of the radiolabelled material. Before analysis, samples were dissolved in 10 µl 95% ethanol. Of glycerol,

2 µl were applied to the FAB probe tip, and 2 µl of the sample solution were added. Samples were analysed using a VG 70SQ mass spectrometer equipped with a caesium ion gun, with glycerol as the sample matrix. The mass spectrometer acceleration voltage was set at 5000 V with a resolution of 2000 (10% valley definition) while the caesium ion gun was operated at 22 kV. Mass spectra were acquired over a mass range of 1350–2800 Da at a scan speed of 20 s decade⁻¹. Data were collected in the continuum mode on a VG 11/250J data system using a gain factor (MYH parameter) of 1000 and analysed on a Kratos Mach 3 data station.

Pharmacokinetic analysis

Amounts of tritium in the pulmonary system (sum of radiolabel in the trachea, bronchi and lung) for each rat were plotted against time. Based on visual inspection of these data, a linear regression analysis was performed on the data from days 3 to 120. A similar approach was used for analysis of excreted tritium in faeces, except that the data were plotted as:

$$[F_{\infty} - F(t)] \text{ versus time } (t),$$

where F_{∞} is the total amount of tritium excreted in faeces at infinite time, defined here as the total amount recovered in faeces through the 89-day collection period, plus that amount remaining in the lungs, liver and spleen (all or nearly all of which may be presumed to be excreted eventually in the faeces; see data for i.v. administration below), and $F(t)$ is the amount excreted in faeces at time t . Based on visual inspection of the faeces excretion data, all data points were used in the linear regression analysis.

For both pulmonary and faecal elimination analysis, the linear regression was performed on the natural log of the ordinate value against time, and the resulting slope of the regression line was divided into -0.693 to obtain the half-life.

Results

Tissue distribution and excretion of ³⁵S in rats after ³⁵S-Moli1901 aerosol inhalation

Rats were exposed to an aerosol of ³⁵S-Moli1901 for 6 min, resulting in a mean ($\pm$ SD) achieved dose of $12.0 \pm 0.1 \mu\text{g kg}^{-1}$. The rats were euthanized in groups of three animals at 0, 24 and 168 h after dosing. The highest concentrations of radiolabel were in lung tissue, with very little radiolabel detectable in the other three tissues examined (table 1). The concentration of radiolabel in lung tissue declined very little over the 7-day post-dosing period, indicating that the elimination half-life of radiolabel in the lung was relatively long.

Concentrations of radiolabel in whole blood, plasma and red blood cells in these nine animals were almost uniformly below the limit of detection (data not shown), indicating that little, if any, radiolabelled material was absorbed into the systemic circulation following aerosol exposure. Of radiolabel, $<0.4 \mu\text{g eq.}$ were recovered in the urine after dosing, while from 15.5 to $38.2 \mu\text{g eq.}$ were recovered in the faeces (data not shown). The low recovery in urine was consistent with the apparent low systemic availability of the radiolabel following inhalation exposure. The recovery in faeces of radiolabel in amounts greater than the achieved doses was probably attributable to p.o. ingestion of radiolabel during exposure, and from post-dosing ingestion of radiolabel groomed from parts of the muzzle in contact with the restraining cone.

Tissue distribution and excretion of ³H in rats after oral administration of ³H-Moli1901

Three male Sprague-Dawley rats were each given a $231 \mu\text{g kg}^{-1}$ dose of ³H-Moli1901 by gavage and immediately placed in metabolism cages for collection of urine and faeces during 7 days following dosing. Urine contained $0.17 \pm 0.02\%$ of

Table 1. Radioactivity in selected tissues of male rats after inhalation exposure to ³⁵S-Moli1901 at a dose of 12 µg kg⁻¹.

Animal no.	Hours after exposure	Lungs		Liver		Spleen		Kidney	
		ng eq. g ⁻¹	% Dose in lung	ng eq. g ⁻¹	% Dose in liver	ng eq. g ⁻¹	% Dose in spleen	ng eq. g ⁻¹	% Dose in kidney
1001	0*	1790	56.3	ND	ND	ND	ND	ND	ND
1002	0*	930	37.3	5	1.4	ND	ND	10	0.6
1003	0*	1280	41.8	ND	ND	ND	ND	ND	ND
Mean		1330	45.2	NC	NC	NC	NC	NC	NC
1004	24	1440	42.7	ND	ND	ND	ND	8	0.4
1005	24	1270	52.6	ND	ND	ND	ND	6	0.4
1006	24	370	11.4	ND	ND	ND	ND	ND	ND
Mean		1030	35.6	NC	NC	NC	NC	NC	NC
1007	168	1350	46.4	ND	ND	ND	ND	ND	ND
1008	168	1210	47.5	ND	ND	ND	ND	ND	ND
1009	168	1400	48.8	ND	ND	ND	ND	ND	ND
Mean		1320	47.6	NC	NC	NC	NC	NC	NC

*Immediately following 6 min of aerosol exposure.
Data are from a single determination for each animal.
ND, not detected; LLOQ was about 1 ng eq. g⁻¹.
NC, not calculated.

the dose, while faeces contained essentially 100% of the dose (figure 2). Most compound-related material was excreted within the first 48 h after dosing. Consistent with the excretion data was the absence of appreciable residual radioactivity in selected tissues (liver, spleen, lung, trachea, kidney) examined on post-dosing

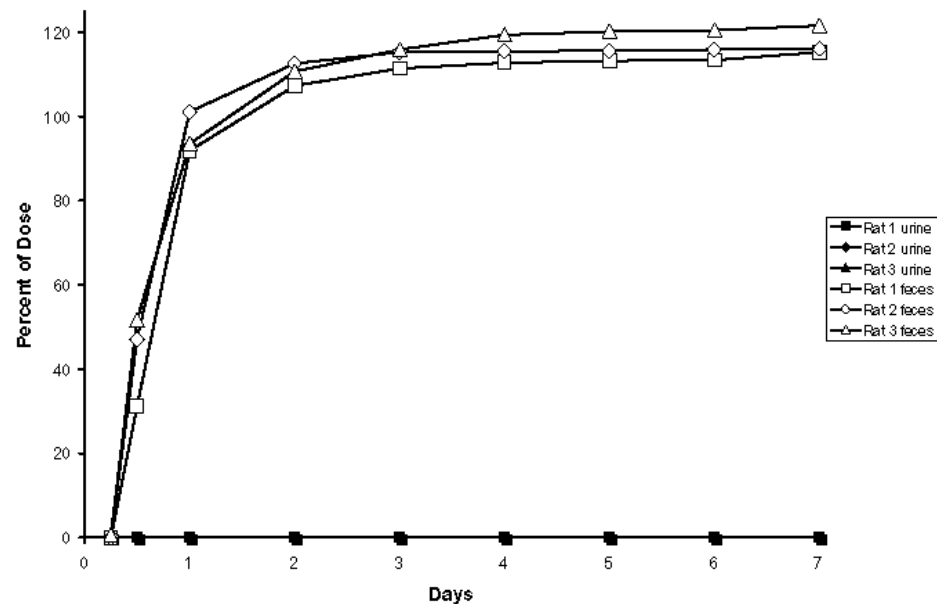


Figure 2. Cumulative excretion of radiolabel from rats after a single 231 µg kg⁻¹ p.o. dose of ³H-Moli1901. Each point is the mean of the values from three rats (single determination per rat per time point).

day 7. Plasma concentrations ranged from 29 to 49 dpm ml⁻¹, equivalent to approximately 0.1 ng ml⁻¹ (data not shown).

Tissue distribution and excretion of radiolabel in rats after intravenous administration of ³H-Moli1901

In an experiment similar to the one described above, three male Sprague-Dawley rats were given 150 µg kg⁻¹ i.v. doses of ³H-Moli1901 and placed in metabolism cages for collection of urine and faeces. At 7 days after dosing, cumulative excretion in urine accounted for a mean of 5.4% of the dose, while excretion in faeces accounted for 54% of the dose. In contrast to the results after p.o. dosing, radiolabel was still being excreted in the faeces at a relatively steady rate through the seventh post-dosing day (figure 3).

On day 7, nearly 33% of the dose was still in the liver, with the spleen, lungs and kidneys containing much smaller amounts of radiolabel (table 2). Spleen, however, contained the highest concentration of radiolabel on a per gram tissue basis, followed by liver and then lungs.

Tissue distribution and excretion of ³H in rats after intratracheal administration of ³H-Moli1901

The aerosol exposure study had indicated that the elimination half-life of radiolabel in lung was relatively long. To measure this half-life accurately, it was desirable to know the original amount deposited in the pulmonary system with better accuracy than could be achieved in an aerosol exposure. Therefore, rats were

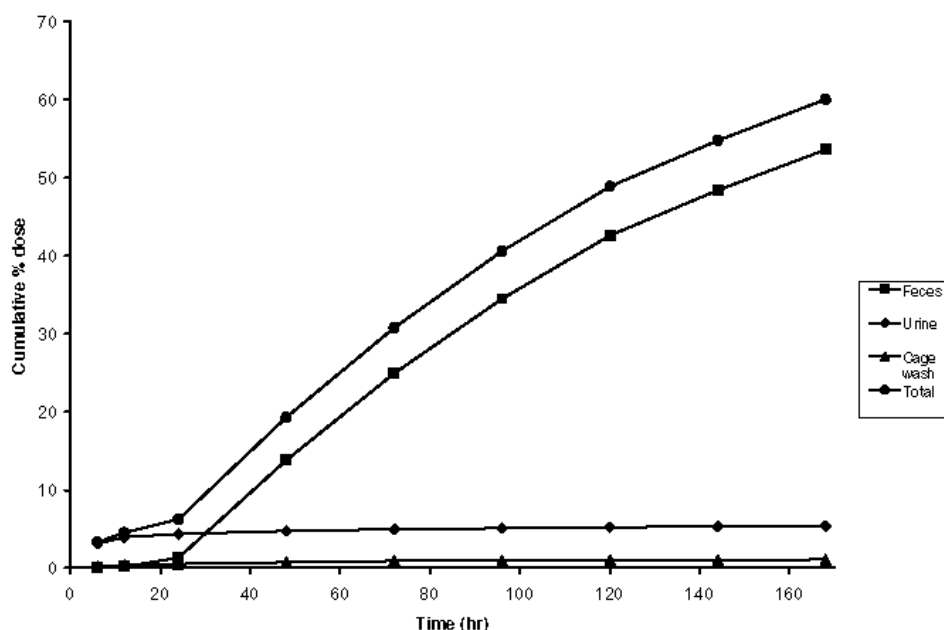


Figure 3. Cumulative excretion of radiolabel from rats after a single 150 µg kg⁻¹ i.v. dose of ³H-Moli1901. Each point is the mean of the values from three rats (single determination per rat per time point).

Table 2. Radioactivity in selected tissues of male Sprague–Dawley rats 168 h after a single 150 µg kg⁻¹ intravenous bolus dose of ³H-Moli1901.

	Tissue concentrations (ng eq. g ⁻¹)		Recovery of radioactivity in tissues (% of dose)	
	Mean	Range	Mean	Range
Liver	717	640–763	32.9	30.7–35.1
Spleen	1089	853–1322	2.9	2.2–3.3
Lung	185	171–207	0.9	0.8–1.0
Kidney	65	60–73	0.5	0.5–0.6
Tail	18	16–22	0.4	0.4–0.5
Trachea	5	0–9	NC	NC
Carcass			6.2	5.3–6.8
Blood	1	0.1		
Plasma	0.2	0.1		
Subtotal			43.9	41.7–45.6

All results are the mean and range for *n* = 3 rats.
NC, results not calculated (< 0.01% of the dose).

given 2.3 µg intratracheal doses of ³H-Moli1901 (matching the estimated achieved dose in the lungs during the aerosol exposure described above), and the amount of tritium remaining in the pulmonary system (trachea, bronchi, lung) through a 120-day post-dosing period was determined. The half-life of elimination of radiolabel from the pulmonary system was 64 days (figure 4). Blood and plasma samples taken from each rat in the study at all post-dosing time points contained < 1 ng ml⁻¹ tritium, with many samples containing undetectable amounts of radiolabel. Liver and spleen concentrations of radiolabel were low throughout the 120-day study, with spleen concentrations peaking at around 7 days (1.5% of

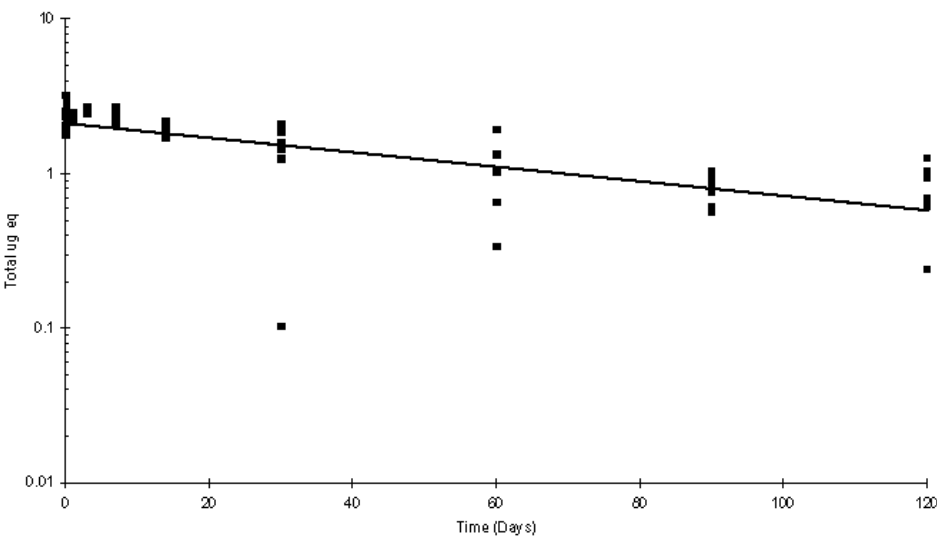


Figure 4. Elimination of radiolabel from rat pulmonary system (trachea, bronchi, lungs) after a 2.3 µg intratracheal instillation dose of ³H-Moli1901. Each symbol represents an individual animal. The solid line is the regression line using all the points.

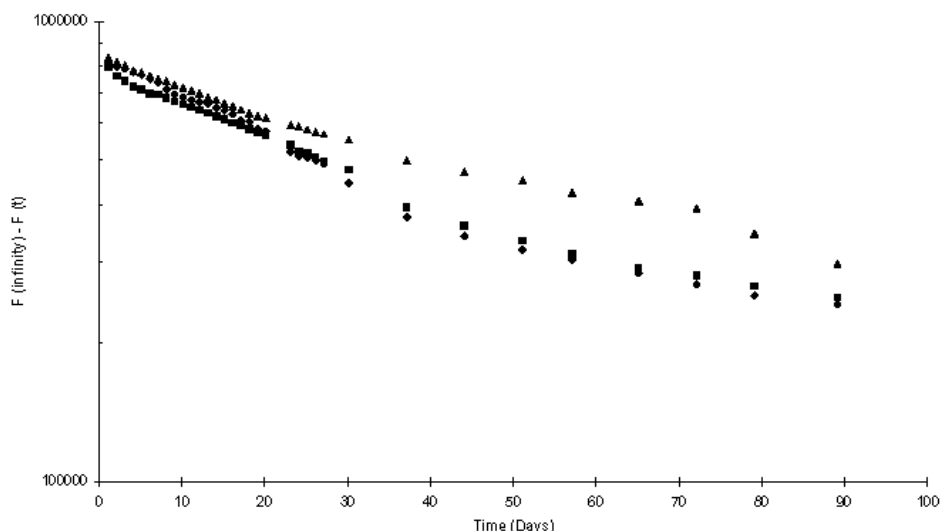


Figure 5. Elimination of radiolabel via faeces from rats given a $2.3\mu\text{g}$ intratracheal dose of ^3H -Moli1901. Each point represents the data from one rat (single determination per rat per time point). The same three rats were used throughout the experiment.

the dose) and liver concentrations peaking at the end of the study (9.3% of the dose).

In addition, three rats given intratracheal doses were housed individually in metabolism cages for collection of urine and faeces during an 89-day post-dosing period. A mean of $1.0 \pm 0.2\%$ of the dose was recovered in the urine over this period, while a mean of $68.2 \pm 4.9\%$ was recovered in the faeces. Radiolabel was eliminated via the faeces at a relatively constant rate from each rat through this period (figure 5). The faecal elimination half-lives were 45, 49 and 63 days in the three rats (mean of 52 ± 9 days), which was in relatively good agreement with the half-life of elimination from lung tissue.

Metabolite profiles

The profiles of metabolites in lung and liver tissue, and excreta were determined by HPLC analysis of extracts of these biological materials. Following a p.o. dose of ^3H -Moli1901, faeces collected over 48 h after the dose contained a single radiolabelled peak that eluted at the retention time of authentic parent compound (data not shown). Similarly, extracts of faeces (24–48- and 48–72-h collections) and liver (at 0.25 and 4 h after the dose) from rats given an i.v. dose of the ^{35}S -labelled compound showed only one prominent radiolabelled peak eluting at the retention time of authentic parent compound. This peak was collected and analysed by FAB-MS. Both tissue extracts exhibited ions at m/z 2012.7, with the correct isotope cluster found for the authentic Moli1901 standard (data not shown). Lung tissue from rats euthanized at 1 h and at 120 days after intratracheal doses of tritiated Moli1901 showed the presence of a single prominent radiolabelled peak eluting at the retention time of parent Moli1901 (figure 6).

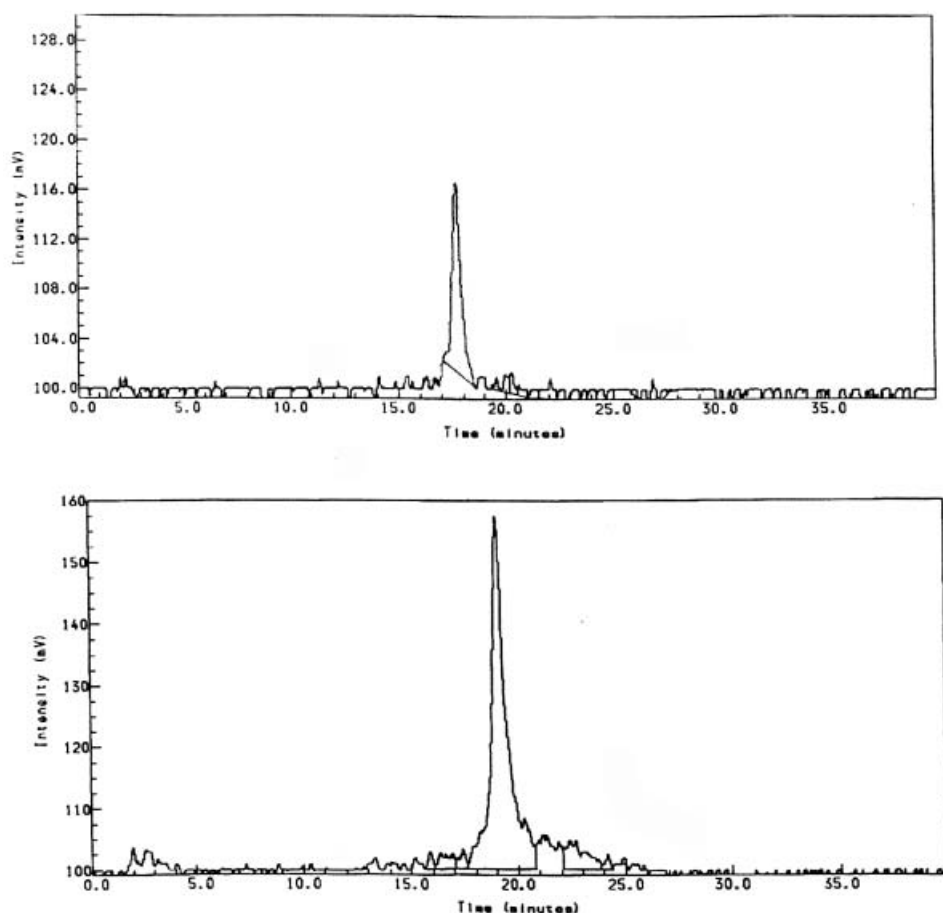


Figure 6. HPLC radiochromatograms of Moli1901-related material extracted from rat lung 60 min (top) and 120 days (bottom) after intratracheal instillation of ^3H -Moli1901.

Tissue distribution of ^{35}S in mice after intravenous administration of ^{35}S -Moli1901

To examine the behaviour of the compound in a different species, CDF-1 female mice were given 40 μg doses of ^{35}S -Moli1901 as either a bolus i.v. dose or a p.o. gavage dose. The mice were euthanized at various times after dosing, and the radiolabel content in tissues was determined (data not shown). As with the rat, very little radiolabel was systemically available after p.o. dosing, whereas after i.v. dosing high concentrations were measured in spleen and liver, followed by lung and kidney; concentrations of radiolabel in these four tissues did not decline appreciably during the 24-h post-dosing period.

Discussion

In rats, exposure to an aerosol of radiolabelled Moli1901 effectively delivered the compound to the lungs. In addition, very little radiolabel entered the systemic circulation and other important tissues and organs following this route of delivery.

Furthermore, little of the radiolabel was cleared from the pulmonary system in the 7 days following aerosol exposure, indicating that the elimination of compound-related material from lung proceeded very slowly. These results prompted experiments in which the compound was given by other routes to improve the understanding of the compound's disposition.

Owing to the nature of an inhalation exposure, some of the aerosolized compound can be swallowed, resulting essentially in a p.o. dose. When radiolabelled Moli1901 was given to rats by p.o. gavage, nearly 100% of the dose was recovered in the faeces. Tissue and blood/plasma concentrations on day 7 were nearly undetectable, indicating that either the compound was poorly absorbed from the gastrointestinal tract, or if it was absorbed, it was very rapidly eliminated.

Intravenous exposure provided insight into the disposition of blood-borne compound. Plasma concentrations of radiolabel declined relatively rapidly and were undetectable by 24 h after dosing. Nevertheless, tissue levels of radiolabel after i.v. dosing were readily measurable at 7 days post-dosing; in particular, nearly 33% of the dose was still present in the liver at this time. The highest concentrations of radiolabel were measured in the spleen, followed by the liver, lungs and kidneys. Common to these four tissues is a relatively high blood flow; the rapid elimination of radiolabel from plasma and the relatively high concentrations in these four tissues suggest that Moli1901 was taken up rapidly, but slowly eliminated by these tissues. A comparison of the results obtained after i.v. and p.o. dosing indicates that absorption of Moli1901 from the gastrointestinal tract must be minimal; after p.o. dosing no radioactivity was detectable in the tissues shown to retain the compound when given i.v. and the faecal excretion kinetics after p.o. dosing differed considerably from those observed after i.v. dosing.

While the aerosol exposure had indicated that compound-related material persisted at least 1 week after dosing, it did not provide an estimate of the elimination rate of material from the pulmonary system. Repeating an aerosol exposure to determine the elimination rate was considered, but the calculation of half-life following an aerosol exposure would have been compromised by the inability to quantitate the delivered dose accurately. By instilling a known amount of radiolabelled Moli1901 into the tracheas of rats, the initial dose was well defined and consistent from animal to animal, albeit by a means that possibly resulted in somewhat different pulmonary distribution than would occur by aerosol delivery. The 64-day elimination half-life from lung tissue was in reasonable agreement with the half-life for elimination of radiolabel via the faeces (mean of 52 days, range 45–63 days), suggesting that elimination from the lung may be the rate-limiting step in elimination from the animal. The reasons for this long half-life remain to be determined, but possible explanations are explored below.

Moli1901 likely integrated into pulmonary cell membranes. Numerous studies indicate that the compound interacts with membrane lipids. Moli1901 has been shown to inhibit the chloride transporter in clathrin-coated vesicles (Stone *et al.* 1984) and to alter membrane structure and permeability in isolated heart mitochondria (Sokolove *et al.* 1989). Its effects on ion transport in cultured human colonic epithelia, which contain apical chloride channels similar to those found in airway cells, are attributed to interactions with membrane lipids (Roberts *et al.* 1991). Moli1901 causes aggregation of rabbit sarcoplasmic reticulum vesicles (Navarro *et al.* 1985) and was shown to bind to the lipid fraction of *Escherichia coli*, as demonstrated by co-sedimentation with intact, but not with lipid-depleted

E. coli (Marki *et al.* 1991). If Moli1901 integrated into, or bound to, pulmonary cell membranes, its elimination from the pulmonary system would be determined by the turnover and subsequent clearance of the pulmonary cells to which it binds.

Given that very little compound-related material was absorbed from the pulmonary system, clearance of Moli1901, either free or bound to cells or cell debris, likely was largely via the mucociliary escalator. Mucociliary flow in rats has been measured at approximately 4.5 mm min^{-1} (Gatto 1993), and thus is not likely the rate limiting step yielding a 64-day half-life. Uptake of radiolabel by alveolar macrophages is a likely endpoint for much of the material. In fact, in a separate study of inhaled radiolabelled Moli1901 in dogs, microhistoautoradiography of lung sections demonstrated even distribution of radiolabel and a significant amount of radiolabel associated with pulmonary alveolar macrophages (McNulty *et al.* 1997). Migration of alveolar macrophages to the upper respiratory tract in rats has been measured at 2.5% of the total alveolar macrophage population per day (Fritsch and Masse 1992), equivalent to a 28-day half-life. The longer half-life for compound-related material (64 versus 28 days for alveolar macrophage clearance) is consistent with the notion that Moli1901 is integrating into airway epithelial cells and non-alveolar macrophages for a time before clearance.

The limited absorption of Moli1901 from the pulmonary system could simply be due to avid binding of the compound to membranes, as postulated above. In addition, the physico-chemical properties of the compound may also contribute to its poor absorption from the pulmonary system. As reviewed by Patton and Platz (1992), the therapeutic peptides leuprolide (nine amino acids), insulin (51) and growth hormone (192) are absorbed in active form from the lungs of humans and animals, with bioavailabilities in humans ranging from 10 to 25%. Thus, Moli1901, at 19 amino acids, is in a size range demonstrated to be bioavailable from the lung. However, its structure, with several intramolecular bonds, is quite different to those of the above-mentioned peptides. Studies of model peptide transport across rat alveolar cell monolayers by Dodoo *et al.* (2000) indicated that passage is primarily paracellular and inversely related to size in terms of molecular radius; possibly the three-dimensional structure of Moli1901, with its multiple intramolecular bonds, is such that it limits its absorption.

Disposition in mice paralleled that in the rat; p.o. administered radiolabel passed through the gastrointestinal tract rapidly, with little radiolabel measurable systemically, whereas i.v. dosing resulted in high concentrations in spleen and liver, from which the compound appeared to be eliminated slowly. The results of the present study are consistent with those of an inhalation study in dogs exposed to aerosolized ^3H -Moli1901 where pulmonary radiolabel concentrations 4 weeks after dosing were still approximately half those observed immediately after exposure (McNulty *et al.* 1997). As in rodents, excretion of radiolabel in dogs was primarily via the faeces (92% of excreted radiolabel). The consistency of Moli1901 disposition across several species suggests that disposition information in these preclinical models may be indicative of the compound's behaviour in man.

The limited systemic exposure and the slow clearance from the pulmonary system have important implications for the safety of the compound, suggesting that the likelihood of any major, non-pulmonary organ toxicities following inhalation exposure to Moli1901 would be substantially less than might be seen with systemically available compounds. On the other hand, the slow clearance of material deposited in the pulmonary system must be considered in assessing the

safety of the compound, since adverse reactions could potentially be long-lasting. To date, preclinical studies on the toxicology of inhaled Moli1901 in rats and dogs indicated no significant toxicities after single aerosol exposures at three different concentrations that achieved total depositions up to 1 mg kg^{-1} (unpublished data). Furthermore, a human safety study of four escalating doses in aerosol (single dose) in 16 healthy volunteers and 16 CF patients did not reveal any serious adverse events at pulmonary depositions of approximate $20 \mu\text{g kg}^{-1}$ (unpublished data).

A Phase I clinical dose escalation and safety study was designed to test the hypothesis that Moli1901 increases chloride secretion by the human nasal epithelium *in vivo*, using nasal transepithelial potential difference measurements as described by Knowles *et al.* (1991). In this latter study, the potential difference in nasal respiratory epithelium of healthy human volunteers was measured after intranasal exposure to solutions of Moli1901. No toxicity was observed in this study. Both an acute and a sustained response to Moli1901 were observed in the form of chloride transport upon perfusion of a chloride-free amiloride solution. The period of the sustained response varied widely from 1 to 4 weeks (Zeitlin *et al.* 1998). Although the half-life of Moli1901 in the human nasal epithelium was not measured in this study and is unknown, these results suggest that the prolonged pharmacokinetic half-life observed in the studies described above in rats and mice and in a separate study in dogs (McNulty *et al.* 1997) may also be observed in human airway epithelium. The ability to correlate the pharmacokinetics and pharmacodynamics of Moli1901 will aid considerably in setting dosing regimens in the clinical setting.

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Inhibitory effect of troleandomycin on the metabolism of omeprazole is CYP2C19 genotype-dependent

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Received 24 April 2002.

1. Eighteen healthy CYP2C19 genotyped male subjects were administered a 20-mg oral dose of omeprazole (OP) alone or received troleandomycin (TAO) 500 mg daily for 2 days before the dose of OP was administered. Blood samples were obtained and OP 5-hydroxyomeprazole (5-OH-OP) and OP sulfone in plasma were determined by reversed-phase HPLC.

2. The mean $C_{\max}$, AUC and CL for OP in poor metabolizers (PMs) were greater with TAO than without TAO. The $C_{\max}$ and AUC of 5-OH-OP in PMs were significantly ($p < 0.05$) less with TAO than without TAO. The differences in 5-OH-OP between heterozygous extensive metabolizers (EMs) with TAO versus without TAO were similar to those observed in PMs, except for the AUC. However, in homozygous EMs, there were no statistical differences for the effect of TAO.

3. The effect of TAO on the metabolism of OP and its two principal metabolites differs in different genotype groups of CYP2C19. CYP3A4 not only plays a dominant role in the formation of OP sulfone, but also it contributes to the 5-hydroxylation of OP. Both CYP2C19 and CYP3A contribute to the further elimination of 5-OH-OP and OP sulfone.

Introduction

Omeprazole (OP), a proton pump inhibitor that inhibits acid secretion by the parietal gastric mucosa cell, is widely used in the treatment of acid-related gastrointestinal disorders (Lindberg *et al.* 1986). It is extensively metabolized in the liver and the major metabolites present in plasma are 5-hydroxyomeprazole (5-OH-OP) and OP sulfone (Regårdh *et al.* 1986, Cederborg *et al.* 1989) (figure 1). *In vivo* and *in vitro* studies have indicated that the formation of 5-OH-OP is mediated predominantly by CYP2C19, with only a minor contribution of CYP3A (Andersson *et al.* 1992, 1993, Sohn *et al.* 1992, Chiba *et al.* 1993, Curi-Pedrosa *et al.* 1993, Balian *et al.* 1995). The production of sulfone seems catalysed primarily by CYP3A, but its further elimination is also dependent on the activity of mephenytoin hydroxylase (Andersson *et al.* 1990, 1993, 1994, Curi-Pedrosa *et al.* 1993, Balian *et al.* 1995). Population studies have shown that hydroxylation of OP correlates positively with *S*-mephenytoin metabolism (Curi-Pedrosa *et al.* 1993).

The *S/R* ratio of mephenytoin has been used to measure the activity of *S*-mephenytoin hydroxylase identified as CYP2C19 (Wrighton *et al.* 1993, Goldstein 1994) and the *S/R* ratios of mephenytoin classifies individuals into poor metabolizers (PMs) and extensive metabolizers (EMs). Polymorphic metabolism exists in

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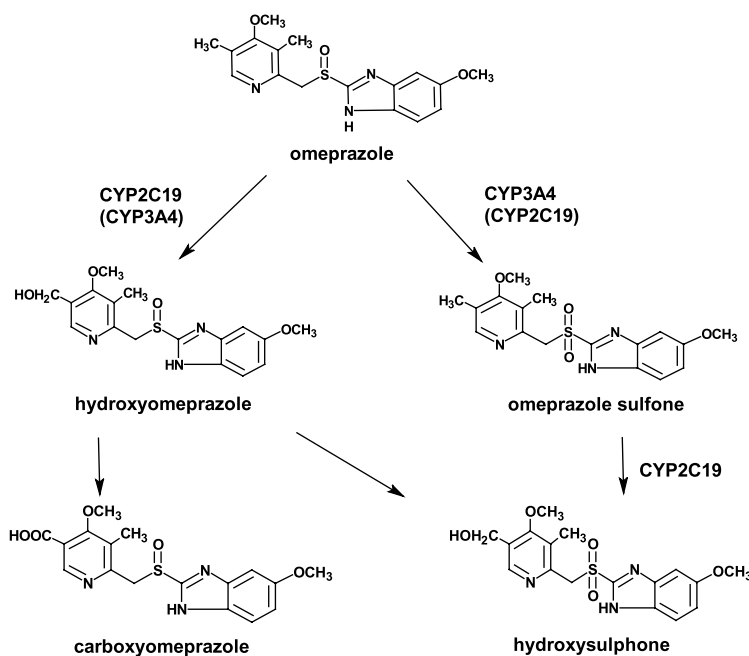


Figure 1. Omeprazole metabolism in man. 5-OH-OP mediated by CYP2C19 is the major metabolic pathway for OP.

the human population with approximately 3% of Caucasians and up to 23% of Oriental populations having poor *S*-mephenytoin hydroxylation activity (Wilkinson *et al.* 1989). The PM phenotype results from the absence of CYP2C19 activity and at least seven inactivating mutations of *CYP2C19* have been reported (Ibeanu *et al.* 1998, 1999). *CYP2C19**2 and *CYP2C19**3, the two major mutant alleles of CYP2C19, comprise almost 100% of the PMs in Oriental populations (de Morais *et al.* 1995, Xiao *et al.* 1997).

Troleandomycin (TAO) is a selective inhibitor of CYP3A. Using the erythromycin breath test as a probe, CYP3A activity is inhibited 80% by the oral administration of 250 mg TAO in healthy subjects, whereas the activity of several other CYP forms, including CYP2C, are unaffected by TAO (Watkins *et al.* 1992, Chang *et al.* 1994).

The current study was designed to clarify the effect of TAO on the metabolism of OP in *CYP2C19* genotyped subjects and to assess the role of CYP3A4 and CYP2C19 in the metabolism of OP.

Material and methods

Subjects

Ninety unrelated healthy Chinese students from Xiang-Ya School of Medicine, Central South University, P. R. China, were recruited and genotyped for *CYP2C19* by the methods of de Morais *et al.* (1994a, b). Six homozygous EMs (*CYP2C19**1/*1), six heterozygous EMs (four of *CYP2C19**1/*2, two of *CYP2C19**1/*3) and six PMs (five of *CYP2C19**2/*2, one of *CYP2C19**3/*3) were selected randomly from the three genotyped subgroups. The subjects enrolled in the study were from 20 to 24 years (mean $\pm$ SD, 21.8 ± 1.0 years) and weighed 52–85 kg (65.1 ± 8.7 kg). The weights of subjects were within 20% of their ideal body weights. Each subject gave written consent before the study, the protocol

of which was approved by the ethics committee of Xiang-Ya School of Medicine, Central South University. All subjects were judged as healthy based on a physical examination and routine laboratory analyses of haematological, renal and hepatic function. All were non-smokers and had refrained from alcohol throughout the study period. None had taken any medication for 3 weeks before and during this study.

Chemicals and drugs

OP, 5-OH-OP, OP sulfone and internal standard H 259/36 were generous gifts from Astra Hässle AB (Mölndal, Sweden). Acetonitrile and methanol of HPLC grade were purchased from the Tedia Co. (Fairfield OH, USA), Inc. (USA). All other molecular biologic products for genotyping of *CYP2C19* were from Sigma Chemical Co. (St Louis, MO, USA). TAO was purchased as an enteric-coated formulation (Pfizer, Inc., Tamsui, Taiwan) and OP as encapsulated enteric-coated granules (Astra, Wuxi, P. R. China).

Study protocol

The study was performed in a crossover design with a 2-week washout. Each subject took 500 mg TAO orally as a single morning dose (or not) for 2 days. On the morning of the second study day, after an overnight fast, each of them took a 20-mg oral dose of OP alone with 200 ml water or 1 h after 500 mg TAO. Food was given 4 h after the ingestion of OP. Venous blood samples (8 ml) were obtained just before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after drug administration. The plasma was separated after centrifugation and stored at -20°C until analysed.

Analytic methods

Peripheral venous blood (8 ml) was obtained from each subject and DNA was isolated from peripheral leukocytes by standard methods (Sambrook *et al.* 1989). Detection of *CYP2C19**2 and *CYP2C19**3 mutant alleles was performed by PCR/RFLP (de Moraes *et al.* 1994a, b). PCR amplification was performed with a PCR-90A AMPLIFIER (Genetic Research Institute, The Academy of Sciences of China). An initial denaturation step at 94°C for 5 min was followed by 37 cycles consisting of denaturation at 94°C for 20 s, annealing at 56°C for 20 s and extension at 72°C for 20 s. The PCR products were digested by *Sma*I and *Bam*HI restriction enzymes to detect the *CYP2C19**2 and *CYP2C19**3 mutant alleles respectively.

The plasma concentration of OP, 5-OH-OP and OP sulfone were determined by HPLC according to the method developed in our laboratory (Shu *et al.* 2000). A total of 100 μl of internal standard (H 259/36, $4.3\ \mu\text{mol l}^{-1}$ stock solution in methanol), 100 μl sodium carbonate buffer ($0.1\ \text{mol l}^{-1}$, pH 9.3), and 5 ml dichloromethane were added to 1 ml serum in a capped tube. After shaking for 3 min, centrifugation was performed at 1500g for 10 min, and the organic layer was transferred to a clean glass tube and evaporated to dryness under a mild stream of nitrogen gas at 40°C . The residue was resuspended in 50 μl of the HPLC mobile phase. The HPLC system consisted of an HP series 1050 pump, online degasser, variable wavelength detector, and manual injector (Hewlett-Packard Co., Palo Alto, CA, USA). The assessment of eluate was performed on a C_8 column ($4.6 \times 150\ \text{mm}$, zorbax 5 μm , Agilent Technologies Inc., Wilmington, DE) at a wavelength of 302 nm at 40°C . The mobile phase consisted of $0.05\ \text{mol l}^{-1}$ phosphate buffer (pH 7.0) and acetonitrile (71:29 v/v), at a flow rate of $1.0\ \text{ml min}^{-1}$. The limit of detection of OP and the two metabolites was $25\ \text{nmol l}^{-1}$. At 3200, 800 and $50\ \text{nmol l}^{-1}$, intraday variation was 0.5, 0.7 and 6.4%, respectively, for OP; 0.6, 0.6 and 5.2%, respectively, for OP sulfone; and 1.2, 2.1 and 8.6%, respectively, for 5-OH-OP. Interday variation ranged from 1.1 to 9.4%. The recovery of OP, OP sulfone and 5-OH-OP ranged from 85 to 104%.

Pharmacokinetic analysis

Pharmacokinetic analysis of OP, OP sulfone and 5-OH-OP was accomplished by a non-compartmental method. The elimination rate constant β was calculated by linear least-square regression of the log-plasma concentration-time curve of the post-distribution phase. Elimination $t_{1/2}$ was calculated as $0.693/\beta$. The serum AUC was calculated by the trapezoidal rule from zero to 12 h (or 24 h) after dose administration. The AUC from zero hour to infinity was calculated according to the formula:

$$\text{AUC}_{(\infty)} = \text{AUC}_{(0-t)} + C_t/K_e.$$

Oral clearance (CL_0) was calculated as follows:

$$\text{CL}_0 = \text{dose}/\text{AUC}_{(\infty)}.$$

Concentrations below the limit of quantification were set either to zero at zero hours or to the quantification limit at all other time points. C_{max} and T_{max} were real-time measurements.

Statistical methods

One-way ANOVA, combined with the least significant difference method (LSD), was used to compare means between different genotyped groups. Statistical evaluation of genotyped groups with TAO versus without TAO was performed using a paired *t*-test. Data are the mean $\pm$ SE. Statistical significance was considered as $p < 0.05$.

Results

No clinically undesirable signs and symptoms possibly attributed to the administration of OP and TAO were recognizable throughout the study. According to the protocol, all subjects completed the study successfully. The mean plasma concentration–time curves of OP, 5-OH-OP and OP sulfone in genotyped groups of CYP2C19 without or with TAO are shown in figures 2–4. The mean plasma OP concentrations were significantly ($p < 0.01$) greater in PMs than in EMs (including heterozygous EMs and homozygous EMs) at corresponding time points after 1-h dosage. The plasma concentrations of OP at 12 h postdose in EMs were undetectable, whereas in all of PMs (figure 2C), OP was detectable even at 24 h postdose. TAO did not change the detectable time points postdose in either PMs or in EMs (figure 2).

The mean plasma concentrations of 5-OH-OP were significantly lower ($p < 0.01$) at 0.75–6 h in PMs than those in EMs (figure 3). The concentration of 5-OH-OP in plasma became lower than the linear range of the assay after the 8 and 12 h of time points in EMs and PMs, respectively. TAO also did not change the detectable time points after drug administration in either PMs or EMs (figure 3). OP sulfone had a similar trend of concentration–time profile to that of OP. From 1 to 12 h postdose, concentrations of OP sulfone in PMs were significantly higher than those in EMs ($p < 0.01$). The plasma concentrations of OP sulfone after 12 h postdose in EMs were undetectable, whereas they were still detectable at 24 h postdose in all of PMs (figure 4A, C). TAO changed the detectable time points of OP sulfone after the postdose in EMs (figure 4A, B), which changed from 12 h without TAO to 8 h with TAO. In contrast, TAO did not affect detectable time points in the PMs.

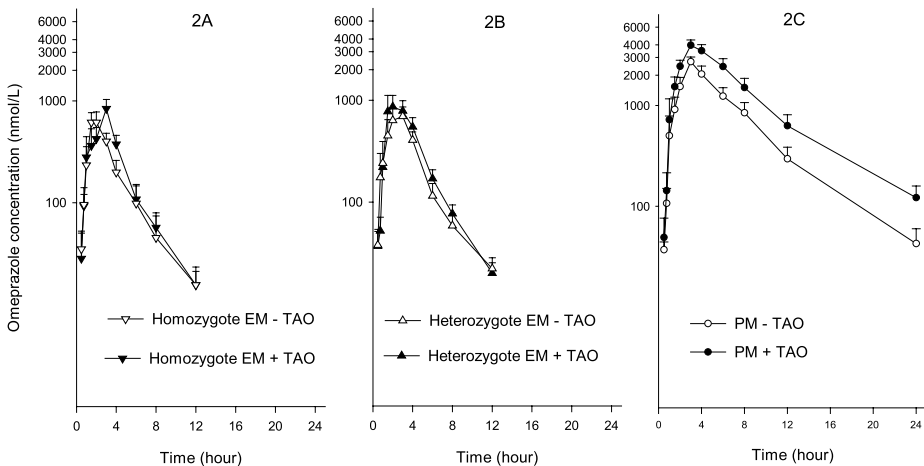


Figure 2. Plasma concentration–time curve for OP after a single 20-mg oral dose of OP: effect of TAO in homozygote EMs (A), heterozygote EMs (B) and PMs (C). Data are the mean $\pm$ SE.

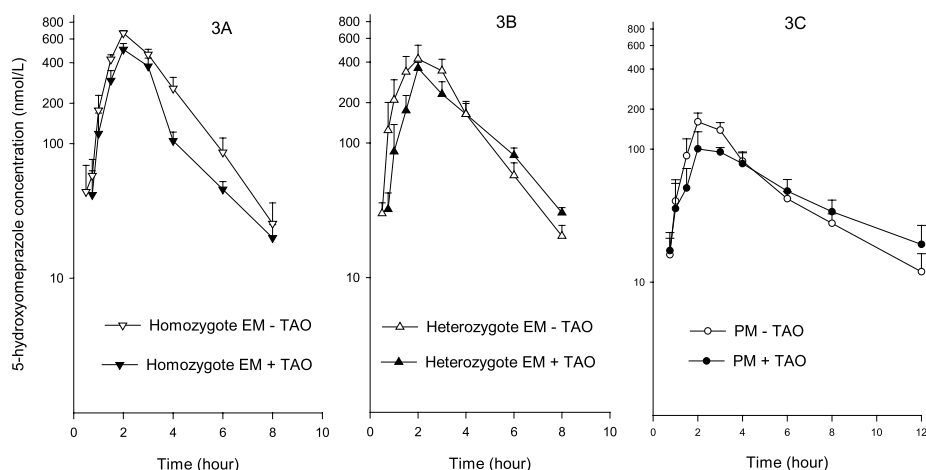


Figure 3. Plasma concentration–time curve for 5-OH-OP after a single 20-mg oral dose of OP: effect of TAO in homozygote EMs (A), heterozygote EMs (B) and PMs (C). Data are the mean $\pm$ SE.

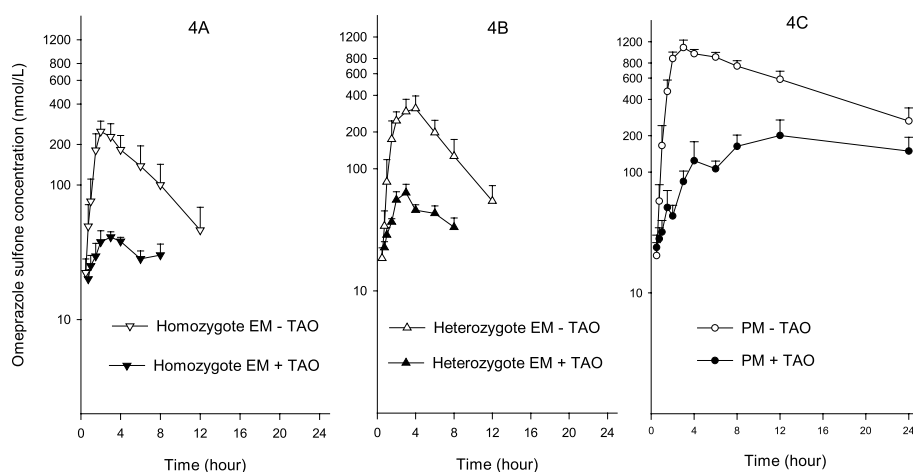


Figure 4. Plasma concentration–time curve for OP sulfone after a single 20-mg oral dose of OP: effect of TAO in homozygote EMs (A), heterozygote EMs (B) and PMs (C). Data are the mean $\pm$ SE.

The pharmacokinetic data of OP and its two major metabolites are summarized in table 1. There were no statistically significant differences in the kinetic data for OP, 5-OH-OP and OP sulfone between heterozygous EMs and homozygous EMs except for the $C_{\max}$'s for 5-OH-OP, which were greater in homozygous EMs than in heterozygous EMs ($p < 0.05$). In contrast, significant ($p < 0.05$ – 0.01) differences between EMs (homozygous EMs and heterozygous EMs) and PMs in the mean kinetic parameters of OP, 5-OH-OP and OP sulfone were observed. The mean AUC and $C_{\max}$'s for OP were approximately three to six times greater in PMs than in EMs, whereas the CL values were seven to eight times smaller in PMs than in EMs. There were similar differences for the kinetic values of OP sulfone between EMs and PMs as observed with OP. The mean AUC and $C_{\max}$'s for OP

Table 1. Effect of TAO on the pharmacokinetics of omeprazole and its two main metabolites in genotyped groups of CYP2C19.

	Without TAO				With TAO			
	EMs (n = 12)	Homozygous EMs (n = 6)	Heterozygous EMs (n = 6)	PMs (n = 6)	EMs (n = 12)	Homozygous EMs (n = 6)	Heterozygous EMs (n = 6)	PMs (n = 6)
Omeprazole								
$C_{\max}$ ($\mu\text{mol l}^{-1}$)	0.84 ± 0.11**	0.87 ± 0.17**	0.82 ± 0.15**	2.94 ± 0.25	1.12 ± 0.15**	1.05 ± 0.14**	1.19 ± 0.28**	4.19 ± 0.44 ^a
$t_{\max}$ (h)	2.13 ± 0.26	1.83 ± 0.25	2.42 ± 0.45	2.83 ± 0.31	2.46 ± 0.2	2.83 ± 0.17	2.08 ± 0.3	3.0 ± 0.26
$t_{1/2}$ (h)	1.49 ± 0.26**	1.16 ± 0.06**	1.81 ± 0.51*	3.12 ± 0.43	1.26 ± 0.11**	1.03 ± 0.17** ^c	1.49 ± 0.08**	3.74 ± 0.51
$AUC_{-\infty}$ ($\mu\text{mol h l}^{-1}$)	2.34 ± 0.32**	2.09 ± 0.55**	2.59 ± 0.36**	15.53 ± 2.51	2.96 ± 0.39**	2.67 ± 0.47**	3.26 ± 0.63** ^a	28.11 ± 4.63 ^a
CL ($\text{ml h}^{-1} \text{ kg}^{-1}$)	546.4 ± 103.4**	676.2 ± 188.7**	416.6 ± 68.5*	71.2 ± 11.9	398.6 ± 53.5**	427.0 ± 75.9**	370.1 ± 80.7**	41.8 ± 9.9 ^a
5-hydroxyomeprazole								
$C_{\max}$ ($\mu\text{mol l}^{-1}$)		0.63 ± 0.03**	0.68 ± 0.01**	0.57 ± 0.06**	0.19 ± 0.01	0.51 ± 0.03** ^b	0.55 ± 0.05** ^c	0.47 ± 0.02** ^a
$t_{\max}$ (h)		2.0 ± 0.11	1.92 ± 0.08	2.08 ± 0.2	2.08 ± 0.2	2.21 ± 0.15 ^a	2.03 ± 0.17	2.33 ± 0.21 ^b
$t_{1/2}$ (h)		1.26 ± 0.1**	1.19 ± 0.05**	1.33 ± 0.21**	2.26 ± 0.32	1.51 ± 0.07** ^a	1.3 ± 0.04** ^d	1.72 ± 0.07** ^a
$AUC_{-\infty}$ ($\mu\text{mol h l}^{-1}$)		1.72 ± 0.14**	1.88 ± 0.11**	1.56 ± 0.25**	0.87 ± 0.12	1.61 ± 0.12**	1.68 ± 0.17** ^c	1.45 ± 0.19**
$AUC_{\text{m}}/AUC_{\text{p}}$		0.74 ± 0.24*	0.85 ± 0.46**	0.65 ± 0.13*	0.07 ± 0.02	0.66 ± 0.09**	0.77 ± 0.16** ^c	0.51 ± 0.09**
0.03 ± 0.01				Omeprazole sulfone				
$C_{\max}$ ($\mu\text{mol l}^{-1}$)	0.38 ± 0.03**	0.3 ± 0.03**	0.47 ± 0.03**	1.22 ± 0.13	0.05 ± 0.01** ^b	0.05 ± 0.01** ^b	0.05 ± 0.01** ^b	0.27 ± 0.06 ^b
$t_{\max}$ (h)	2.58 ± 0.25	2.42 ± 0.27*	2.75 ± 0.44	3.5 ± 0.36	3.33 ± 0.41**	3.5 ± 0.56**	3.17 ± 0.65**	8.92 ± 1.97 ^a
$t_{1/2}$ (h)	3.2 ± 0.45**	3.08 ± 0.83**	3.33 ± 0.45**	10.72 ± 2.12	4.67 ± 0.6** ^b	4.84 ± 0.92** ^a	4.5 ± 0.85** ^a	16.47 ± 3.53 ^a
$AUC_{-\infty}$ ($\mu\text{mol h l}^{-1}$)	2.2 ± 0.4**	1.72 ± 0.49**	2.68 ± 0.61**	18.96 ± 3.05	0.49 ± 0.06** ^b	0.49 ± 0.08** ^a	0.49 ± 0.11** ^a	7.64 ± 2.16 ^a
$AUC_{\text{m}}/AUC_{\text{p}}$	0.94 ± 0.12	0.88 ± 0.17	1.0 ± 0.18	1.33 ± 0.2	0.18 ± 0.02	0.19 ± 0.03	0.17 ± 0.03	0.28 ± 0.06

Data are the mean ± SE.

$AUC_{C_{\infty}}$, area under the plasma concentration–time curve from 0 h to infinity; AUC_{m} , AUC of metabolite from 0 h to infinity; AUC_{p} , AUC of parent drug (omeprazole) from 0 h to infinity.

EMs, includes homozygous EMs and heterozygous EMs.

* $p < 0.05$ as compared with PMs; ** $p < 0.01$ as compared with PMs, respectively.

^a $p < 0.05$ as compared with the same group without TAO; ^b $p < 0.01$ as compared with the same group without TAO; ^c $p < 0.05$ as compared with Heterozygous EMs with TAO; ^d $p < 0.01$ as compared with Heterozygous EMs with TAO.

sulfone were significantly greater ($p < 0.01$) in PMs than in EMs, implying that the further elimination of OP sulfone was dependent on the activity of CYP2C19.

In contrast, the kinetic data for 5-OH-OP exhibited the opposite to that of OP and OP sulfone. The mean $C_{\max}$, AUC, and AUC_m/AUC_p were significantly less ($p < 0.05$ – 0.01) in PMs than in EMs, demonstrating that the hydroxylation of OP to 5-OH-OP is considerably impaired in PMs.

The kinetic values for the $t_{1/2}$ of OP and its two metabolites differed significantly ($p < 0.05$ – 0.01) between EMs and PMs. The mean $t_{1/2}$ was significantly longer in PMs than in EMs, implying that CYP2C19 plays a dominant role in the metabolism of OP and the further elimination of 5-OH-OP and OP sulfone.

The statistical differences of the pharmacokinetic data on OP, 5-OH-OP and OP sulfone among homozygous EMs, heterozygous EMs and PMs pretreated with TAO were not similar to those observed in the absence of TAO. The $t_{1/2}$'s for OP in homozygous EMs were significantly less ($p < 0.05$) than those in heterozygous EMs. Furthermore, the mean $C_{\max}$, AUC, AUC_m/AUC_p and $t_{1/2}$'s for 5-OH-OP in homozygous EMs were significantly greater or less ($p < 0.05$ – 0.01) than those in heterozygous EMs, implying a gene dosage effect of CYP2C19.

Pharmacokinetic data for OP and its two major metabolites showed significant differences between the heterozygous EMs and PMs groups with TAO compared with those without TAO. The mean $C_{\max}$ and AUCs for OP were greater ($p < 0.05$) and the CLs less ($p < 0.05$) in PMs with TAO than without TAO. The AUC of OP in heterozygous EMs was significantly greater ($p < 0.05$) with TAO than without TAO. However, homozygous EMs showed no statistical difference between TAO versus without TAO.

The $C_{\max}$ and AUCs for 5-OH-OP in PMs were significantly less ($p < 0.05$) with TAO than without TAO. In contrast, the $T_{\max}$ and $t_{1/2}$ data were significantly greater ($p < 0.05$) with TAO than without TAO. There were similar differences ($p < 0.05$ – 0.01) in heterozygous EMs to those observed in PMs with TAO versus without TAO except for the AUC. The AUC for PMs was significantly smaller ($p < 0.05$) with TAO versus Without TAO ($14.2\% \pm 2.6\%$, from 0.87 ± 0.12 to 0.75 ± 0.12), suggesting that CYP3A4 may make about a 14% contribution to 5-hydroxylation of OP.

The $C_{\max}$ and AUC for OP sulfone were significantly less ($p < 0.05$ – 0.01) in all groups with TAO than without TAO. In contrast, the $t_{1/2}$ data were significantly greater ($p < 0.05$ – 0.01) with TAO than without TAO. The $T_{\max}$ values were significantly greater ($p < 0.01$) only in PMs with TAO than without TAO.

Discussion

S-mephenytoin hydroxylation polymorphism is now known to affect the metabolism of several clinically important drugs such as diazepam, imipramine, propranolol, proguanil and OP (Goldstein 2001). *S*-mephenytoin hydroxylation is catalysed by CYP2C19 and PMs occur to the extent of 3% of Caucasians and up to 23% of Oriental populations. Therefore, the clinical significance of this polymorphism appears extremely important for Oriental populations rather than for Caucasians. In the present study, significant differences in the metabolism of OP were observed among different genotyped groups. The mean AUC and $t_{1/2}$ were

greater and the mean CL was less in genotyped PMs than in genotyped EMs, consistent with previous *in vivo* studies (Sohn *et al.* 1992, Ieiri *et al.* 1996).

The differences in kinetic values of OP sulfone between EMs and PMs were similar to those observed with OP. The mean AUC and $C_{\max}$'s for OP sulfone were significantly greater in PMs than in EMs. These observations support previous studies that the generation of OP sulfone seems to be catalysed primarily by CYP3A4, but its further elimination also appears dependent on the activity of CYP2C19 (Andersson *et al.* 1990, 1993, 1994, Curi-Pedrosa *et al.* 1993, Balian *et al.* 1995).

In contrast, the kinetic data of 5-OH-OP was opposite to that of OP and OP sulfone. The mean $C_{\max}$, AUC, and AUC_m/AUC_p were significantly less in PMs than in EMs, demonstrating that the hydroxylation of OP to 5-OH-OP is considerably impaired in PMs. The kinetic behaviour of OP correlated with S-mephenytoin hydroxylation metabolism.

The mean values of $t_{1/2}$ for OP and its two metabolites were significantly greater in PMs than in EMs, also implying that the hydroxylation activity of CYP2C19 plays a dominant role in the metabolism of OP and the further elimination of 5-OH-OP and OP sulfone. Thus, the results of our study are in agreement with previous *in vitro* and *in vivo* studies (Andersson *et al.* 1990, 1994, Curi-Pedrosa *et al.* 1993, Balian *et al.* 1995).

Except for the $C_{\max}$'s for 5-OH-OP, there were no statistically significant differences in the kinetic data for OP, 5-OH-OP and OP sulfone between heterozygous EMs and homozygous EMs in our study. A possible explanation of these results is that the number of subjects in this study is too small to reach a definitive conclusion; the other possible explanation is that some kinetic data overlapped in a few of the homozygous and heterozygous EM subjects. Furthermore, at least two distinct enzymes participate in the metabolism of OP and its two principal metabolites may be another factor bearing on this result. However, a gene-dose effect for CYP2C19 are apparent in the kinetic data for OP and 5-OH-OP among genotyped groups when pretreated with TAO, implying that CYP3A4 partially contributes to the metabolism of OP and 5-OH-OP mediated by CYP2C19 as previously demonstrated.

TAO is an effective inhibitor of CYP3A4. Such inhibitory effects are probably caused in part by the *in vivo* formation of an inactive cytochrome CYP-TAO metabolite complex (Pessayre *et al.* 1981, Kinirons *et al.* 1993). After the oral administration of 250 mg TAO in healthy subjects, CYP3A activity is inhibited 80%, whereas the activity of several other CYP forms, including CYP2C, are unaffected (Watkins *et al.* 1992, Chang *et al.* 1994). TAO should be used at the lowest possible dose to minimize the risk of both adverse reactions and unspecific inhibition of other CYPs. In a previous *in vivo* study (Böttiger *et al.* 1997), the optimal dose of ketoconazole, a CYP3A4-selective inhibitor, was investigated for OP metabolism in phenotyped groups of CYP2C19 and a 200-mg dose of ketoconazole was sufficient for an inhibitory effect on CYP3A4 activity. We did not investigate the optimal dose of TAO in the current study. However, a 500 mg daily dose of TAO has been used in clinical practice and no adverse effects have been reported in any of the subjects. Whether TAO affects the absorption of OP is unclear. For avoiding this influence, OP was given 1 h after TAO administration.

In our previous *in vitro* study (Shu 2000), CYP2C19 appears to be a high-affinity enzyme for OP 5-hydroxylation, whereas CYP3A4 is a low-affinity enzyme

for OP sulfoxidation. Therefore, the effect of the CYP3A4-selective inhibitor TAO on OP 5-hydroxylation was less at 4 μM OP in EMs than that at 20 and 100 μM OP in EMs. In contrast, 5-OH-OP formation at every concentrations of OP in PMs was almost completely inhibited by this inhibitor. In fact, the OP concentrations *in vitro* were substantially very higher than that under *in vivo* conditions. Therefore, CYP3A4 mainly contributes to the 5-hydroxylation mostly refers to the PMs as well as some heterozygous EMs.

The activity of CYP3A4, inhibited by TAO, was reduced and resulted in greater C_{max} and AUCs and a lower CL for OP in PMs, whose activity of CYP2C19 was genetically impaired. The AUC for OP in heterozygous EMs was significantly greater with TAO than in its absence, and homozygous EMs showed no statistical difference with TAO versus without TAO, which also implied a gene-dose effect for CYP2C19 for the metabolism of OP.

The C_{max} and AUCs for 5-OH-OP in PMs were significantly less with TAO than in its absence. These data confirm that CYP3A4 participates in the 5-hydroxylation of OP and are in agreement with previous *in vitro* studies (Andersson *et al.* 1993, Yamazaki *et al.* 1997). Furthermore, similar differences in heterozygous EMs with TAO versus without TAO were observed except for the AUC. In a few heterozygous EMs, the concentration of 5-OH-OP at two to four time points decreased substantially in the presence of TAO, implying that the decreased activity of CYP3A in the presence of the inhibitor may alter the OP hydroxylation index and result in a discordance of CYP2C19 phenotype and genotype as previous reported (Kimura *et al.* 1998, Williams *et al.* 2000).

The $t_{1/2}$'s for 5-OH-OP and OP sulfone were significantly greater with TAO than in its absence, suggesting that CYP3A4 contributes to the further metabolism of both metabolites, consistent with previous *in vitro* studies (Andersson *et al.* 1993). The C_{max} and AUC for OP sulfone in all subjects were significantly less with TAO than in its absence, demonstrating that CYP3A4 plays a predominant role in the formation of OP sulfone, consistent with previous *in vivo* and *in vitro* studies.

In conclusion, the inhibitory effect of TAO on the metabolism of OP and its two principal metabolites differed in different CYP2C19 genotype groups, showing a gene dose effect of CYP2C19 (PMs > heterozygous EMs > homozygous EMs). CYP3A4 not only plays a dominant role in the formation of OP sulfone, but also participates in the 5-hydroxylation of OP. Both CYP2C19 and CYP3A4 participate in the further elimination of 5-OH-OP and OP sulfone.

Acknowledgement

This project was supported by the China Medical Board Grants 99-697 and 01-755, and the National Natural Science Foundation of China Grant F30130210.

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mRNA and protein expression of dog liver cytochromes P450 in relation to the metabolism of human CYP2C substrates

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Received 1 August 2002

1. Interpretation of novel drug exposure and toxicology data from the dog is tempered by our limited molecular and functional knowledge of dog cytochromes P450 (CYPs). The aim was to study the mRNA and protein expression of hepatic dog CYPs in relation to the metabolism of substrates of human CYP, particularly those of the CYP2C subfamily.

2. The rate of 7-hydroxylation of *S*-warfarin (CYP2C9 in humans) by dog liver microsomes (mean $\pm$ SD from 12 (six male and six female) dogs) = 10.8 ± 1.9 fmol mg⁻¹ protein min⁻¹) was 1.5–2 orders of magnitude lower than that in humans.

3. The rate of 4'-hydroxylation of *S*-mephenytoin, catalysed in humans by CYP2C19, was also low in dog liver (4.6 ± 1.5 pmol mg⁻¹ protein min⁻¹) compared with human liver. In contrast, the rate of 4'-hydroxylation of the *R*-enantiomer of mephenytoin by dog liver was much higher. The kinetics of this reaction (range of K_m or $K_{0.5}$ 15–22 μ M, V_{max} 35–59 pmol mg⁻¹ protein min⁻¹, $n = 4$ livers) were consistent with the involvement of a single enzyme.

4. In contrast to our findings for *S*-mephenytoin, dog liver microsomes 5'-hydroxylated omeprazole (also catalysed by CYP2C19 in humans) at considerably higher rates (range of K_m 42–64 μ M, V_{max} 22–46 pmol mg⁻¹ protein min⁻¹, $n = 4$ livers).

5. For all the substrates except omeprazole, a sex difference in their metabolism was observed in the dog (dextromethorphan N-demethylation: female range = 0.7–0.9, male = 0.4–0.8 nmol mg⁻¹ protein min⁻¹ ($p < 0.02$); *S*-warfarin 7-hydroxylation: female = 9–15.5, male = 8–12 fmol mg⁻¹ protein min⁻¹ ($p < 0.02$); *R*-mephenytoin 4'-hydroxylation: female = 16–35, male = 11.5–19 pmol mg⁻¹ protein min⁻¹ ($p < 0.01$); omeprazole 5'-hydroxylation: female = 15–20, male 13–22 pmol mg⁻¹ protein min⁻¹ ($p > 0.2$)).

6. All dog livers expressed mRNA and *CYP3A12*, *CYP2B11*, *CYP2C21* proteins, with no sex differences being found. Expression of *CYP2C41* mRNA was undetectable in the livers of six of 11 dogs.

7. Correlation analysis suggested that CYP2B11 catalyses the N-demethylation of dextromethorphan (mediated in humans by CYP3A) and the 4'-hydroxylation of mephenytoin (mediated in humans by CYP2C19) in the dog, and that this enzyme and CYP3A12 contribute to *S*-warfarin 7-hydroxylation (mediated in humans by CYP2C9).

8. In conclusion, we have identified a distinct pattern of hepatic expression of the *CYP2C41* gene in the Alderley Park beagle dog. Furthermore, marked differences in the metabolism of human CYP2C substrates were observed in this dog strain compared with humans with respect to rate of reaction, stereoselectivity and CYP enzyme selectivity.

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Introduction

The dog is used extensively by the pharmaceutical industry for the pharmacological and toxicological testing of new drugs. However, compared with humans, much less is known about the drug-metabolizing abilities of the animal. There are marked species differences in the expression and activities the cytochrome P450 (CYP) superfamily of drug-metabolizing enzymes (Guengerich 1997). Thus, it is important to characterize the expression and function of canine forms of CYP and compare them with those from humans.

From a clinical standpoint, the five most important forms of CYP involved in human drug metabolism are CYP1A2, CYP2D6, CYP3A4, CYP2C9 and CYP2C19 (www.imm.ki.se/cypalleles). The metabolism of a limited number of substrates of these enzymes has been studied in dog liver or intestinal microsomes (Gee *et al.* 1984, Court *et al.* 1987, Ohta *et al.* 1989, Sharer *et al.* 1995, Priekšaritanont *et al.* 1996, Chauret *et al.* 1997, Zhao and Ishizaki 1997, Roussel *et al.* 1998). Some metabolic reactions occur at similar rates to human liver, for example the O-demethylation of dextromethorphan (Roussel *et al.* 1998), but for the majority of substrates there are substantial species differences in their metabolism. To date, eight dog CYPs have been identified: CYP1A1 (Uchida *et al.* 1989), CYP1A2 (Ohta *et al.* 1989), CYP2B11 (Graves *et al.* 1990), CYP2C21 (Uchida *et al.* 1990), CYP2C41 (Blaisdell *et al.* 1998), CYP2D15 (Sakamoto *et al.* 1995), CYP3A12 (Ciaccio and Halpert 1989) and CYP3A26 (Fraser *et al.* 1997). A limited number of human CYP substrates have been shown to be metabolized by these dog forms. The CYP2C subfamily is of particular note in the context of the dog-human comparison. Blaisdell *et al.* (1998) reported that the *CYP2C41* gene is expressed polymorphically in that it was present in only four of the 25 dogs studied. In contrast, the *CYP2C21* gene is found in all dogs. Furthermore, this species seems to be deficient in its ability to metabolize tolbutamide (Gee *et al.* 1984, Sharer *et al.* 1995), a human CYP2C9 substrate. However, other substrates of this enzyme behave differently in the dog. Thus, the metabolism of celexicob appears to be polymorphically distributed (Paulson *et al.* 1999), whereas the 4-hydroxylation of phenytoin, although occurring at a lower rate in the livers of adult dogs (Tanaka *et al.* 1998) compared with humans (Schmider *et al.* 1997a), shows no evidence of polymorphism.

In view of the above findings, we have studied the metabolism of additional substrates of the human CYP2C subfamily in dog liver and examined the relationship between catalytic activity and mRNA and protein expression of individual forms of dog CYPs.

Materials and methods

Drugs and chemicals

3-Methoxymorphinan was a gift from Roche (Basle, Switzerland), *S*-warfarin and 7-hydroxywarfarin were from GlaxoSmithKline (Amersham, UK), omeprazole, 5'-hydroxymeprazole were from AstraZeneca (Molndal, Sweden), and *R*- and *S*-mephentyoin and 4'-hydroxymephentyoin were from Professor G. R. Wilkinson (Vanderbilt University, Nashville, TN, USA). Dextromethorphan hydrobromide and chlorzoxazone were purchased from Sigma Chemical Co., laudanoline and 7-ethoxycoumarin were from Aldrich Chemical Co. and sodium phenobarbitone was from BDH Ltd (all Poole, UK). All other chemicals and solvents were obtained commercially and were of the highest grade available.

Dog liver

Livers were obtained from six male and six female adult beagle dogs (age range 7–13 months; mean weight (range) at necropsy: male = 10.8 (10.0–12.5) kg, female = 8.9 (8.0–10.5) kg) bred at AstraZeneca, Alderley Park. Animals had been used as controls in drug safety studies and had all been administered saline vehicle.

Preparation of microsomes

Three volumes of ice-cold homogenization buffer comprising 154 mM KCl and 50 mM Tris (pH 7.4) were added to each sample of frozen dog liver (v/w), and kept on ice until the liver was thawed. Tissue was homogenized using a Polytron PT 3100 homogenizer, and the homogenate was centrifuged at 9000g for 20 min at 4 °C. The supernatant was separated and centrifuged at 100 000g for 60 min at 4 °C. Microsomal pellets were resuspended in an ice-cold solution of potassium phosphate (50 mM) containing glycerol (20% v/v) (pH 7.4) (0.5 ml g⁻¹ tissue). Aliquots (0.5 ml) were stored at -80 °C until use. Total protein concentration for each microsome preparation was determined using a Sigma Diagnostics Micro Protein Determination Kit (Sigma-Aldridge, Poole, UK). Mean (range) recovery of protein from male dog liver was 17.3 (15.3–18.4) mg total protein g⁻¹ liver, and from female dog liver 17.1 (13.0–23.5) mg total protein g⁻¹ liver.

Dog CYP immunoassays

Dog microsome standards and antisera for CYP3A12, CYP2C21 and CYP2B11 were obtained from Gentest (Woburn, MA, USA). There was no cross-reactivity with respect to these forms of CYP or with CYP1A1 and CYP1A2 (Gentest data). The cross-reactivity of the CYP2C21 antibody to CYP2C41 is not known. A modified version of the ELISA method supplied with the microsomes was used. Briefly, dog liver microsomes were solubilized in buffer containing 1% NaCl (w/v), 1% cholic Acid (w/v) and 1% Triton-X 100 (v/v) to appropriate concentrations. Microsome standards were added to Maxisorp ELISA plates (Fisher, Loughborough, UK) over the concentration range 1.95–1000 ng per well (10 µl). The amount of the individual dog CYPs was specified in the technical notes supplied with the microsomes. A total of 500 ng test dog liver microsomal protein per well (10 µl) were added to the same Maxisorp plate. A total of 190 µl carbonate/bicarbonate buffer (Sigma) was added to each well of the plate, and was incubated at 37 °C for 1 h. Samples were removed and each well of the plate was washed four times with 200 µl buffer (0.1% v/v Tween-20 in PBS) using a 96-well plate washer (Dynatech Laboratories, Chantilly, VA, USA). Dog CYP antisera were diluted 1:1000 in Superblock buffer (Perbio Science UK Ltd, Cheshire, UK). A total of 100 µl diluted primary antibody were added to each well and the plate was incubated at 37 °C for 1 h, and washed as described previously. Anti-rabbit IgG-HRP secondary antibody (Perbio) was diluted 1:7500 in Superblock buffer. A total of 100 µl diluted secondary antibody were added to each well and the plate was incubated at 37 °C for 1 h, and washed as described previously. A total of 100 µl Turbo-TMB were added to each well and the plate was incubated at room temperature for 10–30 min. The colour development was stopped by the addition of 100 µl 1 M sulphuric acid (Fisher). The absorbance at 450 nm of each of the reactions was read using a Victor 2 microplate reader (Perkin Elmer Life Sciences Ltd, Cambridge, UK). All data analysis was performed using Origin v6.0 software (Microcall, Norcross, GA, USA).

Preparation and quantitation of dog CYP mRNA

Total mRNA was isolated from liver using an RNAqueousTM-4PRC kit (Ambion Europe Ltd, Huntingdon, UK), treated with DNase I (Panvera, Madison, WI, USA), and reverse transcribed using GibcoTM random primers and a Gibco Superscript II kit according to the manufacturer's instructions (Invitrogen Ltd, Paisley, UK). The relative expression of mRNA for each CYP was determined using TaqMan[®] 'Real Time' PCR. TaqMan[®] primers and probes were designed using cDNA sequences for CYP2C21, CYP2C41, CYP2B11 and CYP3A12 in the public domain (Accession Nos AF049909, AF016248, M92447 and X54915), and Primer Express[®] software (PE Biosystems, CA, USA). Primer and probe sequences were (5'–3') 2B11 forward: CCTCCAGGACCCACCTT; reverse: CAAGCGTTTCCAAAGACAATG; probe: CACTCCATGACCGCCAACATCATCTG; 3A12 forward: GAATTGGCCCACTCACCTGAT; reverse: AGTGGGCAATGCCTTATTGG; probe: AAAGTCGCATCAATCTCCTCGCAGTTT; 2C21 forward: TCAGGCAGAGGC-CATTTTCC; reverse: TTTTCTCCGTTGCTGAAAACAA; probe: TTGTTGGACTGGACCA-TACAGGGATTAGGA; 2C41 forward: CCAGTGGGTTTGGCCATG; reverse: GCCTTTCCTCACACGGGAA; probe: CCCACCCACCTACCAGCTCTGCTTC. Primers and probes were supplied by PE Biosystems (Applied Biosystems, Warrington, UK) and all probes were FAM and TAMRA labelled. The Applied Biosystems TaqMan Ribosomal RNA control reagents Vic Probe literature (1999) states that the 18s rRNA primer and probe sets are conserved between man, rat mouse, *Xenopus*, *Saccharomyces* and *Giardia*. We hypothesized they would be conserved with dog 18s rRNA too. We tested the primers against dog cDNA and found they amplified cDNA linearly over a 200-fold range. TaqMan PCR reactions were not duplexed.

cDNA was amplified by a TaqMan PCR reaction in a 25- μ l volume using TaqMan universal PCR master mix (PE Biosystems), F and R primer (300 nM), and probe (50 nM). 18s rRNA normalization reactions were performed using TaqMan ribosomal RNA control reagents and 200 ng cDNA in a 25- μ l volume. 18s and CYP reactions were performed in separate wells but on the same plate using the same stocks of cDNA. Amplifications were carried out using an ABI PRISM 770D Sequence Detection System and by standard methods supplied by PE Biosystems. Primers for 18s rRNA were used as an endogenous normalization control as recommended by the PE Biosystems' technical literature. The relative expression of each mRNA species was determined using standard curves generated for each CYP primer and probe set and using cDNA prepared from the total mRNA of dog. All data were analysed using Microsoft Excel[®].

CYP2C21 and CYP2C41 were also amplified by high-fidelity standard PCR using an Advantage-HF2 PCR Kit (BD Biosciences Clontech, Cowley, UK). PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK), and then cut with Nde I or Bbs I restriction endonucleases (New England BioLabs, Hitchin, UK). The primer design, amplification conditions and analysis were as described by Blaisdell *et al.* (1998). The restriction enzyme cut PCR products were analysed by agarose gel electrophoresis. Standard PCR, coupled with restriction enzyme analysis, was performed before TaqMan PCR as a simple, cost-effective way to assess whether the liver of the Alderley Park beagle dog was polymorphic in its expression of *CYP2C41* mRNA. Following confirmation of expression of *CYP2C41* mRNA, we chose to quantify the expression by TaqMan PCR.

Microsomal incubation conditions

The incubation mixture comprised substrate (in 50 μ l 0.2 M potassium phosphate, pH 7.4), incubation buffer (0.2 M potassium phosphate buffer, pH 7.4, 100 μ l), an NADPH-generating system consisting of 4 μ mole glucose 6-phosphate, 0.4 μ mole NADP, 0.4 Units glucose 6-phosphate dehydrogenase and 2 μ mole MgCl₂ dissolved in incubation buffer (50 μ l), and the microsomal suspension (50 μ l containing 0.25–0.6 mg protein), in a final volume of 250 μ l. The reaction was started by the addition of microsomes. Incubations were carried out at 37 °C in a shaking water bath. For enzyme kinetic studies, incubations were performed under conditions of linearity with respect to time and protein concentration. Enzyme kinetic data were obtained in microsomes from four to six livers. Except for incubations with omeprazole, the reaction was stopped by the addition of 25 μ l 60% (w/v) perchloric acid. After addition of internal standard (laudanosine (125 ng) for the analysis of 3-methoxymorphinan, 7-ethoxycoumarin (0.68 ng) for that of 7-hydroxywarfarin, and phenobarbitone (125 ng) for that of 4'-hydroxymephenytoin), the mixture was centrifuged (9000g for 5 min), and an aliquot of the supernatant was injected onto the hplc. Following incubation of microsomes with omeprazole, the reaction was stopped with 5 ml methyl *t*-butylether and chlorzoxazone (300 ng) was added as internal standard. The mixture was vortexed for 1 min and centrifuged at 2000g for 5 min. The upper organic layer was evaporated to dryness and the residue reconstituted in 200 μ l mobile phase, an aliquot of which was injected onto the hplc.

Hplc analysis of metabolites

3-Methoxymorphinan. A RCM CN reverse-phase column (10 cm $\times$ 5 mm i.d.) fitted with a CN guard column (Waters Ltd, Watford, UK) was used. The eluent was passed through a Model 820FP fluorescence detector (Jasco, Great Dunmow, UK) with excitation and emission wavelengths set at 270 and 312 nm, respectively. The mobile phase was acetonitrile:water (10:90 v/v) containing 0.025% (v/v) triethylamine and adjusted to pH 3 with orthophosphoric acid (70% w/v). Chromatography was performed at ambient temperature and at a flow rate of 3 ml min⁻¹. The intra- and interassay coefficients of variation at a concentration of 0.8 nmol ml⁻¹ were 3.6% ($n=6$) and 10.5% (12), respectively. The lowest detectable rate of 3-methoxymorphinan formation was 30 pmol mg⁻¹ protein min⁻¹.

7-Hydroxywarfarin. A RCM C₁₈ reverse-phase column (10 cm $\times$ 5 mm i.d.) fitted with a C₁₈ guard column was used. The eluent was passed through a Model 820FP fluorescence detector with excitation and emission wavelengths set at 320 and 415 nm, respectively. The mobile phase was acetonitrile:0.5% orthophosphoric acid in water (40:60 v/v). Chromatography was performed at ambient temperature and at a flow rate of 2 ml min⁻¹. The interassay coefficient of variation at a concentration of 3.25 pmol ml⁻¹ was 3.3% ($n=8$). The lowest detectable rate of 7-hydroxywarfarin formation was 5.1 fmol mg⁻¹ protein min⁻¹.

4'-Hydroxymephenytoin. A RCM C₁₈ reverse-phase column (10 cm $\times$ 5 mm i.d.) fitted with a C₁₈ guard column was used. The eluent was passed through a Spectroflow 757 UV detector with the wavelength set at 224 nm. The mobile phase was acetonitrile:water (17:83 v/v). Chromatography was performed at

ambient temperature and at a flow rate of 2 ml min^{-1} . The interassay coefficient of variation at a concentration of 0.1 nmol ml^{-1} was 8.2% ($n = 14$). The lowest detectable rate of 4'-hydroxymephenytoin formation was $10 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$.

5'-Hydroxyomeprazole. The column and detector were the same as those for 4'-hydroxymephenytoin. The wavelength was set at 230 nm. The mobile phase was methanol:acetonitrile:water (33:9:58 v/v) containing 0.055% (v/v) triethylamine adjusted to pH 7 with orthophosphoric acid (70% v/v). Chromatography was performed at ambient temperature and at a flow rate of 1 ml min^{-1} . The interassay coefficient of variation at a concentration of 0.2 nmol ml^{-1} was 15% ($n = 14$). The lowest detectable rate of 5'-hydroxyomeprazole formation was $5 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$.

Data analysis

Analysis of the kinetics of metabolite formation was performed using Microsoft Excel[®] Solver, an iterative non-linear least-squares regression analysis, with a weighting of $1/(\text{rate})^2$ and based on initial estimates of K_m and $V_{\max}$ obtained from an Eadie-Hofstee plot. All combinations of one- and two-site models incorporating the Michaelis-Menten equation:

$$v = \frac{V_{\max} S^n}{K_{0.5}^n + S^n}$$

where v is the velocity of the reaction, $V_{\max}$ is the maximum velocity of the reaction, K_m is the substrate concentration at which $v = V_{\max}/2$, and S is the substrate concentration, and/or the Hill equation (Cornish-Bowden 1995):

$$v = \frac{V_m S}{K_m + S}$$

where n is the Hill coefficient and $K_{0.5}^n$ is the apparent Michaelis-Menten constant, were fitted to the data. Goodness of fit in models containing increasing number of parameters were compared by applying the Akaike Information Criteria (AIC) (Yamaoka *et al.* 1978).

Data from male and female dogs were compared using a two-tailed Student's *t*-test, with $p < 0.05$ being accepted as statistically significant. The rates of individual metabolic reactions were correlated with CYP2C21, CYP2C41, CYP2B11 and CYP3A12 expression in the livers from 12 dogs using the Spearman rank test.

Results

CYP mRNA expression

The expression of *CYP3A12*, *CYP2B11*, *CYP2C21* and *CYP2C41* mRNA and that of CYP3A12, CYP2B11 and CYP2C21/2C41 proteins in the livers of 12 dogs is shown in figure 1. All livers showed expression of the first three forms of CYP mRNA, with no sex differences being found. Expression of *CYP2C41* mRNA was undetectable in the livers of six of 11 dogs. However, *CYP2C41* mRNA was expressed in both sexes.

Dextromethorphan N-demethylation

Microsomes from all 12 dogs (six male) N-demethylated dextromethorphan to form 3-methoxymorphinan. No metabolite was formed when substrate was incubated with heat-inactivated microsomes. The kinetics of this reaction were then investigated over a wide range of substrate concentrations. In microsomes from five of the six livers studied, the data were best described by two Michaelis-Menten functions representing one high-affinity, low-capacity site and one low-affinity, high-capacity site (figure 2). In microsomes from the sixth liver (male), atypical curvature was observed at high substrate concentrations, and the most parsimonious model was that incorporating one Michaelis-Menten and one Hill function. Values for the kinetic parameters are shown in table 1. At a substrate

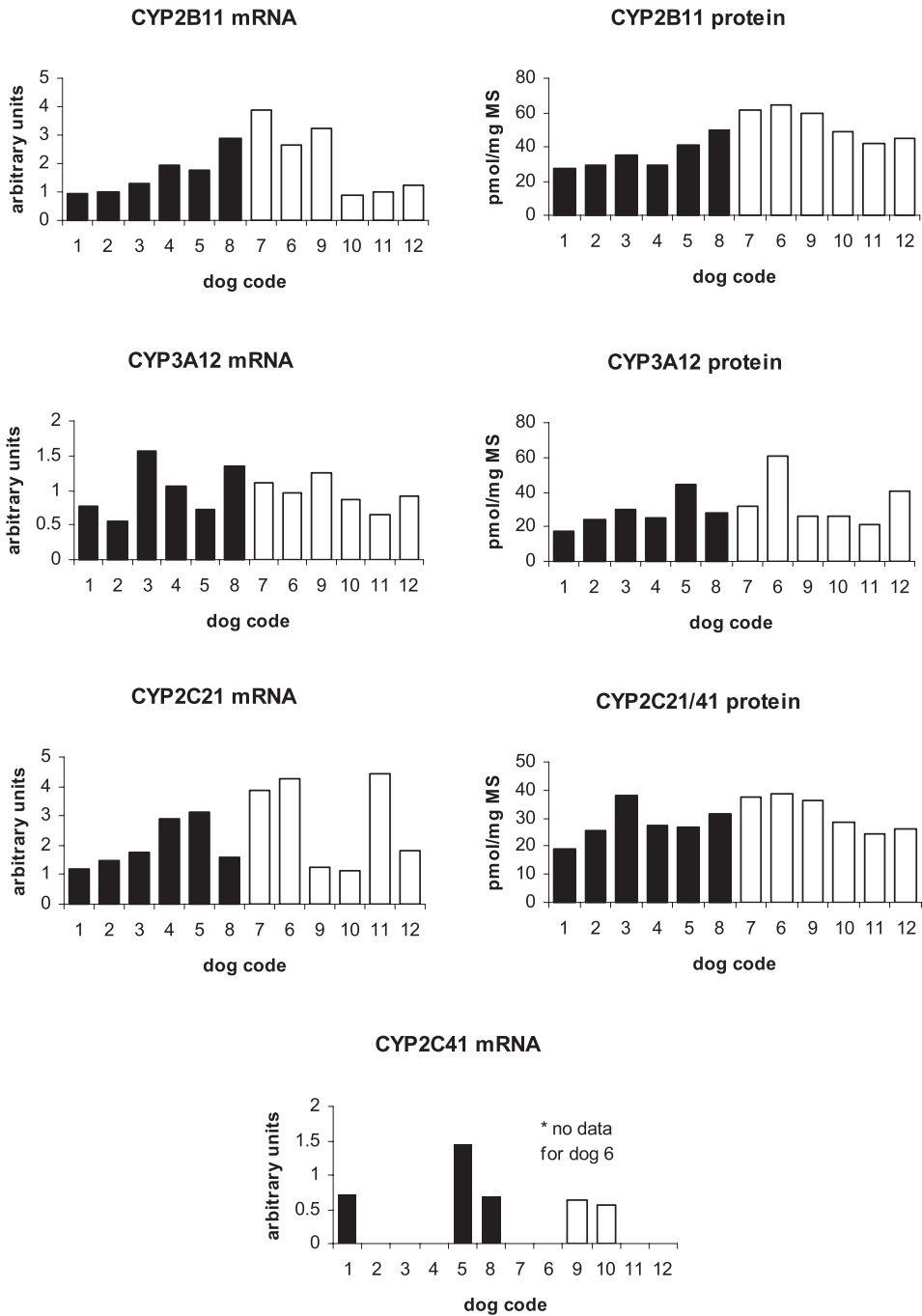


Figure 1. Expression of CYP mRNA and protein in the livers of the 12 dogs tested. The antibody to CYP2C did not discriminate between CYP2C21 and CYP2C41. MS, microsomal protein. The expression of dog CYP mRNA was normalized by calculating the amplified equivalents (ng) of cDNA for 18S rRNA and each respective CYP mRNA, and then the ratio of CYP/18S RNA. The nanogram units cancel out and thus the ratio is in arbitrary units (AU). This method is described as the Standard Curve Method in Applied Biosystems User Bulletin #2. Closed panels, males; open panels, females.

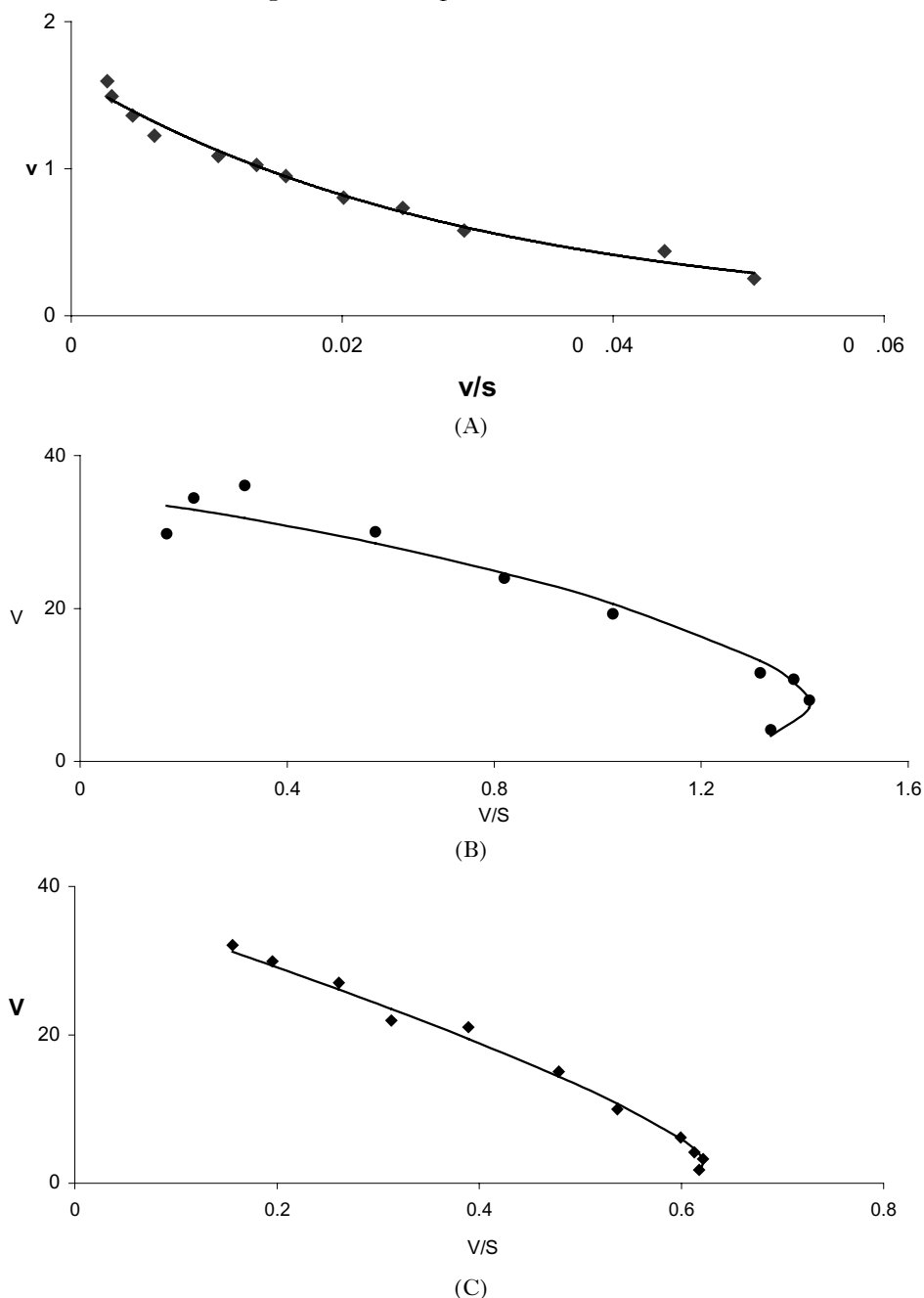


Figure 2. Representative Eadie-Hofstee plots of (A) the N-demethylation of dextromethorphan (substrate concentration range 5–1500 μM , incubation time 10 min, protein concentration 0.25 mg ml^{-1}) by dog liver microsomes, (B) the 4'-hydroxylation of *R*-mephenytoin (1–200 μM , 30 min, 0.5 mg ml^{-1}) and (C) the 5'-hydroxylation of omeprazole (2.5–200 μM , 30 min, 0.6 mg ml^{-1}). Data points are the average of two estimations and each plot contains data from a single dog. Lines of best fit of the various models to the data are shown. Data are best described by (A) two Michaelis-Menten functions, and (B) and (C) single Hill functions.

Table 1. Enzyme kinetic data for the metabolism of dextromethorphan, *R*-mephenytoin and omeprazole by dog liver microsomes.

	Site 1		Site 2	
	K_m or $K_{0.5}$ (μM)	$V_{\max}$ ($\text{pmol mg}^{-1} \text{protein min}^{-1}$)	K_m (μM)	$V_{\max}$ ($\text{pmol mg}^{-1} \text{protein min}^{-1}$)
Dextromethorphan	15.7 ± 7.69	847 ± 551	720.4 ± 1028.6	2290 ± 1804
N-demethylation				
<i>R</i> -Mephenytoin	18.4 ± 3.19	43.7 ± 10.8		
4'-hydroxylation				
Omeprazole	50.5 ± 9.7	33.2 ± 10.9		
5'-hydroxylation				

Site 1, high affinity, low capacity; site 2, low affinity, high capacity.
Data are the mean $\pm$ SD from four to six livers (comprising equal numbers of male and female dogs).

concentration of $16 \mu\text{M}$ (mean K_m), the relative contribution of the high-affinity site to total metabolite formation was 89%.

At a substrate concentration of $20 \mu\text{M}$ (within the range of the high-affinity K_m 's) the rate of dextromethorphan N-demethylation by microsomes from the six female dogs (0.76 ± 0.14 (SD) $\text{nmol mg}^{-1} \text{protein min}^{-1}$) was significantly greater than that in the six male dog microsomes ($0.55 \pm 0.15 \text{ nmol mg}^{-1} \text{protein min}^{-1}$) ($p < 0.02$). The rate of N-demethylation varied two- to threefold ($0.39\text{--}0.93 \text{ nmol mg}^{-1} \text{protein min}^{-1}$) between the livers.

S-warfarin 7-hydroxylation

The assay used detected the dog liver microsomal formation of 7-hydroxy-warfarin from *S*-warfarin. However, the amount of metabolite generated was extremely small and only about double the lower limit of determination of the assay ($5.1 \text{ fmol mg}^{-1} \text{protein min}^{-1}$). At a substrate concentration of $4 \mu\text{M}$ the mean ($\pm$ SD) rate of metabolite formation was $9.25 \pm 1.62 \text{ fmol mg}^{-1} \text{protein min}^{-1}$ in male dog liver microsomes and $12.3 \pm 2.1 \text{ fmol mg}^{-1} \text{protein min}^{-1}$ in female microsomes compared with $787 \text{ fmol mg}^{-1} \text{protein min}^{-1}$ in the single human liver tested as a positive control. Although the absolute rates of this reaction were low in both female and male dog liver microsomes, the sex difference was statistically significant. ($p < 0.02$).

R- and S-mephenytoin 4'-hydroxylation

Microsomes from all 12 dog livers catalysed the 4'-hydroxylation of both *R*- and *S*-mephenytoin. The reaction was stereoselective and the mean rate ($\pm$ SD) of 4'-hydroxylation of *R*-mephenytoin was $20 (\pm 6.5) \text{ pmol mg}^{-1} \text{protein min}^{-1}$ and that of *S*-mephenytoin $4.6 (\pm 1.5) \text{ pmol mg}^{-1} \text{protein min}^{-1}$ at a substrate concentration of $20 \mu\text{M}$. The amount of metabolite formed from *S*-mephenytoin was only about double the lower limit of determination of the assay. Accordingly, subsequent kinetic studies were only performed using the *R*-isomer, with microsomes from four dog livers (two male).

In microsomes from two (one male) of the dogs studied (figure 2), the data were best described by a single site Michaelis–Menten model. However, a single Hill

function best fitted the data from the two (one male) other dogs studied. Values for $V_{\max}$, K_m , $K_{0.5}$ and n are shown in table 1. At a substrate concentration of 20 μM , the mean rate of *R*-mephenytoin 4'-hydroxylation by liver microsomes from female dogs ($n=6$) ($24.6 \pm 1.1 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$) was significantly higher than that from male dog microsomes ($n=6$) ($15.4 \pm 3.2 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$) ($p < 0.01$). *R*-mephenytoin 4'-hydroxylase varied about threefold ($11.6\text{--}34.1 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) in the 12 livers studied.

Omeprazole 5'-hydroxylation

Microsomes from all 12 livers catalysed the 5'-hydroxylation of omeprazole. Like *R*-mephenytoin, the enzyme kinetic profile differed between livers. Thus, the kinetics of the reaction were best described by a single site Michaelis–Menten model in one (female) dog, but by a single-site Hill function in three (two male) dogs (figure 2). Values for $V_{\max}$, K_m , $K_{0.5}$ and n are shown in table 1. At a substrate concentration of 50 μM , the mean rates of omeprazole 5'-hydroxylation by microsomes from male ($n=6$) ($19.1 \pm 3.7 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$) and female livers ($n=6$) ($16.6 \pm 2.3 \text{ pmol mg}^{-1} \text{ protein min}^{-1}$) were not significantly different. Omeprazole 5'-hydroxylation varied about twofold ($13\text{--}23 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) in the 12 livers studied.

Correlations

Significant correlations between CYP2B11 mRNA and protein expression, and dextromethorphan *N*-demethylase and *R*-mephenytoin 4'-hydroxylase activity and were observed (table 2). There were also significant although less strong correlations between warfarin 7'-hydroxylase activity and the expression of CYP2B11 and CYP3A12 proteins. No other statistically significant correlations were found.

Discussion

The activity of the cytochrome P450 enzyme system in the livers of the dogs studied was evaluated first by monitoring the *N*-demethylation of dextromethorphan. This reaction, catalysed in humans by CYP3A (Schnider *et al.* 1997b), was

Table 2. Spearman rank correlation coefficients and p values for the relationships between enzyme activities and CYP mRNA and protein expression in the 12 dog livers studied.

	CYP2B11		CYP2C21		CYP2C41	CYP3A12	
	mRNA	protein	mRNA	protein	mRNA	mRNA	protein
Dextromethorphan <i>N</i> -demethylation	0.718 $p < 0.01$	0.921 $p < 0.001$	0.305	0.609	−0.094	0.532	0.441
<i>S</i> -warfarin 7-hydroxylation	0.420	0.685 $p < 0.05$	0.196	0.308	0.224	0.175	0.594 $p < 0.05$
<i>R</i> -mephenytoin 4-hydroxylation	0.364	0.734 $p > 0.01$	0.245	0.538	−0.320	0.175	0.252
Omeprazole 5-hydroxylation	0.028	−0.196	0.357	0.259	−0.085	−0.042	0.266

Only $p < 0.05$ are shown.

used as a positive control, since dog liver has been shown to metabolize other known substrates of human CYP3A (Sharer *et al.* 1995). All livers were catalytically active. The two-site kinetic profile seen in all the six (three male) livers studied is consistent with contributions from one enzyme with high affinity and low capacity for dextromethorphan and one with low affinity and high capacity. Only one site has been identified for the N-demethylation of dextromethorphan by human liver microsomes (von Moltke *et al.* 1988) with a K_m (259 μ M) comparable with the low-affinity site in dog liver.

One of the main aims of this study was to extend knowledge of the metabolism of human CYP2C substrates by dog liver. Clinically, warfarin is probably the most important CYP2C9 substrate, and the 7-hydroxylation of its *S*-enantiomer is catalysed by this enzyme (Yamazaki and Shimada 1997). Our data indicate that the dog is profoundly deficient in its ability to catalyse this reaction, the rate of metabolite formation being approximately 1.5–2 orders of magnitude less than that seen in human liver by us and Yamazaki and Shimada (1997). The metabolism of tolbutamide, another important human CYP2C9 substrate, is also impaired in dog compared with human liver, although the difference (sixfold) (Chauret *et al.* 1997) is not as great as for warfarin. These findings raise a serious concern over the use of the dog in the pharmacological and toxicological testing of human CYP2C9 substrates, of which there are an emerging number. Another factor that needs to be considered is that different human CYP2C9 substrates may be metabolized by different forms of CYP in the dog. For example, the rate of 4'-hydroxylation of phenytoin, a third CYP2C9 substrate, is actually greater in dog compared with human liver (Shimada *et al.* 1997). However, in humans, CYP2C19 is also involved to a small extent (Yasumori *et al.* 1999), and thus the dog orthologue of this enzyme may be responsible for metabolizing phenytoin.

The data on species differences in the 4'-hydroxylation of *S*-mephenytoin, catalysed in humans by CYP2C19, are conflicting. Sharer *et al.* (1995), Shimada *et al.* (1997) and Chauret *et al.* (1997) reported no difference in 4'-hydroxylase activity between dog and human liver. In contrast, Yasumori *et al.* (1993) reported a very low rate for this reaction in dog liver. Our data supported the results of the latter study, which adds further evidence that the dog is impaired in its ability to metabolize some substrates of the human CYP2C subfamily. This finding for *S*-mephenytoin raises the same concerns as for CYP2C9, namely in the use of the dog for the pharmacological and toxicological testing of human CYP2C19 substrates.

In contrast, the rate of 4'-hydroxylation of *R*-mephenytoin by dog liver microsomes was much greater. The kinetic data were consistent with a single enzyme contributing to the reaction. Whether or not the Hill behaviour seen in two of the livers is real or artefactual requires further study. Our data confirm that the 4'-hydroxylation of mephenytoin is stereoselective in the dog with metabolism favouring the *R*-enantiomer (Yasumori *et al.* 1993). This pattern of stereoselectivity is opposite to that seen in humans, where 4'-hydroxylation of the *S*-enantiomer predominates (Wilkinson *et al.* 1989).

Probably the most important substrate of CYP2C19 is omeprazole, a drug widely used to treat gastrointestinal disorders. 5'-Hydroxylation of the drug is catalysed by this enzyme in humans (Andersson *et al.* 1993), but the reaction has not been studied in dog liver. In contrast to the 4'-hydroxylation of *S*-mephenytoin, dog liver microsomes catalysed the production of 5'-hydroxyomeprazole at readily measurable rates, albeit at well over an order of magnitude lower rate than

in human liver (Andersson *et al.* 1993). Visual inspection of Eadie-Hofstee plots of the reaction indicate that it appeared to be catalysed by a single form of CYP.

For all substrates except omeprazole, a significant sex difference in their metabolism by dog livers was found, females showing a greater metabolic capacity than males. Sex differences in human liver CYP2C activity have not been reported. Clearly, when screening drugs during development, equal numbers of male and female dogs should be used, and any sex difference characterized.

The relatively small extent of variation (two- to threefold) between dog livers in the rates of metabolism of the substrates tested is not unexpected, since animals were bred and housed in controlled laboratory conditions. Greater interindividual variability has been reported in the activities of the CYP2C subfamily in humans (Shimada *et al.* 1994).

We were able to quantify the expression of mRNA from *CYP2B11*, *CYP2C21*, *CYP2C41* and *CYP3A12*, as well as that of protein from CYP2B11, CYP2C21/41 and CYP3A12. There were no sex differences in expression of mRNA or protein with respect to any of these CYP enzymes. One striking finding from these data was that six of the 11 dogs studied did not display significant expression of hepatic *CYP2C41* mRNA. This polymorphic pattern confirms the work of Blaisdell *et al.* (1998) who showed that 21 of 25 dogs studied did not express mRNA for this CYP form. This apparent difference in the proportion of 'non-expressors' should not be over interpreted, since the number are too small to define the exact prevalence of each phenotype in population terms.

A logical extension of this work was to define which if any of these forms of dog CYP catalysed the metabolism of the human, mainly CYP2C substrates that were tested. Recombinant forms of these dog CYPs were not available for this purpose. Chemical inhibitors used to define the role of human forms of CYP in metabolism have not been validated in the dog. However, another approach to defining the involvement of individual enzymes is to perform correlation analysis. The highest correlations recorded were those between the high-affinity site of dextromethorphan N-demethylation and both CYP2B11 mRNA and protein expression, providing indirect evidence that CYP2B11 catalyses this pathway at low substrate concentrations. By implication, the low-affinity site may be mediated by CYP3A12, since in humans CYP3A mainly catalyses this reaction at comparable substrate concentrations (Schmider *et al.* 1997). Another highly significant correlation was that between the rate of *R*-mephenytoin 4'-hydroxylation and CYP2B11 protein expression. This is in agreement with findings that in humans this reaction is partially catalysed by CYP2B6 as well as by CYP3A (D. A. Flockhart, personal communication). That these correlations were so statistically significant is particularly striking given the small number of dogs studied and the relatively limited interindividual variability in enzyme expression and activity. The rate of omeprazole 5'-hydroxylation catalysed by CYP2C19 in humans did not correlate with the expression of CYP2B11, 2C21, 2C41 or 2A12. *S*-mephenytoin 4'-hydroxylation is also catalysed by CYP2C19 in humans, but the finding that the rate of metabolite formation in dog liver is very much less than that of 5'-hydroxyomeprazole suggests that different CYPs are responsible for these reactions in the latter species. Although warfarin 7-hydroxylase activity was extremely low in dog liver, it correlated significantly with CYP2B11 and CYP3A12 expression, suggesting that these enzymes rather than CYP2C21/41 contribute to the reaction in the dog.

In conclusion, we have identified a distinct pattern of hepatic expression of the *CYP2C41* gene in the Alderley Park beagle dog. Furthermore, marked differences in the metabolism of human CYP2C substrates were observed in this dog strain compared with humans with respect to rate of reaction, stereoselectivity and CYP selectivity.

Acknowledgements

This study was partly funded by a grant from AstraZeneca.

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Homology modelling of human CYP1A2 based on the CYP2C5 crystallographic template structure

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Received 1 July 2002

1. The results of homology modelling of human cytochrome P4501A2 (CYP1A2) based on the CYP2C5 crystal structure are reported. It exhibits improved sequence homology relative to that of CYP102.

2. It was demonstrated that many selective substrates for CYP1A2 could fit within the putative active site of the enzyme, and in orientations which agree with documented evidence for CYP1A2-mediated metabolism.

3. Furthermore, a number of amino acid residues lining the haem pocket have been shown, via site-directed mutagenesis, to have an influence on substrate metabolism, and these experimental findings from the literature are consistent with the modelled interactions for selective substrates.

4. The binding affinities of several CYP1A2 substrates have also been calculated from the CYP1A2 active site interactions and they agree closely with experimental values.

Introduction

Enzymes of the cytochrome P450 (CYP) superfamily of haem-thiolate proteins are involved in the Phase 1 metabolism of a vast number and variety of organic chemicals, both endogenous and exogenous (Lewis 2001). In *Homo sapiens*, it is known that cytochrome P450s of the CYP1, CYP2 and CYP3 families are associated with the oxidation of many drugs and other xenobiotics (Rendic and DiCarlo 1997, Anzenbacher and Anzenbacherova 2001). Of these, the CYP1 family enzymes (CYP1A1, CYP1A2 and CYP1B1) mediate over 10% of all Phase 1 drug oxidations, with CYP1A2 representing the most important enzyme of this family for the oxidative metabolism of exogenous compounds in human liver (Eaton *et al.* 1995, Hammons *et al.* 1997, Rendic and DiCarlo 1997). However, CYP1A2 can also catalyse 2-hydroxylation of the endogenous steroid oestradiol, whereas CYP1B1 preferentially mediates 4-hydroxylation of oestradiol (Kerlan *et al.* 1992), which is an activating pathway as opposed to the former route of metabolism. Selective marker substrates of CYP1A2 include: caffeine, phenacetin and 7-methoxyresorufin (Rendic and DiCarlo 1997), where the former has been employed in an important *in vivo* study (Horn *et al.* 1995) showing that the modulation in CYP1A2 levels may be linked with the oestrus cycle. More recently,

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Table 1. A selection of substrates for human CYP1A2 showing their metabolic characteristics.

Compound	Route of metabolism	K_m (μ M)	Reference
1. Caffeine	N-3 demethylation	180	Tassaneeyakul <i>et al.</i> (1992)
2. 7-Methoxyresorufin	O-demethylation	0.21	Turesky <i>et al.</i> (1998)
3. Phenacetin	O-deethylation	48	Parikh <i>et al.</i> (1999), Tassaneeyakul <i>et al.</i> (1993)
4. MeIQ	N-hydroxylation	13	Parikh <i>et al.</i> (1999)
5. Aflatoxin B ₁	8,9-epoxidation	31	Gallagher <i>et al.</i> (1996)
6. Tacrine	7-hydroxylation	14	Becquemont <i>et al.</i> (1998)
7. Naproxen	O-demethylation	160	Rodrigues (1999)
8. Oestradiol	2-hydroxylation	20	Kerlan <i>et al.</i> (1992), Yamazaki <i>et al.</i> (1998)
9. Melatonin	6-hydroxylation	85.5	Facciola <i>et al.</i> (2001)
10. Furafylline ^a	8-methyl hydroxylation	3 (K_i)	Clarke <i>et al.</i> (1994)
11. PhIP	N-hydroxylation	55	Zhao <i>et al.</i> (1994)
12. IQ	N-hydroxylation	33	Hammons <i>et al.</i> (1997)

^a Mechanism-based selective inhibitor of CYP1A2.

PhIP, 2-amino-1-ethyl-6-phenylimidazo[4,5-*b*]pyridine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 4-methyl IQ; K_m , Michaelis constant (μ M) for substrate metabolism.

human CYP1A1 and CYP1A2 have been reported to mediate in the 3,4-epoxidation of coumarin (Born *et al.* 2002), which is generally regarded as an metabolic activation pathway. Table 1 provides a summary of typical CYP1A2 substrates that have been investigated in this current study. Known inhibitors of human CYP1A2 include furafylline, fluvoxamine and α -naphthoflavone (Sesardic *et al.* 1990, Fuhr *et al.* 1993, Kunze and Trager 1993, Clarke *et al.* 1994, Pelkonen *et al.* 1998, Zhai *et al.* 1998, Rodrigues 1999) with the former mechanism-based inhibitor constituting a substrate analogue, and bearing structural similarities with both caffeine and theophylline. We and others have reported previously (Lewis and Lake 1996, Lozano *et al.* 1997, Lewis *et al.* 1999) that substrates and inhibitors fit into the active site of a CYP1A2 model derived from CYP102, and the present study aims to develop improved models for CYP1A enzymes based on the recently available mammalian P450 crystal structure (Williams *et al.* 2000) as this template shows greater sequence identity (30%) than that exhibited with CYP102 (24%).

Materials and methods

Using the CYP2C5 crystal structure (pdb code: 1dt6) as a template, a model of human CYP1A2 was generated via protein sequence homology following a satisfactory alignment of the two protein sequences (figure 1). In figure 1, we have aligned several CYP1 family sequences with that of CYP2C5 as this enables the pooling of information from residue modification. Some residues in the CYP1 family have been investigated using site-directed mutagenesis and other site-specific probes, such that there is some information regarding likely residues involved in substrate binding and redox partner interaction, and these are summarized in table 2. The amino acid sequence homology between human CYP1A2 and rabbit CYP2C5 was 30% for the matched residues in the alignment and, moreover, construction of the CYP1A2 model required very few amino acid insertions, all of which involved only small portions of peptide 1–3 residues in length, and these were added via loop-searching of the protein databank. There were key residue differences within the putative active site which, presumably, account for the altered substrate selectivity between the two enzymes. For example, the sequence identities for substrate recognition sites (SRS) 1, 2 and 6 are 16, 8 and 10%, respectively. However, for SRS4 the sequence identity is 50%, and for SRS5 the sequence identity is 40%; both of which are higher than the total identity of the two enzymes. It should be noted, however, that the SRS regions have been reported (Gotoh 1992) from sequence analysis of CYP2 family proteins and, therefore, their location in the CYP1 family has been inferred from alignment of the two families. The raw model was then energy minimized

	membrane anchor region 10				20
cyp2c5	MDP		VVVLVLGLCC	LLLLS.IWKQ	NSGR.G.KL.
cyplalhum	MLFP	I...SMSATE	FLLASVIFCL	VFWVIRASRP	QVPK.GLKN.
cyplalmou	MYGLP	A...FVSATE	LLLAVTVFCL	GFWVVRATRT	WVPK.GLKT.
cyplalrab	..MVSDFGLP	T...FISATE	LLLASAVFCL	VFWVAGASKP	RVPK.GLKR.
cyplalrat	..MPSVYGFP	A...FTSATE	LLLAVTTFCL	GFWVVRVTRT	WVPK.GLKS.
cyplaltro	...MVLMLIP	I.IGSVSASE	GLVAMVTLCL	VYMIMKYMHT	EIPE.GLKR.
cypla2ham	MALSQY	T...SLS.TE	LVLATAIFCI	VFWVARALRT	QVPK.GLKT.
cypla2hum	MALSQS	V...PFSATE	LLLASAIFCL	VFWVLKGLRP	RVPK.GLKS.
cypla2mou	MAFSQY	I...SLA.PE	LLLATAIFCL	VFWMVRASRT	QVPK.GLKN.
cypla2rab	AMSPA	A...PLSVTE	LLLVSAVFCL	VFWAVRASRP	KVPK.GLKR.
cypla2rat	MAFSQY	I...SLA.PE	LLLATAIFCL	VFWVLRGTRT	QVPK.GLKS.
cypla6pla	..MMLMLP	F.IGSVSVSE	SLVAMTTMCL	VYLILKFFQT	EIPE.GLRR.
cyplblhum	MGTSLSPNDP	WPLNPLSIQQ	TLLLLLSVL	ATVHVQORLL	RQRRRLRSA

	30	40	50	60	70
cyp2c5	PPGPTPFPII	GNILQIDAKD	ISKSLTKFSE	CYGPVFTVYL	GMKPTVVVLHG
cyplalhum	PPGPWGWLPI	GHMLTLG.KN	PHLALSRMSQ	QYGDVLQIRI	GSTPVVVLSSG
cyplalmou	PPGPWGLPFI	GHMLTVG.KN	PHLSLTRLSQ	QYGDVLQIRI	GSTPVVVLSSG
cyplalrab	LPGPWGWPLL	GHVLTG.KN	PHVALARLSR	RYGDVFQIRL	GSTPVVVLSSG
cyplalrat	PPGPWGLPFI	GHVLTG.KN	PHLSLTKLSQ	QYGDVLQIRI	GSTPVVVLSSG
cyplaltro	LPGPKPLPII	GNVLEVH.NN	PHLSLTAMSE	RYGSVFQIQI	GMRPVVVLSSG
cypla2ham	PPGPWGLPFL	GMCLTLG.KN	PHLSLTKLSK	QYGDVLQIRI	GSTPVVVLSSG
cypla2hum	PPEPWGWPLL	GHVLTG.KN	PHLALSRMSQ	RYGDVLQIRI	GSTPVLVLSR
cypla2mou	PPGPWGLPFI	GHMLTVG.KN	PHLSLTRLSQ	QYGDVLQIRI	GSTPVVVLSSG
cypla2rab	LPGPWGWPLL	GHLLTLG.KN	PHVALARLSR	RYGDVFQIRL	GSTPVVVLSSG
cypla2rat	PPGPWGLPFI	GHMLTLG.KN	PHLSLTKLSQ	QYGDVLQIRI	GSTPVVVLSSG
cypla6pla	LPGPKPLPII	GNVLGLG.SK	PYLSLTAMSK	RYGHVFQIQI	GMRPVVVLSSG
cyplblhum	PPGPFAWPLI	GHAAAVG.QA	AHLSFARLAR	RYGDVFQIRL	GSCPIVVVLNG

	80	90	100	SRS1	110	120
cyp2c5	YEAVKEALVD	LGEEFAGTGS	VPILEKVSQG	LGIAFS..NA	KTWKEMRRFS	
cyplalhum	LDTIRQALVR	QGDDFKGRPD	LYTFTLISNG	QSMSFSPDSG	PVWAARRRLA	
cyplalmou	LNTIKQALVR	QGDDFKGRPD	LYSFTLITNG	KSMTFNPDSG	PVWAARRRLA	
cyplalrab	LDTIKQALVR	QGDDFKGRPD	LYSFSEVTKG	QSMIFGSDSG	PVWAARRRLA	
cyplalrat	LNTIKQALVK	QGDDFKGRPD	LYSFTLIANG	QSMTFNPDSG	PLWAARRRLA	
cyplaltro	SETVRQALIK	QGEDFAGRPD	LYSEKFINDG	KSLAFSTDKA	GVWRARRKLA	
cypla2ham	LDTIRQALVR	QGDDFKGRPD	LYSFTLITNG	KSMTFNPDCG	PVWAARRRLA	
cypla2hum	LDTIRQALVR	QGDDFKGRPD	LYTSTLITDG	QSLTFSTDG	PVWAARRRLA	
cypla2mou	LNTIKQALVR	QGDDFKGRPD	LYSFTLITNG	KSMTFNPDSG	PVWAARRRLA	
cypla2rab	LDTIKQALVR	QGDDFKGRPD	LYSSSFITEG	QSMTFSPDSG	PVWAARRRLA	
cypla2rat	LNTIKQALVK	QGDDFKGRPD	LYSFTLITNG	KSMTFNPDSG	PVWAARRHLA	
cypla6pla	TGTVRQALIK	QGDEFAGRPD	LYSFRFINAG	KSLAFSTDQA	GVWRARRKLA	
cyplblhum	ERAIHQALVQ	QGSADFADRA	FASFRVVSQG	RSMAGHYSE	H.WKVQRRRA	

Figure 1. Multiple sequence alignment between CYP2C5 and a number of CYP1 family proteins. Sequences from different species have been indicated as follows: hum, human; mou, mouse; tro, trout; ham, hamster; rab, rabbit; pla, plaice. The numbering system used is that of the CYP2C5 sequence and substrate recognition sites (Gotoh 1992) are shown as shaded boxes. The membrane anchor peptide comprising the first 20 residues is shown as an open box. Residues that have been the subject of site-directed mutagenesis experiments, photoaffinity labelling or are associated with allelic variants of CYP1 enzymes have been emboldened. (Continued)

	130	140	150	160
cyp2c5	LMTLRNFGMG	..KRS....	IEDRIQEEAR	CLVEELRKTN ASP..CDPTF
cypla1hum	QNLKSFSIA	SDPASSTSCY	LEEHVSKEAE	VLISTLQELM AGPGHFNFPYR
cypla1mou	QNALKSFSIA	SDPTSASSCY	LEEHVSKEAN	YLVSKLQKVM AEVGHFDPYK
cypla1rab	QNALNSFSVA	SDPASSSSCY	LEEHVSQEA	NLISKFQELM AAVGHFDPYR
cypla1rat	QNALKSFSIA	SDPTLASSCY	LEEHVSKEAE	YLISKFQKLM AEVGHFDPFK
cypla1tro	MSALRSFATL	EGTTP EYSCA	LEEHVCKEGE	YLVKQLTSVM DVSGSFDPFR
cypla2ham	QDALKSFSIA	SDPTSASSCY	LEDHVIKEAN	HLVSKLQKLT AEVGHFEPVN
cypla2hum	QNALNTFSIA	SDPASSSSCY	LEEHVSKEAK	ALISRLQELM AGPGHFDPN
cypla2mou	QDALKSFSIA	SDPTSASSCY	LEEHVSKEAN	HLVSKLQKAM AEVGHFEPVS
cypla2rab	QDSLKSFSIA	SNPASSSSCY	LEEHVSQEA	NLIGRFQELM AAVGRFDPYS
cypla2rat	QDALKSFSIA	SDPTSVSSCY	LEEHVSKEAN	HLISKFQKLM AEVGHFEPVN
cypla6pla	YSALRSFSTL	EGTTP EYSCV	LEEHICKEGE	YLIKQLNTVM KADGSFDPFR
cyplb1hum	HSMMRNFFTR	.QPRSRQV..	LEGHVLSEAR	ELVALLVRGS ADGAFLDPRP
	170	180	190	200 <i>SRS2</i> 210
cyp2c5	ILGCAPCNVI	CSVIFHNRFD	YKDEEF.LKL	MESINENVRILSSPWLQVYNN
cypla1hum	YVVSVTNVI	CAICFGRRYD	HNHQE..LLS	LVNLNNNFGEVVG.SGNPADF
cypla1mou	YLVSVANVI	CAICFGQRYD	HDDQE..LLS	IVNLSNEFGEVTG.SGYPADF
cypla1rab	YVMSVANVI	CAMCFGRRYD	HDDQE..LLS	LVNLNDEFQKVAA.SGSPADF
cypla1rat	YLVSVANVI	CAICFGRRYD	HDDQE..LLS	IVNLSNEFGEVTG.SGYPADF
cypla1tro	HIVSVANVI	CGMCFGRRYS	HDDQE..LLG	LVNMSDEFQGVVG.SGNPADS
cypla2ham	QVVESVANVI	GAMCFGKNFP	RKSEE..MLR	IVKGSSDFVENVS.SGNAVDF
cypla2hum	QVVSVANVI	GAMCFGQHFP	ESSDE..MLS	LVKNTHFVETAS.SGNPLDF
cypla2mou	QVVESVANVI	GAMCFGKNFP	RKSEE..MLN	IVNNSKDFVENVT.SGNAVDF
cypla2rab	QLVSAARVI	GAMCFGRRFP	QGSEE..MLD	VVRNSSKFVETAS.SGSPVDF
cypla2rat	QVVESVANVI	GAMCFGKNFP	RKSEE..MLN	LVKSSKDFVENVT.SGNAVDF
cypla6pla	HIVSVANVI	CGMCFGRRYD	HDDQE..LVS	LVTLSDEFGRVVG.SGNPADF
cyplb1hum	LTVVAVANVM	SAVCFGCRYS	HDDPE..FRE	LLSHNEEFGRTVG.AGSLVDV
	220	230 <i>SRS3</i>	240	250 260
cyp2c5	FPALLDYFPG	IHKTLKLNAD	YIKNFIMEKV	KEHEKLLDVN N..P..RDFI
cypla1hum	IPILRYLPNP	SLNAFKDLNE	KFYSEFMQKMV	KEHYKTFEKG H..I..RDIT
cypla1mou	IPVLRYLPNS	SLDAFKDLND	KFYSEFMKKLI	KEHYRTFEKG H..I..RDIT
cypla1rab	FLILRYLPNP	ALDTFKDLNE	RFYSFTQERV	KEHCRSFEKG H..I..RDIT
cypla1rat	IPILRYLPNS	SLDAFKDLNK	KFYSEFMKKLI	KEHYRTFEKG H..I..RDIT
cypla1tro	IPILRYLPNR	TMKRFMDIND	RFNTFVQNII	SEHYESYDKD N..I..RDIT
cypla2ham	FPILRYLPNP	DLKRFGGEND	NFVLFLQKTV	QEHYQDFNKN S..I..QDIT
cypla2hum	FPILRYLPNP	ALQRFKAFNQ	RFLWFLQKTV	QEHYQDFDNKN S..V..RDIT
cypla2mou	FPVLRYLPNP	ALKRKFTEFND	NFVLFLQKTV	QEHYQDFNKN S..I..QDIT
cypla2rab	FPILRYLPNR	PLQRFKDFNQ	RFLRFLQKTV	REHYEDFDRN S..I..QDIT
cypla2rat	FPVLRYLPNP	ALKRKFKNFND	NFVLFLQKTV	QEHYQDFNKN S..I..QDIT
cypla6pla	IPILQYLPNS	EMKNFLRINE	HFTEFVQKIV	TEHYTTFNKD N..I..RDIT
cyplb1hum	MPWLQYFPNP	VRTVFREFEQ	LNRNFSNFIL	DKFLRHCESL RPGAAPRDM

Figure 1. Continued.

	270	280	290 <i>SRS4</i>	300
cyp2c5	DCFLIKMEQE.....NNL.EFTLESIVIAVS		DLFGAGTETT	STTLRYSLLL
cyplalhum	DSLIEHCQEK.QLDENANV.QLSDEKIINIVL		DLFGAGFDTV	TTAISWSLMY
cyplalmou	DSLIEHCQDR.KLDENANV.QLSDDKVITIVL		DLFGAGFDTV	TTAISWSLMY
cyplalrab	DSLIIKHRYVD.RLDENANV.QVSDEKTVGIVL		DLFGAGFDTV	TTAISWSLMY
cyplalrat	DSLIEHCQDR.RLDENANV.QLSDDKVITIVF		DLFGAGFDTI	TTAISWSLMY
cyplaltro	DSLIDHCEDR.KLDENANI.QVSDEKIVGIVN		DLFGAGFDTI	STALSWAVVY
cypla2ham	GALFKHSE.N.SKD..SGG.LIPQEKIVNIVN		DLFGAGFDTV	TTAITWSILL
cypla2hum	GALFKHSE.N.SKD..SGG.LIPQEKIVNIVN		DIFGAGFDTV	TTAISWSLMY
cypla2mou	SALFKHSE.N.YKD..NGG.LIPEEKIVNIVN		DIFGAGFDTV	TTAITWSILL
cypla2rab	GALFKHSEKN.SKA..NSG.LIPQEKIVNIVN		DIFGAGFDTI	TTAISWSLMY
cypla2rat	GALFKHSE.N.YKD..NGG.LIPQEKIVNIVN		DIFGAGFETV	TTAIFWSILL
cypla6pla	DSLIDHCEDR.KLDENSNV.QMSDEKIVGIVN		DLFGAGFDTV	STALSWSVMY
cyplblhum	DAFILSAEKKAAGDSHGGLDLENVPATIT		DIFGASQDTL	STALQWLLLL
	310	320	330	340
cyp2c5	LLKHPEVAAR	VQEEIERVIG	RHRSPCMQDR	SRMPYTDAMI
cyplalhum	LVMNPRVQRK	IQEELDTVIG	RSRRPRLSDR	SHLPYMEAFI
cyplalmou	LVTNPRVQRK	IQEELDTVIG	RDRQPRLSDR	PQLPYLEAFI
cyplalrab	LVTKPRIQRK	IQEELDAVVG	RARRPRFSDR	PQLPYLEAVI
cyplalrat	LVTNPRIQRK	IQEELDTVIG	RDRQPRLSDR	PQLPYLEAFI
cyplaltro	LVAYPEIQER	LHQELKEKVG	MIRTPRLSDK	INLPLEAFI
cypla2ham	LVTWPNVQRK	IHKELDTVIG	RDRQPLLSDR	LQLPYMEAFI
cypla2hum	LVTKPPIQRK	IQKELDTVIG	RERRPRLSDR	PQLPYLEAFI
cypla2mou	LVTWPNVQRK	IHEELDTVVG	RDRQPRLSDR	PQLPYLEAFI
cypla2rab	LVTNPRRQRK	IHEELDAVVG	RARQPRLSDR	PQLPYLEAFI
cypla2rat	LVTPEPKVQRK	IHEELDTVIG	RDRQPRLSDR	PQLPYLEAFI
cypla6pla	LVAHPEIQER	LYQEIEDKVG	LDRMPLLSK	PNLPFLEAFI
cyplblhum	FTRYPDVQTR	VQAELDQVVG	RDRLPCMGRQ	PNLPYVLAFL
	350 <i>SRS5</i>			
				HEIQRFDLL
				LETFRHSSFFV
				LETFRHSSFFV
				METFRHTSFL
				LETFRHSSFFV
				LEIFRHSSFFL
				LELYRYTSFV
				LETFRHSSFFL
				LEIYRYTSFV
				LEIFRHSSFFV
				LEIYRYTSFV
				LEILRHSSFFL
				YEAMRFSSFFV
	360	370	380	390
cyp2c5	PTNLPHAVTR	DVRFRN.YFI	PKGTDIITSL	TSVLHDEKAF
cyplalhum	PFTIPHSTTR	DTSLKG.FYI	PKGRCVFNQ	WQINHDQKLW
cyplalmou	PFTIPHSTTR	DTSLNG.FYI	PKGCCVFNQ	WQVNHDRELW
cyplalrab	PFTIPHSTTR	DTSLGG.FYI	PKGRCVFNQ	WQNNHDPQLW
cyplalrat	PFTIPHSTIR	DTSLNG.FYI	PKGHCVFNQ	WQVNHDQELW
cyplaltro	PFTIPHCTIK	DTSLNG.YFI	PKDTCVFINQ	WQVNHDPLDW
cypla2ham	PFTIPHSTTR	DTSLNG.FYI	PKDRCIFINQ	WQVNHDEKQW
cypla2hum	PFTIPHSTTR	DTTLNG.FYI	PKKCCVFNQ	WQVNHDPELW
cypla2mou	PFTIPHSTTR	DTSLNG.FHI	PKERCIYINQ	WQVNHDEKQW
cypla2rab	PFTIPHSTTR	DTTLNG.FHI	PKECCIFINQ	WQINHDPLQW
cypla2rat	PFTIPHSTTR	DTSLNG.FHI	PKERCIFINQ	WQVNHDEKQW
cypla6pla	PFTIPHCTTK	DTSLNG.YFI	PKDTCVFINQ	WQINHDPELW
cyplblhum	PVTIPHATTA	NTSVLG.YHI	PKDTCVFNQ	WSVNHDPLKW
				P.NPENFDPA

Figure 1. *Continued.*

	410	420	430	440	450
cyp2c5	HFLDESGN.F	KK..SDYFMP	FSAGKRMCVG	EGLARMELFL	FLTSILQNFK
cyplalhum	RFLTPDGA.I	DKVLSEKVII	FGMGKRKCIG	ETIARWEVFL	FLAILLQRVE
cyplalmou	RFLTPSGT.L	DKRLSEKVTL	FGLGKRKCIG	ETIGRSEVFL	FLAILLQQIE
cypla1rab	RFLTPSGA.V	DKALTEKVLL	FGLGKRKCIG	ETIGRLEVFL	FLATLLQQVE
cypla1rat	RFLTSSGT.L	DKHLSEKVIL	FGLGKRKCIG	ETIGRLEVFL	FLAILLQQME
cypla1tro	RFLSADGTEL	NKLEGEKVLV	FGMDKRRSIG	EAIGRNEVFL	FLAILLQRLR
cypla2ham	RFLTNDNTVI	NKTLSEKVML	FGLGKRKCIG	EIPAKWEVFL	FLAILLQQLE
cypla2hum	RFLTADGTAI	NKPLSEKMML	FGMGKRKCIG	EVLAKWEIFL	FLAILLQQLE
cypla2mou	RFLTNNNSAI	DKTQSEKVML	FGLGKRKCIG	EIPAKWEVFL	FLAILLQHLE
cypla2rab	RFLTADGAAI	NKPLSEKVTL	FGLGKRKCIG	ETLARWEVFL	FLAILLQRLE
cypla2rat	RFLTNDNTAI	DKTLSEKVML	FGLGKRKCIG	EIPAKWEVFL	FLAILLHQLE
cypla6pla	RFLSADGSEV	NKLDGEKVMA	FAMGKRKCIG	EVIARNEVYL	FLAIIIQKLH
cyplb1hum	RFLDKDGL.I	NKDLTSRVM	FSVGKRKCIG	EELSKMQLFL	FISILAHQCD
	460	SRS6470	480		
cyp2c5	LQSLVEPKDLD	ITAVVNGFVS	VPPSYQLCFI	PI	
cyplalhum	FSVP.LGVKVD	MTPIYGLTMK	HACCEHFQMQ	LRS	
cyplalmou	FKVS.PGEKVD	MTPTYGLTLK	HARCEHFQVQ	MRSSGPQHLQA	
cypla1rab	FSVS.PGTTVD	MTPIYGLTMK	HARCEHFQAK	LRFEA	
cypla1rat	FNV.S.PGEKVD	MTPAYGLTLK	HARCEHFQVQ	MRSSGPQHLQA	
cypla1tro	FQEK.PGHPLD	MTPEYGLTMK	HKRCQLKASM	RPWGQEE	
cypla2ham	FSVP.PGQKVD	LTPTYGLTMK	PQTCKYIQAW	PRFSK	
cypla2hum	FSVP.PGVKVD	LTPIYGLTMK	HARCEHVQAR	LRF SIN	
cypla2mou	FSVP.PGVKVD	LTPNYGLTMK	PGTCEHVQAW	PRFSK	
cypla2rab	FSVP.PGVKVD	LTPIYGLTMK	HPRCEHVQAR	PRFSDQ	
cypla2rat	FTVP.PGVKVD	LTPSYGLTMK	PRTCEHVQAW	PRFSK	
cypla6pla	FLPI.PGEKLD	MTPEYGLTMK	HKRC.HLKAT	MRARNAVE	
cyplb1hum	FRAN.PNEPAK	MNFSYGLTIK	PKSF.KVNV	LRESMELLD	SAVQNLQAKETCQ

Figure 1. Continued.

Table 2. A summary of site-directed mutagenesis and residue modification within the CYP1A subfamily.

CYP	Change	2C5	SRS	Comments	Reference
1A2h	R108K	T97	1	close to binding site	Hadjokas <i>et al.</i> (2002)
1A2h	I117T	V106	1	close to binding site	Lainé <i>et al.</i> (1994)
1A2h	F226I	V205	2	π - π stacking contact	Parikh <i>et al.</i> (1999)
1A2r	K250L	K231	—	redox interaction site ^c	Kraïnev <i>et al.</i> (1992)
1A2r	R251L	T232	—	redox interaction site ^c	Kraïnev <i>et al.</i> (1992)
1A2r	K253L	L234	3	redox interaction site ^c	Kraïnev <i>et al.</i> (1992)
1A2r	F313Y	F292	4	in binding site	Furuya <i>et al.</i> (1989a)
1A2r	A315S	A294	4	in binding site	Furuya <i>et al.</i> (1989b)
1A2h	D320A	E297	4	conserved acidic residue	Parikh <i>et al.</i> (1999)
1A2r	E318A	E297	4	conserved acidic residue	Shimizu <i>et al.</i> (1991, 1994)
1A2h	T321S	T298	4	conserved distal threonine	Parikh <i>et al.</i> (1999)
1A2h	I386L	L363	5	in binding site	Joseph <i>et al.</i> (2000)
1A1h	I462V ^a	L437	—	proximal heme contact	Kawajiri <i>et al.</i> (1992)
1A1r	T501 ^b	V473	6	hydrogen bond contact	Cvrk and Strobel (1998)

^a Allelic variant associated with increased lung cancer susceptibility.^b Part of tripeptide affinity labelled with azidocumene.^c Possible redox partner binding site: other likely interaction sites with reductase have also been reported for CYP1A1 (Shen and Strobel 1992).

h, Human; r, rat; SRS, substrate recognition site (Gotoh 1992).

General references: Lewis *et al.* (1999) and Lewis (2001).

using molecular mechanics for 100 iterative cycles in order to achieve a low energy conformation and a satisfactory protein geometry containing no conformationally disallowed regions. Individual substrate and inhibitor molecules were fitted within the CYP1A2 putative active site via interactive docking. This procedure used the location of potential hydrogen bond-forming pairs of atoms on both the protein and small molecules, together with prior knowledge of the preferred site of metabolism gained from the literature (Rendic and DiCarlo 1997). All molecular modelling procedures were carried out using the Sybyl software package (Tripos Associates, St Louis, MO, USA) implemented on a Silicon Graphics Indigo² 10000 graphics workstation operating under UNIX.

Results and discussion

Table 1 shows a selection of typical human CYP1A2 substrates and their sites of metabolism, together with information relating to the selective CYP1A2 inhibitor, furafylline. From the docking studies described above and due to their molecular planarity, it is found that all of these compounds can fit (figures 2–4) the putative active site of human CYP1A2, where interactions with complementary

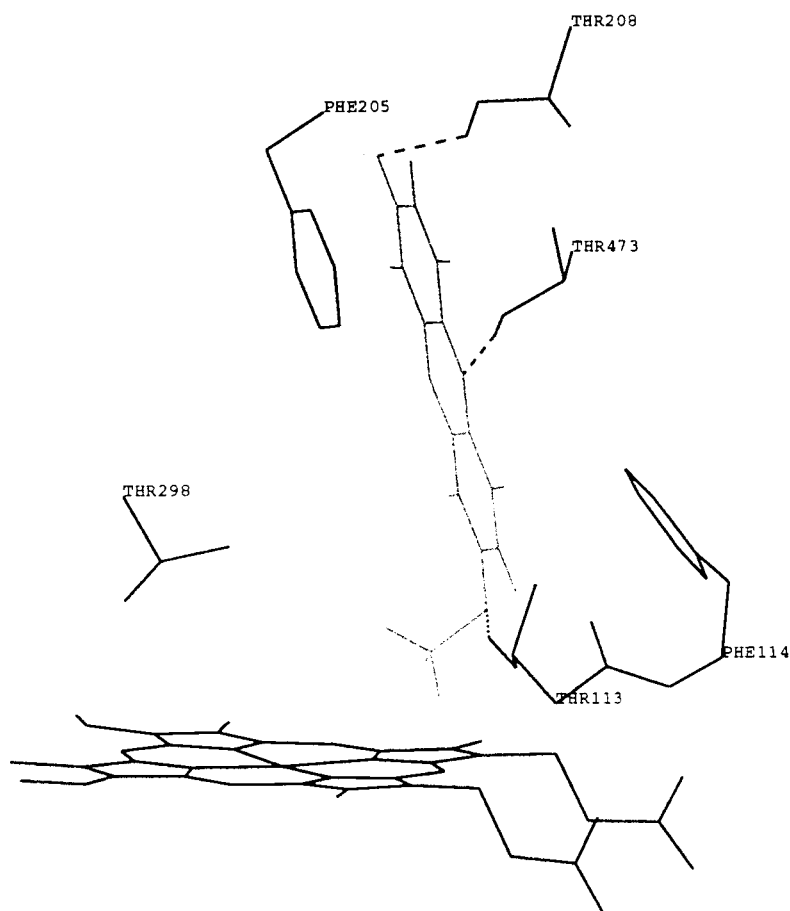


Figure 2. Marker substrate, 7-methoxyresorufin (grey), is shown orientated within the putative active site of human CYP1A2 via a combination of π - π stacking and hydrogen-bonded interactions (dashed lines). The site of metabolism (O-demethylation) is indicated via an arrow close to the substrate's methyl group. Residues are numbered according to the alignment with CYP2C5, as shown in figure 1. The distance between the 7-methoxy group and the haem iron is 3.548 Å.

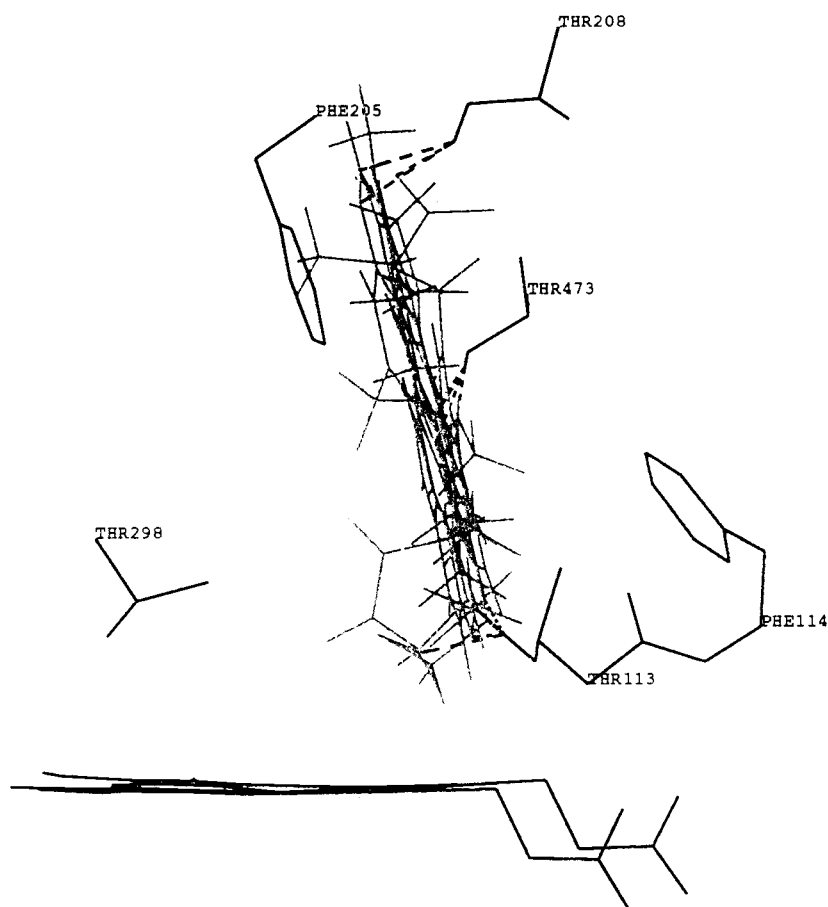


Figure 3. Template of superimposed substrate molecules (grey) is shown within the putative active site of human CYP1A2 where there is a reinforcement of the favourable contacts with hydrogen-bonded and π - π stacking residues. Residues are numbered according to the alignment with CYP2C5 shown in figure 1. Hydrogen bonds are depicted as dashed lines. The distance between the flanking aromatic rings in the active site is 5.426 Å, whereas the total depth of the substrate template is 3.728 Å.

amino acid residues are involved in orientating each substrate for metabolism at the experimentally observed position. The distance between the two flanking aromatic rings (Phe114 and Phe205) in the CYP1A2 active site (figures 2–4) is 5.426 Å, whereas the total depth of the substrate overlay is 3.728 Å, thus allowing sufficient room for the occupancy of typical CYP1A2 substrates. Table 2 provides a summary of site-directed mutagenesis information for CYP1 family enzymes, and a number of these positions are equivalent to those encountered in the putative active site of the current CYP1A2 model. These include residues Phe226 (Parikh *et al.* 1999) and Thr501 (Cvrk and Strobel 1998), which appear to act as substrate contacts via π - π stacking and hydrogen bonding, respectively. In fact, the majority of CYP1A2 substrates investigated in this work can form complementary interactions with these two residues, as can be appreciated from an inspection of figure 2–4. Moreover, selective inhibitors of CYP1A2 such as furafylline (Sesardic *et al.* 1990) and rutaecarpine (Ueng *et al.* 2002) also appear to bind within the putative

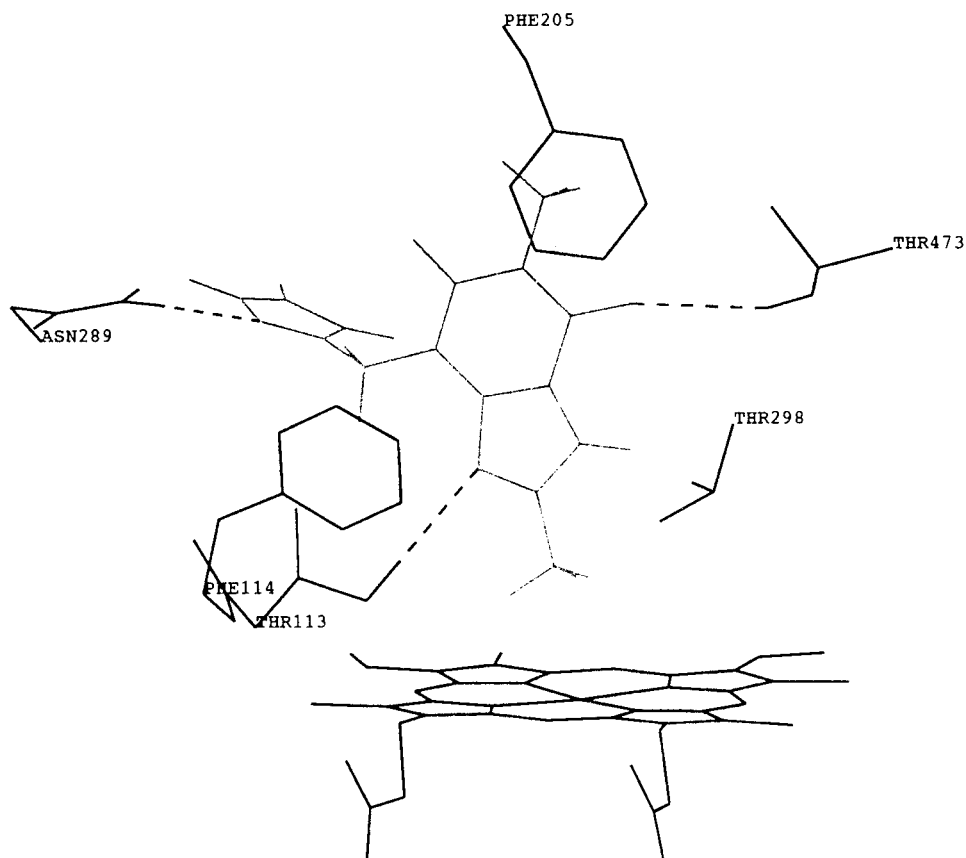


Figure 4. Selective mechanism-based inhibitor, furafylline (grey), shown orientated within the putative active site of CYP1A2 where a combination of hydrogen bonding and π - π stacking interactions orientate the molecule for 8-methyl hydroxylation. Residues are numbered according to the alignment with CYP2C5 shown in figure 1, and hydrogen bonds are displayed as dashed lines. The distance between the 8-methyl group and the haem iron is 4.202 Å.

active site in a similar manner to that of the preferred substrates (cf. figures 2 and 4, for example) and in accordance with their known mechanism of inhibition. In the case of furafylline, the mechanism for inhibition has been reported (Kunze and Trager 1993) to involve 8-methyl hydroxylation, whereas rutaecarpine represents a structural analogue of 9-hydroxy ellipticine.

Table 3 shows a dataset of physicochemical properties for CYP1A2 substrates, whereas the modelled distances between the sites of metabolism on fitted substrates and the haem iron are presented in table 4. It is possible to make use of various substrate properties to derive QSAR expressions (Lewis *et al.* 2002) relating to binding affinity, and we have found that inclusion of the number of active site hydrogen bonds provides an improvement in the correlation with CYP1A2 binding energy substrate from $R=0.95$ to 0.97 (Lewis *et al.* 2001, 2002) although this results in the use of four variables for the 11 compounds investigated. The specific interactions between substrates and active site amino acid residues facilitate a calculation of the overall binding energy, and table 6 shows that there is a very good correlation (98.5%) with experimental values for 12

Table 3. Properties of CYP1A2 substrates.

Compound	log <i>P</i>	<i>pK</i> _a	log <i>D</i> _{7.4}	<i>M</i> _r	<i>K</i> _m (μM)	Δ <i>G</i> _{bind} (kcal mol ^{−1})
1. Caffeine	0.01	neutral	0.01	194.22	180	−5.3118
2. PhIP	2.23	8.69 ^b	0.92	224.29	55	−6.0422
3. 7-Methoxyresorufin	3.15 ^c	neutral	3.15 ^c	227.23	0.21	−9.4577
4. Phenacetin	1.57	neutral	1.57	179.24	48	−6.1260
5. IQ	1.84	9.18 ^b	0.06	198.25	33	−6.3568
6. MeIQ	2.40 ^c	9.77 ^b	0.06 ^c	212.28	13	−6.9307
7. 4-Aminobiphenyl	2.86	4.61 ^b	2.86	169.24	30	−6.4156
8. 7-Ethoxyresorufin	3.61 ^c	9.85 ^c	1.19 ^c	241.26	1.7	−8.1839
9. Aflatoxin B ₁	2.20 ^c	neutral	2.20 ^c	312.29	31	−6.2231
10. Theophylline	1.40 ^c	8.8 ^b	−0.02	180.20	455	−4.7405
11. Tacrine	2.71	9.8 ^b	0.46	198.27	14	−6.8851
12. Oestradiol	2.69	neutral	2.69	272.37	20	−6.6653
13. Naproxen	3.18	4.15 ^a	0.33	230.26	160	−5.3843
14. Melatonin	1.45 ^c	neutral	1.45 ^c	232.31	85.5	−4.7704

Range of log *P* = 0.01–3.61.

Average log *P* = 2.17 (aflatoxin B₁ has a log *P* close to the average.

^a Acidic; *M*_r, relative molecular mass; Δ*G*_{bind} = *RT* ln *K*_m, where *R* is the gas constant and *T* is the absolute temperature; ^b basic; *K*_m, Michaelis constant for the CYP1A2-mediated reaction (μM); PhIP, 2-amino-1-ethyl-6-phenylimidazo[4,5-*b*]pyridine; ^c calculated value (Pallas System CompuDrag Ltd, Budapest); log *D*_{7.4}, logarithm of the distribution coefficient at pH 7.4; log *P*, logarithm of the octanol/water partition coefficient; *pK*_a = −log *K*_a, where *K*_a is the acid/base dissociation constant; IQ, 2-amino-3-methylimidazo-[4,5-*f*]quinoline; MeIQ, 4-methyl IQ.

References to physicochemical data: Newton and Kluza (1978), Hansch and Leo (1979), Sangster (1989), Suzuki and Kudo (1990) and Elks and Ganellin (1990).

Table 4. Distances between sites of metabolism and haem iron for human CYP1A2.

Compound	Route of metabolism	Reference	Distance (Å)
1. Caffeine	N-demethylation	Tassaneeyakul <i>et al.</i> (1992)	4.8
2. PhIP	N-hydroxylation	Zhao <i>et al.</i> (1994)	3.2
3. 7-Methoxyresorufin	O-demethylation	Turesky <i>et al.</i> (1998)	3.5
4. Phenacetin	O-demethylation	Parikh <i>et al.</i> (1999)	3.5
5. MeIQ	N-hydroxylation	Parikh <i>et al.</i> (1999)	5.9
6. Aflatoxin B ₁	8,9-epoxidation	Gallagher <i>et al.</i> (1996)	4.7
7. Tacrine	7-hydroxylation	Becquemont <i>et al.</i> (1998)	5.3
8. Naproxen	O-demethylation	Rodrigues (1999)	3.5
9. Oestradiol	2-hydroxylation	Yamazaki <i>et al.</i> (1998)	6.3
10. Melatonin	6-hydroxylation	Facciola <i>et al.</i> (2001)	4.5
11. Furfaylline	9-methyl hydroxylation	Clarke <i>et al.</i> (1994)	4.2
12. IQ	N-hydroxylation	Hammons <i>et al.</i> (1997)	5.9

PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQ, 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline; IQ, 2-amino-4-methylimidazolo-[4,5-*f*]quinoline.

CYP1A2 substrates using *K*_m information for an estimation of the enzyme–substrate binding affinity. Although the *K*_D’s for substrate dissociation with the enzyme would be preferable, these were not available in the literature. Consequently, it was considered appropriate to make use of Michaelis constant data (*K*_m) on the assumption that *K*_m ≅ *K*_D, which would be the case when breakdown of the enzyme–substrate complex is rate-limiting. For a number of cytochrome P450 substrates for which the data are available, it is found that the *K*_m’s are relatively close to the relevant *K*_D and, consequently, it is felt that this approximation is reasonable. For example, for midazolam in CYP2A4, the *K*_m is 8.7 μM, whereas *K*_D is 8.8 μM. Moreover, for bufuralol, dextromethorphan and imipramine

Table 5. Baseline lipophilicity relationship† for CYP1A2 substrates.

Compound	$\log P$	ΔG_{part}	ΔG_{bind}
1. Caffeine	0.08	-0.1135	-5.3118
2. Phenacetin	1.57	-2.2270	-6.1260
3. IQ	1.84	-2.6100	-6.3568
4. MeIQ	2.40 ^c	-3.4043	-6.9307
5. 7-Ethoxyresorufin	3.61 ^c	-5.1207	-8.1839
6. Melatonin	1.45	-2.0568	-5.7704
7. Tacrine	2.71	-3.8440	-6.8851

QSAR expression*	n	s	R	F
$\Delta G_{\text{bind}} = 0.601\Delta G_{\text{part}}(\pm 0.068) - 4.871$	7	0.2590	0.97	77.17

* Other compounds listed in tables 1, 3 and 4 appear as outliers to this relationship due to additional components to the overall binding energy.

† This type of relationship is between substrate binding affinity and lipophilicity (Lewis and Dickens 2002).

IQ, 2-amino-3-methylimidazo-[4,5-*f*]quinoline; MeIQ, 4-methyl IQ; n , number of points; s , standard error; R , correlation coefficient; F , variance ratio; c , calculated value (Pallas System, CompuDrug Ltd, Budapest); $\Delta G_{\text{part}} = -RT \ln P$, where P is the octanol/water partition coefficient (see table 3 legend); $\Delta G_{\text{bind}} = RT \ln K_m$, where K_m is the Michaelis constant (μM) for substrate metabolism (see table 3 legend); R , gas constant ($1.987 \text{ cal mol}^{-1} \text{ K}^{-1}$); T , absolute temperature (taken as 310 K for reactions at 37 °C).

metabolized by CYP2D6, the K_m 's are 10.8, 2.0 and 2.15 μM , respectively. These compare very favourably with the measured K_D 's of 8.6, 2.76 and 2.4 μM for the three substrates, respectively. Furthermore, Bauer *et al.* (2001) have described enzyme-substrate binding energies in (ΔG) terms of K_m 's using the expression:

$$\Delta G = RT \ln K_m,$$

where R is the gas constant and T is the absolute temperature.

In figure 2, the known substrate 7-methoxyresorufin is shown docked within the putative active site of CYP1A2 where a combination of hydrogen bond and π - π stacking interactions cooperatively assist in orientating the substrate for O-demethylation. In particular, there are complementary hydrogen-bonded interactions between the substrate and at least two threonine residues present in the putative active site. Of these, the latter (Thr501) has been probed using photo-affinity labelling in rat CYP1A1 (Cvrk and Strobel 1998), whereas one of the phenylalanine residues (Phe226) has been the subject of site-directed mutagenesis in human CYP1A2 (Parikh *et al.* 1999). Consequently, there is some evidence for the active site contacts with 7-methoxyresorufin which orientate the molecule for O-demethylation, and where the methyl group of the substrate lies close to the haem iron at a distance of 3.55 Å with respect to the methylene group hydrogens (table 4).

Other selective substrates for CYP1A2 also fit well within the putative active site. For example, caffeine is a known marker substrate for CYP1A2 where N-demethylation is the main route of metabolism. However, of the three possible N-demethylations, it is found that N-3 demethylation is the major route of caffeine metabolism mediated via human CYP1A2 (Tassaneeyakul *et al.* 1992, 1994). Although it is possible to rotate the caffeine molecule within the CYP1A2 active site to show how favourable contacts with amino acid residues can give rise to all

Table 6. Agreement between calculated and experimental ΔG_{bind} values for 11 CYP1A2 substrates.

Compound	$\log P$	ΔG_{part}	ΔG_{hb}	$\Delta G_{\pi-\pi}$	ΔG_{rot}	$\Delta G_{\text{bind}}^{\text{calc}}$	$\Delta G_{\text{bind}}^{\text{expt}}$
1. Caffeine	0.08	-0.1135	-6.0	-0.9	1.8	-5.2135	-5.3118
2. Melatonin	1.45 ^c	-2.0568	-4.0	-0.9	1.2	-5.7568	-5.7704
3. 7-Methoxyresorufin	3.15 ^c	-4.4682	-6.0	-0.9	1.8	-9.5682	-9.4577
4. Phenacetin	1.57	-2.2270	-4.0	-0.9	1.2	-5.9270	-6.1260
5. Tacrine	2.71	-3.8440	-2.0	-0.9	0.0	-6.7440	-6.8851
6. Oestradiol	2.69	-3.8157	-4.0	-0.9	1.8	-6.9157	-6.6653
7. MeIQ	2.40 ^c	-3.4043	-4.0	-0.9	1.8	-6.5043	-6.9307
8. Naproxen	0.33	-0.4681	-4.0	-0.9	0.0	-5.3681	-5.3843
9. PhIP	2.23	-3.1632	-4.0	-0.9	1.8	-6.2632	-6.0422
10. Aflatoxin B ₁	2.20 ^c	-3.1206	-4.0	-0.9	1.8	-6.2206	-6.2231
11. IQ	1.84	-2.6100	-4.0	-0.9	1.2	-6.3100	-6.3568

MeIQ, 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*f*]pyridine; IQ, 2-amino-2-methyl-imidazo[4,5-*f*]quinoline c; calculated value (Pallas System, CompuDrug Ltd, Budapest); $\Delta G_{\text{part}} = -RT \ln P$, where P is the octanol/water partition coefficient (see table 3 for source of data); $\Delta G_{\text{bind}}^{\text{calc}} = \Delta G_{\text{part}} + \Delta G_{\text{hb}} + \Delta G_{\pi-\pi} + \Delta G_{\text{rot}}$, where ΔG_{hb} is the average hydrogen bond free energy (-2 kcal mol^{-1}), $\Delta G_{\pi-\pi}$ is the average $\pi-\pi$ stacking free energy ($-0.9 \text{ kcal mol}^{-1}$) and ΔG_{rot} is the average loss in free energy due to restricted bond rotation ($0.6 \text{ kcal mol}^{-1}$); $\Delta G_{\text{bind}}^{\text{expt}} = RT \ln K_m$, where K_m is the Michaelis constant (μM) for substrate metabolism (see table 3 for K_m values).

The agreement between experimental and calculated ΔG_{bind} values is 98.5% for the 10 compounds shown and the slope of the correlation is approximately unity (value of slope = 0.998).

three N-demethylations, the overall binding interaction leading to N-3 demethylation is likely to be stronger due to the presence of an additional hydrogen bond, and this is consistent with the apparent K_m 's (Tassaneeyakul *et al.* 1994) as the K_m data for N-1 and N-7 demethylations are 28 and 43 mM, respectively: the difference in ΔG 's being roughly equivalent to the average hydrogen bond energy. Furthermore, it is also possible to fit other substrates, such as: phenacetin aflatoxin B₁, MeIQ, oestradiol, melatonin and naproxen within the putative site of CYP1A2 to produce a superimposed template (figure 3) overlaid with caffeine and 7-methoxyresorufin, as mentioned previously, such that there is a reinforcement of common interactions with hydrogen bond forming residues and those involved with $\pi-\pi$ stacking. The method used for each substrate docking being the same as that described in the Materials and methods. Figure 3 shows the appearance of this template of eight substrates lying in the haem pocket of CYP1A2. Although other CYP1A2-selective compounds fit this template, eight were included in figure 3 for the sake of clarity. In each case, the distance between the site of metabolism and haem iron lies within the range of 3–5 Å in line with that shown in P450 crystal structures (Poulos *et al.* 1987, Cupp-Vickery and Poulos 1995, Li and Poulos 1997), and the relevant information for CYP1A2 substrates is provided in table 4. Similar findings are evidenced for selective inhibitors such as furafylline and rutaecarpine, where there are interactions with the same cluster of amino acid residues as that encountered for substrates of the CYP1A2 enzyme. For example, figure 4 shows how furafylline may bind to the active site of CYP1A2, where hydrogen bonded and $\pi-\pi$ stacking interactions cooperatively assist in orientating the molecule for 8-methyl hydroxylation, in agreement with experimental findings (Kunze and Trager 1993).

The results of previously reported (Lewis *et al.* 2001, 2002) quantitative structure–activity relationships (QSARs) for a related set of substrates binding

to human CYP1A2 have indicated that both the polarity and planar shape of molecules, together with the frontier orbital energy values (E_{LUMO} or ΔE), are important. However, the inclusion of a number of hydrogen bond contacts in the active site provides an improvement ($R = 0.97$ compared with 0.95) in the fit with experimental data, indicating that this may be a key feature of active site binding. Furthermore, compound lipophilicity also correlates with experimental binding affinities ($R = 0.97$) as shown in table 5, albeit for a relatively small number of substrates. Some compounds are clearly outliers to this expression, however, and this is found for those cases where additional hydrogen bonds or π - π stacking interactions are formed within the active site of CYP1A2. Nevertheless, when combined with estimated hydrogen bond and π - π stacking energies, the partitioning energy (ΔG_{part}) obtained from log P 's gives a good correspondence ($R = 0.985$) with experimental binding energies (ΔG_{bind}) derived from K_{m} 's for 10 substrates of CYP1A2, as shown in table 6.

In this case, the expression for substrate binding energy, ΔG_{bind} , comprises contributions from partitioning, ΔG_{part} , hydrogen bonding, ΔG_{hb} and π - π stacking, $\Delta G_{\pi-\pi}$, together with the loss in rotational bond energy, ΔG_{rot} . Similar findings have been reported for CYP2 substrates also (Lewis 2002). Consequently, it is possible to estimate the relative binding affinities of CYP1A2 substrates based on active site interactions and desolvation of the haem environment accompanying substrate binding to the enzyme. However, in order to generate realistic evaluations of likely binding affinities for CYP1A2 substrates, it is important to construct a model for the enzyme's active site such that the relevant contacts with interacting amino acid residues can be analysed. Together with compound lipophilicity, the binding interactions can then be used to formulate the overall binding energy although, in some cases, the number of rotatable bonds in the molecule needs to be taken into account.

Conclusions

The construction of human CYP1A2 using the CYP2C5 template provides an enzyme model that can be satisfactorily probed with both substrates and inhibitors. The results show a favourable consistency with available experimental evidence from site-directed mutagenesis and from known substrate metabolism. Moreover, the model provides useful information for the generation of QSARs involving CYP1A2-mediated metabolism for typical substrates, especially where the hydrogen-bonded and π - π stacking interactions between enzyme and substrate are concerned. The CYP2C5-derived model thus represents a basis for further studies on the rationalization of enzyme selectivity and substrate metabolism within the CYP1 family, and should help to explain the regioselectivity of other compounds such as propranolol (Ching *et al.* 1996) where more than one route of metabolism is mediated by CYP1A2. This model represents an advance on our previous studies using CYP102 as a structural template (Lewis and Lake 1996, Lewis *et al.* 1999) because of the significantly higher primary sequence homology between CYP1A2 and CYP2C5 (30%) as opposed to that of CYP102 and CYP1A2 (24%). In addition, the new model agrees closely with the reported evidence from site-directed mutagenesis and other related studies which have identified likely amino acid residues involved in substrate binding. Moreover, the model has been employed in the estimation of binding energies, based on key active site inter-

actions, which produce values close to those obtained from experimental metabolic data.

Acknowledgements

The financial support of GlaxoSmithKline Research and Development Ltd, Merck Sharp & Dohme Ltd and the University of Surrey Foundation Fund is gratefully acknowledged by DFVL.

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Hydrolysis by lactase phlorizin hydrolase is the first step in the uptake of daidzein glucosides by rat small intestine *in vitro*

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Received 1 August 2002

1. Isoflavones are naturally occurring oestrogenic compounds found in plants, where they exist in the glycosylated form. A proportion of ingested glycosides appears to be absorbed in the upper gastrointestinal tract, where enterocytes play an important role in their metabolism.

2. One hypothesis is that ingestion may involve hydrolysis by the lumenally exposed enzyme lactase phlorizin hydrolase (LPH), an enzyme expressed specifically at the small intestinal brush border.

3. Using an everted sac preparation of rat jejunum and an inhibitor of LPH, we investigated the absorption of daidzein-*O*⁷-glucoside (daidzin) and the effect of LPH inhibition on this process. It was demonstrated that LPH plays a major role in the deglycosylation of daidzin.

4. The hydrolysis product, daidzein, is absorbed by epithelial cells and glucuronidated to daidzein-*O*⁷-glucuronide, which is subsequently exported primarily to the serosal (vascular) side of the tissue rather than to the luminal side.

5. A small but significant proportion of the intact glycoside is also transferred to the serosal compartment, and in the presence of an LPH inhibitor this was enhanced with a corresponding reduction in deglycosylation and glucuronidation.

6. The results indicate that LPH plays an important role in the metabolism of glycosylated phytochemicals, and that the expression and activity of this enzyme in the small intestine can modify the profile of metabolites appearing in the circulation.

Introduction

Isoflavone phyto-oestrogens are present in significant quantities in soya products and they may offer protection against chronic human diseases such as cancers of the breast and prostate, and coronary heart disease, mediated by various mechanisms (Adlercreutz and Mazur 1997, Ruiz-Larrea *et al.* 1997, Setchell and Cassidy 1999, Hwang *et al.* 2000, Wiseman *et al.* 2000). Unfermented soya foods contain predominantly isoflavone glycosides, although low levels of aglycones are also present. Until recently, it was thought that glycoside absorption was restricted to the large intestine, following hydrolysis by the colonic microflora (Griffiths and Barrow 1972). However, more recent reports provide evidence that flavonoid glycosides may be taken up by the small intestinal mucosa (Hollman *et al.* 1995, 1996, Gee *et al.* 2000). Evidence that genistin may use similar pathways has been

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provided (Andlauer *et al.* 2000a) in isolated rat small intestine. Metabolites of isoflavones, principally glucuronic acid conjugates, have been detected in human plasma within 30 min after a meal of unfermented soya (Morton *et al.* 1997, King and Bursill 1998), results consistent with their absorption from the small intestine.

Further information on isoflavone bioavailability and pharmacokinetics is therefore essential to an understanding of the role of the parent compound and its metabolites in the reported biological properties. The precise chemical form and concentration in the target tissue will determine efficacy in terms of anti-carcinogenic, antioxidant and other activities in the body. Such a relationship has been demonstrated for the flavonoid quercetin by Day *et al.* (2000a), who reported that the position of conjugation with glucuronic acid affects the inhibition of the pro-oxidant enzyme xanthine oxidase.

We have previously shown that the lactase site of LPH, a membrane-spanning enzyme in the brush border of the small intestine, is capable of hydrolysing flavonoid glucosides (Day *et al.* 2000b). Andersson *et al.* (2000) have also reported that lactase activity can be inhibited in the pig proximal small intestine using NB-DGJ. In the present study we have used cannulated everted sacs of rat proximal small intestine, a model that allows access to both the mucosal and serosal compartments whilst minimizing cross contamination. Tissue was incubated with either daidzein-*O*⁷-glucoside (daidzin) or the aglycone, daidzein, and samples assayed by ELISA and HPLC to assess the uptake and metabolism of these compounds. An inhibitor of the lactase site of LPH was used to probe the mechanisms of absorption at the jejunal brush border.

Materials and methods

Cannulated everted sac model

Male Wistar rats (about 180g) were obtained from a licensed animal supplier, and housed in groups in solid bottomed cages in an environmentally controlled animal facility. All aspects of animal care complied with the ethical guidelines and technical requirements of the UK Home Office. The animals received a commercial pelleted diet (Type RM1 Expanded, Special Diet Services, Whitam, UK) and water *ad libitum* until they reached a weight of approximately 300g. Cannulated everted sacs of rat proximal jejunum were prepared from segments (5 cm) of male Wistar rat small intestine as described previously (Gee *et al.* 1998) and filled with Krebs bicarbonate buffer (pH 7.3, 0.5 ml). Daidzin (Extrasynthese, Genay, France) and daidzein (Apin Chemicals, Abingdon, UK), were checked for purity by HPLC and dissolved in ethanol containing 2–3% v/v dimethylsulfoxide, then diluted with Krebs bicarbonate buffer (pH 7.3) to give final concentrations of 1, 10 and 100 µM. The ethanol concentration in all solutions was 0.8% v/v. Sacs were suspended in organ baths containing 8 ml Krebs buffer without or with isoflavonoid, and incubated for 15 min at 37°C with continuous aeration using oxygen:carbon dioxide (95:5). After incubation the sacs were rinsed thoroughly with physiological saline (0.9% NaCl). The serosal solutions were then withdrawn from the sacs, transferred to Eppendorf tubes and immediately stored at –20°C. The mucosal solutions were also collected at the end of the incubation period and stored as described above. Samples were analysed by ELISA and HPLC as described below.

To establish the optimum concentration of lactase hydrolase inhibitor, sacs were incubated as above in organ baths containing [¹⁴C]-lactose (Amersham Biosciences, Little Chalfont, UK; final concentration 2 mM; 4.63 kBq ml^{–1}) in Krebs buffer (8 ml) without or with 10, 50, 100 or 500 µM *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ; Calbiochem, Nottingham, UK) an inhibitor of lactase (Andersson *et al.* 2000). After incubation, sacs were rinsed and the serosal solutions drained from them and transferred to plastic vials (20 ml; Packard, Zaventem, Belgium). Liquid scintillation cocktail (Zinsser Quicksafe A, Zinsser Analytic, Maidenhead, UK; 9.5 ml) was added to the serosal solutions and their radioactivity determined in an automatic scintillation spectrometer. The tissue from each sac was placed in glass vials (8 ml) and dried before weighing, acid digestion and liquid scintillation counting (Gee *et al.* 2000).

In a further experiment, incubations were carried out with daidzin or daidzein (100 μ M) in the absence or presence of NB-DGJ (250 μ M). A control incubation was also carried out in Krebs buffer with inhibitor present but no isoflavonoid. Mucosal and serosal fluids were collected, frozen and analysed by ELISA and HPLC as appropriate.

ELISA determination of daidzein

Triplicate subsamples of serosal solutions from the cannulated sac experiments, (1–40 μ l, depending on the concentration of the substrate) were placed in Eppendorf tubes and Krebs buffer added to adjust the final volume to 200 μ l. Recombinant β -glucuronidase from *E. coli* K12 (EC 3.2.1.31) (25 U/25 μ l; Roche Diagnostics; Lewes, UK) or Sigma (Poole, UK) was added at this previously optimized concentration and the tubes incubated at 37°C for 2 h. Samples were then extracted twice with ethyl acetate (1.0 ml). The organic phase was retained and evaporated to dryness at 50°C under nitrogen. Extracts were reconstituted for ELISA analysis by dissolving them in assay buffer (see below). Subsamples from incubations with 100 μ M isoflavonoid were also extracted, as described above, but without hydrolysing the samples with β -glucuronidase.

Analysis of the samples by ELISA was performed according to Creeke *et al.* (1998) using a fresh polyclonal anti-daidzein serum raised in the same manner as described. This antiserum had the following cross reactivity: daidzein 100%, daidzin 1%, formononetin 10%, and genistein, genistin and daidzein-*O*⁷-glucuronide <0.1%. Anti-rabbit IgG conjugated with horse radish peroxidase (HRP) was purchased from Sigma, and horse-radish peroxidase substrate (based on tetramethylbenzidine) was purchased from Vetoquinol (Bicester, UK). Optical densities were determined at 450 nm using a Dynex Technologies MRXII microtitration plate reader supplied with Revelation software (Thermo Bioanalysis/Dynex Technologies, London, UK). The amount of daidzein present in the serosal samples was determined from standard curves as described previously (Creeke *et al.* 1998).

HPLC and LC/MS analysis

Mucosal samples from the control (no isoflavonoid) and test daidzein and daidzin (10 and 100 μ M) incubations were prepared for HPLC analysis using the method of DuPont *et al.* (2000). Briefly, samples (3.0 ml) were applied to preconditioned polyamide columns (MN Polyamide SC6, 1 g; Macherey-Nagel GmbH, Düren, Germany) and the column eluted with methanol followed by methanol/ammonium hydroxide (99.5:0.5% v/v) to isolate neutral and acidic fractions, respectively. Both fractions were then evaporated to dryness and reconstituted in methanol (1.0 and 0.2 ml).

In addition, serosal solutions from incubations in the absence and presence of the isoflavonoids (100 μ M) were prepared with or without β -glucuronidase (25 U) treatment as follows. Duplicate aliquots of serosal fluid (50 or 100 μ l) were spiked with genistein (250 ng, 5 μ l) as an internal standard. An equivalent volume of Krebs buffer was added to one subsample followed by an equal volume of methanol containing 0.8 mM ascorbic acid. β -Glucuronidase (25 U/25 μ l) was added to the second aliquot and the sample was incubated at 37°C for 2 h. An equal volume of the methanol/ascorbic acid solution was again added. Both subsamples were evaporated and reconstituted in methanol (0.2 ml) before HPLC.

Mucosal and serosal samples were analysed by HPLC according to the method of Price *et al.* (1998) as modified by Day *et al.* (2000b) with detection at 260, 280 and 320 nm. Under these conditions, separation of daidzin, daidzein-*O*⁷-glucuronide, daidzein and the internal standard genistein was achieved.

Peak identification was confirmed by co-elution of peaks with authentic standard compounds by matching UV spectra, and by positive-ion electrospray LC/MS, using a Micromass Quattro II (Manchester, UK) mass spectrometer equipped with a Z-sprayTM ion source. Samples were introduced using a Hewlett Packard 1050 HPLC (Agilent Technologies, Stockport, UK) equipped with a diode array detector. Eluent flow (1 ml min⁻¹) was split between the diode array detector and the mass spectrometer ion source in the approximate ratio 8:1. The electrospray capillary voltage was set to 3.5 kV and the cone voltage to 28 V. Source block temperature was 140°C and desolvation temperature 350°C. Nitrogen was used as the drying and nebulizing gas at flows of 400 and 20 l h⁻¹, respectively.

Selected ion-monitoring measurements were performed on mass channels 255.07 (daidzein [M + H]⁺), 271.06 (genistein [M + H]⁺), 417.12, 431.10 (daidzin [M + H]⁺, [M + Na]⁺), 439.10 and 453.08 (daidzein-*O*⁷-glucuronide [M + H]⁺, [M + Na]⁺) and with a scan window of 0.2 amu, dwell times of 0.1 s/channel and an interchannel delay of cycle time of 0.03 s. Diode array spectra were scanned from 190 and 450 nm, with an interval of 2 nm. Instrument control, data acquisition and processing were performed using a Micromass MassLynxTM version 3.4 system.

Daidzein-*O*⁷-glucuronide was chemically synthesized (Needs and Williamson 2001) and the authenticity of the compound confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy.

Statistics

The statistical significance of differences between means was assessed by paired *t*-tests.

Results

Analysis of serosal samples by ELISA, following glucuronidase treatment, demonstrated proportional uptake of both the glycoside and aglycone over the concentration range 0–100 μM (figure 1). The uptake of daidzein was approximately four times that of daidzin. Analysis of similarly treated serosal samples by HPLC confirmed the presence of a glucuronidated metabolite. Values for total daidzein equivalents after deglucuronidation, obtained by either method, were in good agreement (figure 1). In cases where recovered serosal volumes from incubations with 100 μM daidzin and daidzein were sufficient ($n = 6$ and 7), samples were also analysed by HPLC without enzyme treatment. This provided more detailed information on the isoflavonoid proportions and the nature of the major metabolite, which was identified as daidzein- O^7 -glucuronide (table 1). The identity of each of the individual compounds was confirmed by co-elution with authentic standards, enzyme treatment, UV spectrum and LC/MS analysis. A representative HPLC chromatogram in which all three forms of daidzein, the glycoside, aglycone and glucuronide were present is shown in figure 2.

Analysis of mucosal solutions following incubation with daidzein (100 μM) also demonstrated the presence of daidzein- O^7 -glucuronide in these solutions indicating that once formed in the enterocytes, this glucuronide can be exported to either mucosal or serosal compartments, although most (about 8–11-fold) is found in the latter (table 1). When the concentration of daidzein in the incubations was lowered to 10 μM , the glucuronide metabolite was also detected in the serosal or mucosal solutions at correspondingly lower concentrations, with once again the greater proportion being transferred to the serosal side (data not shown).

Low levels of daidzein- O^7 -glucuronide were also detected in mucosal solutions following incubation with daidzin (100 μM , table 1). However, in the case of incubations with 10 μM daidzin (data not shown), amounts were below the HPLC

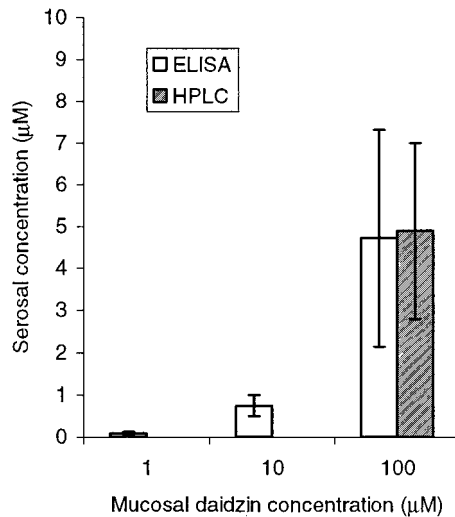


Figure 1. Serosal appearance of daidzein conjugates from mucosal daidzin or daidzein in cannulated everted sac preparations of rat proximal jejunum. ELISA determination was at 1, 10 and 100 μM substrate concentrations; HPLC determination was at 100 μM only. At substrate concentrations of 100 μM , $n = 10$; $n = 5$ elsewhere. Histograms are means $\pm$ SD.

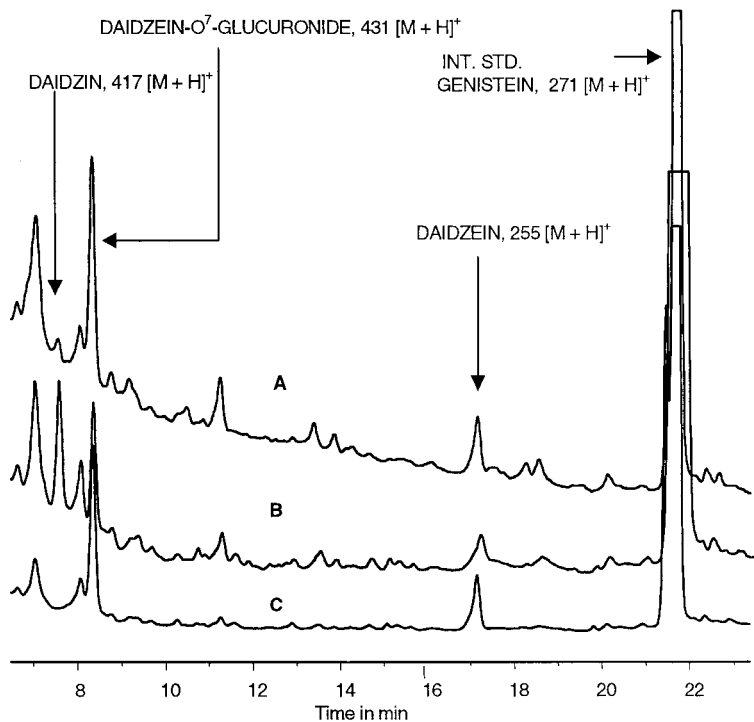


Figure 2. Representative HPLC chromatograms showing the composition of serosal samples following incubation of rat everted sacs with (A) daidzin (100 μ M), (B) daidzin (100 μ M) plus *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ, 250 μ M) and (C) daidzein (100 μ M). The peaks were confirmed by mass spectrometry and the mass of the $[M + H]^+$ are shown along side the respective HPLC peak.

detection limits. In addition to unmetabolized daidzin, the aglycone daidzein was also detected in mucosal solutions from incubations with daidzin (10 and 100 μ M incubations; data for 100 μ M only in table 1), demonstrating β -glucosidase activity at the intestinal brush border.

To ascertain whether LPH was responsible for the observed deglycosylation of daidzin, we used NB-DGJ to suppress the lactase site of LPH. Having ascertained that in the everted sac model maximal but incomplete (60%) inhibition of [^{14} C]-lactose hydrolysis occurred at 250 μ M (figure 3), we used this concentration in subsequent experiments. At this level of NB-DGJ, deglycosylation of daidzin was inhibited by approximately 50% ($p < 0.05$; table 1), which was consistent with the reduction in mucosal hydrolysis of lactose reported above. There was a tendency for the export of daidzein-*O*⁷-glucuronide to the mucosal compartment to be lower, but this was not statistically significant. In contrast, but not unexpectedly, there was no effect of NB-DGJ on daidzein metabolism (table 1). There were also no significant differences in the amount of daidzein-*O*⁷-glucuronide or daidzein on the serosal side when everted sacs were incubated with daidzein in the presence or absence of lactase inhibitor NB-DGJ.

Interestingly, incubation of everted sacs with daidzin in the presence NB-DGJ (table 1) caused a significant change in the profile of isoflavonoid compounds appearing at the serosal side. Daidzin levels increased approximately sixfold

Table 1. Effect of lactose hydrolase inhibition (by NBDG J) on daidzin and daidzein (100µM) uptake by everted sacs of rat proximal jejunum after 15 min.

Substrate	Mucosal concentration (µM) (HPLC)			Serosal concentration (µM) (HPLC)			Serosal concentration (µM)	
	Daidzin	Daidzein- <i>O</i> ⁷ -glucuronide	Daidzein	Daidzin	Daidzein- <i>O</i> ⁷ -glucuronide	Daidzein	(HPLC) Daidzein glucuronide conjugates†	(ELISA) Daidzein glucuronide conjugates†
Daidzin	71 ± 14	0.19 ± 0.15	1.58 ± 0.40	0.2 ± 0.2	1.8 ± 0.6	0.6 ± 0.6	4.9 ± 2.1	4.7 ± 2.6
Daidzin + NB*DGJ	69 ± 12	0.08 ± 0.02	0.76 ± 0.14*	1.1 ± 0.3*	0.6 ± 0.1*	1.0 ± 0.5	4.0 ± 0.8	2.6 ± 0.9
Daidzein	n.d.	1.17 ± 0.44	71 ± 7	n.d.	12.9 ± 4.5	1.5 ± 1.2	14.3 ± 4.1	18.6 ± 10.8
Daidzein + NB-DGJ	n.d.	1.52 ± 0.73	68 ± 13	n.d.	14.3 ± 4.7	1.7 ± 0.2	18.5 ± 5.8	16.0 ± 4.3

n.d., Not detected; NB-DG J, *N*-(*n*-butyl)-deoxygalactonojirimycin (250 µM); † total daidzein after β-glucuronidase treatment.

* *p* < 0.05 when comparing isoflavone plus inhibitor with isoflavone alone; data are means (*n* = 5) ± *SD*.

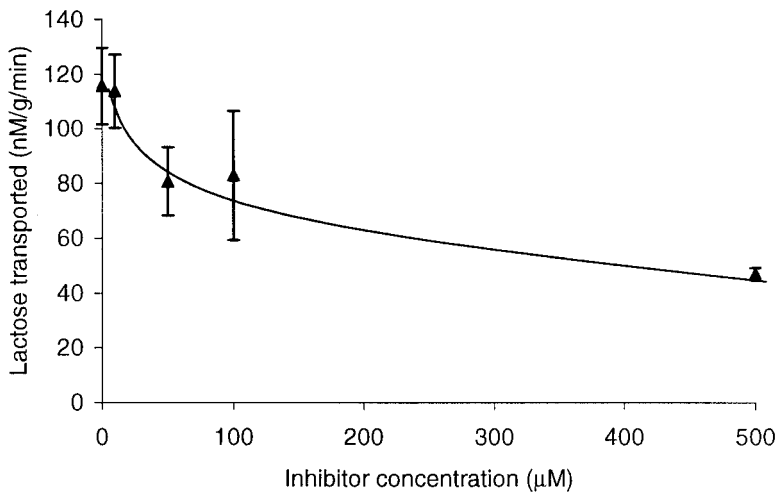


Figure 3. Effect of NB-DGJ on the hydrolysis of ^{14}C -lactose and subsequent uptake of liberated glucose by everted sacs of rat proximal jejunum. Error bars represent SEM; $n = 5$.

($p < 0.05$) and daidzein- O^7 -glucuronide decreased threefold ($p < 0.05$). Under these conditions, the aglycone rather than the glucuronide became the principal serosal isoflavonoid component. There was no significant change in the serosal concentration of the aglycone daidzein. In addition, both HPLC and ELISA analyses (table 1) showed no significant change in the total amount of isoflavonoid transferred to the serosal compartment.

These results indicate that both daidzein and daidzin can be transported across the rat small intestine. They also indicate that LPH plays a major role in the initial metabolism of dietary isoflavonoid glucosides, but glucoside transfer may also occur. Inhibition of LPH activity markedly altered the profile of isoflavonoid compounds appearing on the serosal side potentially affecting systemic antioxidant and other biological activities.

Discussion

Isoflavone metabolites appear in human and rat plasma within 30 min of ingesting soya (Morton *et al.* 1997, King and Bursill 1998, Piskula 2000), indicating that some absorption of isoflavones occurs in the small intestine. This early rise in isoflavone metabolite plasma levels has previously been assumed to reflect the absorption of aglycones, which are present in low amounts in unprocessed soya foods (Barnes *et al.* 1994). Early absorption of daidzein and genistein from the stomach has indeed been reported in rats (Piskula *et al.* 1999), but recent reports of studies with human volunteers consuming foods containing the structurally related flavonol glycosides have shown these compounds can be absorbed from the small intestine (Hollman *et al.* 1995, 1996). In addition, Piskula *et al.* (1999) and Andlauer *et al.* (2000b) showed absorption of isoflavonoid glucosides from the small bowel. Andlauer *et al.* used a perfused preparation to investigate the absorption of isoflavone glucosides from a homogenate of tofu and demonstrated the metabolism of these glucosides, as well as serosal localization of unmetabolized glucosides, daidzin and genistin.

In the current studies, we have demonstrated that daidzein, like genistein and quercetin, is readily absorbed into cells of the rat small intestine where it is glucuronidated and exported to either the vascular or luminal sides, the major chemical form appearing at the serosal side being daidzein-*O*⁷-glucuronide. LC/MS and HPLC analyses of serosal solutions also indicated that a small amount of daidzin was transferred across the epithelium intact, and this finding is consistent with the data of Andlauer *et al.* (2000a, b)

Our results also demonstrate that the first step in the metabolism of the glucoside daidzin is hydrolysis to the aglycone at the brush border. Although Andlauer *et al.* (2000a, b) have reported such a hydrolytic step, they offered no explanation of the mechanism. The present study has shown that lactase phlorizin hydrolase (LPH) is involved and, given the transmembrane localization of this brush border enzyme and the position of the lactase hydrolase site at the mucosal surface (Wacker *et al.* 1992), that cleavage occurs extracellularly. Interestingly, inhibition of the lactose hydrolase (LH) site of the LPH complex markedly reduced luminal hydrolysis without affecting the total amount of isoflavonoid transferred to the serosal compartment. However, there was a significant alteration to the profile of isoflavonoid-derived compounds appearing in the serosal solution. One possible explanation for this is that NB-DGJ also inhibits other enzyme systems present in the enterocyte capable of metabolizing isoflavone glycosides, such as small intestinal cytosolic β -glucosidase (Day *et al.* 1998). However, our data demonstrate that under these conditions, intact daidzin can be transported into the enterocyte and exported to the serosal side. The data are also consistent with the hypothesis that the sugar transporter SGLT1, which has been implicated in the transport of flavonoid glucosides (Hollman *et al.* 1995, Gee *et al.* 1998, 2000), may also be capable of transporting isoflavonoid glycosides. Under circumstances in which LH is inhibited, raised levels of glycosides at the brush border could increase substrate concentrations for the sodium-dependent glucose transporter (SGLT1), potentially elevating transport of the glycoside by this route.

The data reported herein confirm previous findings that small intestinal epithelial cells are involved in the initial metabolism of isoflavones and flavonoids. This metabolism results in the extensive glucuronidation of these phytochemicals. Interestingly, most studies of the biological activities of isoflavonoids, in particular genistein, have shown the aglycone form to be most active (Adlercreutz and Mazur 1997). Whereas glucuronidation is considered a detoxification mechanism for xenobiotic compounds, conjugation with glucuronic acid does not necessarily always eliminate the biological activity of a particular compound. In some cases the activity may be merely modified (Burchell and Coughtrie 1989), as in the case of retinoic acid glucuronides or morphine- β -6-glucuronide compared with their respective aglycones (Kren and Martinkova 2001). In contrast, quercetin glucuronides have been reported to inhibit the pro-oxidant enzyme xanthine oxidase (Day *et al.* 2000a). Isoflavone glucuronides of daidzein and genistein have also been reported to be weakly oestrogenic and affect cells of the immune system (Zhang *et al.* 1999), and the use of glucuronides as pro-drugs is now being reported (Houba *et al.* 2001). We conclude that lactase phlorizin hydrolase may play a key role in isoflavone bioavailability since inhibition of this enzyme significantly reduces deglycosylation and alters the profile of isoflavonoid compounds crossing the intestinal mucosa, thus modifying their potential biological activities at target tissues.

Acknowledgements

This work was supported by the Office of Science and Technology through the Core Strategic Grant of the BBSRC.

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Characterization of the cytochrome P450 enzymes involved in the *in vitro* metabolism of ethosuximide by human hepatic microsomal enzymes

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Received 5 August 2002

1. The relative roles of human hepatic cytochrome P450 (CYP) subfamilies participating in ethosuximide metabolism have been studied *in vitro* using humanized heterologous CYP microsomal systems expressing either CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1 or CYP3A4.

2. Ethosuximide was incubated with each expression system at 37 °C, and its hydroxylated metabolite was quantified by HPLC assay.

3. K_m and V_{max} values for metabolite formation were estimated for CYP3A4 and CYP2E1. The K_m values for the metabolite formed were 1.40 and 0.24 mM for CYP3A4 and CYP2E1, respectively. The V_{max} values were 0.65 and 0.14 nmol mg⁻¹ protein min⁻¹ for CYP3A4 and CYP2E1, respectively.

4. These parameters could not be measured for other enzymes, since metabolite concentrations formed were below the HPLC detection limits.

5. Immuno-inhibition studies using specific antibodies against CYP3A4 or CYP2E1 revealed that ethosuximide metabolite levels decreased when the amount of added CYP3A4 or CYP2E1 antibody increased, with anti-CYP3A4 antibodies yielding a greater inhibitory effect.

6. Simulations of scaled-up *in vivo* ethosuximide CL_{hepatic} mediated by CYP3A4 and CYP2E1 based on the *in vitro* CL_{int}' values, which were calculated from the foregoing respective V_{max} and K_m values, project that well over 90% of *in vivo* CL_{hepatic} is due to CYP3A4. These results support an important role for CYP3A in human ethosuximide metabolism, and a minor role for CYP2E1.

Introduction

Ethosuximide [(*R,S*)-2-ethyl-2-methylsuccinimide, I] (figure 1) is a racemic antiepileptic drug primarily indicated for the treatment of absence seizures (Pisani and Bialer 1995). The primary metabolite of ethosuximide has been shown to be stereoisomeric forms of 2-(1-hydroxyethyl)-2-methylsuccinimide (II) (figure 1) (Horning *et al.* 1973, Pettersen 1980, Maurer 1990, Millership *et al.* 1993, 1995, Pisani and Bialer 1995), accounting for 40–60% of administered drug excreted into urine in rats and humans (Horning *et al.* 1973, Millership *et al.* 1993, Pisani and Bialer 1995). Other forms of the drug detected in urine include unchanged drug (about 10% of administered drug), glucuronide conjugates (20–40%), two other monohydroxylated forms (III and IV, about 7 and 2–3%, respectively) and a

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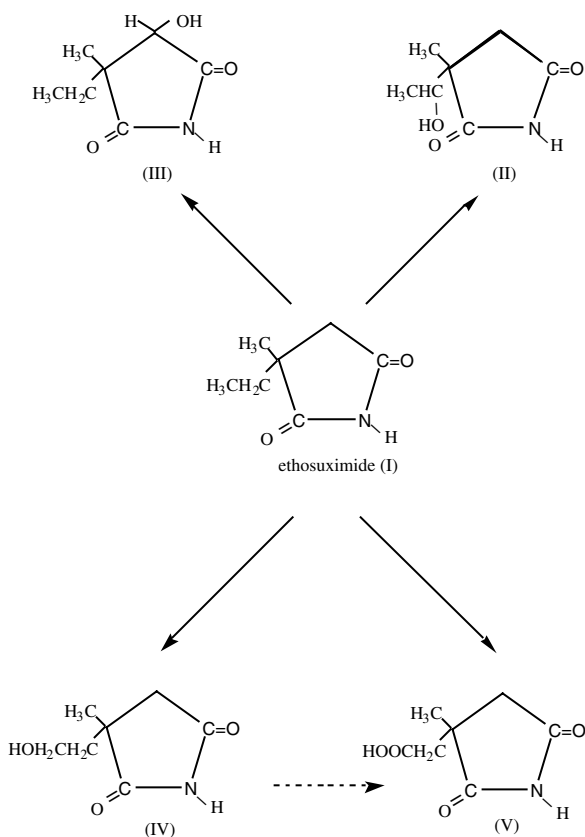


Figure 1. Structures of ethosuximide and its metabolites, (I) ethosuximide, (II) 2-(1-hydroxyethyl)-2-methylsuccinimide, (III) 2-ethyl-3-hydroxy-2-methylsuccinimide, (IV) 2-(2-hydroxyethyl)-2-methylsuccinimide and (V) 2-carboxymethyl-2-methylsuccinimide.

carboxylic acid form (V) detected at lower levels (Horning *et al.* 1973, Pettersen 1980, Pisani and Bialer 1995) (figure 1).

Ethosuximide has been investigated as a possible probe for phenotyping CYP3A activity (Bachmann *et al.* 1992, Bachmann and Jauregui 1993). Ethosuximide is well suited for use as a probe molecule in that it can be given orally, its clearance can be estimated from a single plasma or saliva sample (Bachmann and Jauregui 1993) and it is not significantly bound by plasma proteins. *In vivo* studies with rats (Bachmann *et al.* 1992) and humans (Bachmann and Jauregui 1993), as well as an *in vitro* study with isolated rat liver microsomes (Sarver *et al.* 1998), have all indicated that ethosuximide is principally oxidized by CYP3A. The present study has been undertaken to determine if the primary role of CYP3A in ethosuximide metabolism could be further validated through *in vitro* metabolism with reconstituted human microsomal systems expressing selected CYP enzymes, and through inhibition measurements with specific CYP subfamily antibodies.

Materials and methods

Human SUPERSOMES™ (CYP3A4, CYP2E1, CYP1A2, CYP2A6, CYP2D6 and CYP2C9) were purchased from Gentest Corporation (Woburn, MA, USA). NADP⁺, normal rabbit serum, specific

rabbit antisera for human CYP1A1/1A2, CYP2E1 and CYP3A4, and 3,3-dimethylglutarimide were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade water was generated on a MilliQ system made by Millipore (Bedford, MA, USA). Ethosuximide (2-ethyl-2-methylsuccinimide, $C_7H_{11}NO_2$) and all other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) and were of analytical grade or the highest grade available.

Assays

All samples were analysed by a reverse phase, gradient HPLC assay procedure (Sarver *et al.* 1998) on a Waters system with a Waters 600 controller, Waters 996 Photodiode Assay Detector, Waters 717 Autosampler and a Waters Novapak 3.9 mm $\times$ 15 cm C18 column. Analyte peaks were detected at a fixed wavelength of 195 nm. The injection volume of every sample was 100 μ l. Mobile phase components consisted of MilliQ water as mobile phase A and acetonitrile as mobile phase B. Each component of the mobile phase was drawn through a 0.4- μ m nylon filter and vacuum degassed before use. The mobile phase flow rate was 2.0 ml min⁻¹ with a gradient elution as follows: linear ramp from 3.0% B at 0 min to 35% B at 8.4 min; linear ramp from 35.0% B at 8.4 min to 3.0% at 9.0 min; held steady at 3.0% B from 9.5 min to 10.5 min. The condition was then held at 3.0% B for an 8-min washout period before injecting the next sample. Standards were prepared by mixing 250 μ l 0.2 g ml⁻¹ TCA, 400 μ l 0.1 M potassium phosphate buffer (containing 0.01 M EDTA, pH 7.4), 50 μ l of an NADPH-generating system solution (see below), 50 μ l microsomal suspensions, 250 μ l 200 μ g ml⁻¹ 3,3-dimethylglutarimide solution as internal standard, and 50 μ l of varying concentrations of ethosuximide to give final concentrations of 0, 0.0005, 0.001, 0.002, 0.004, 0.010, 0.015, 0.020, 0.050, 0.10, 0.15, 0.20, 0.40, 0.60, 0.80 and 1.00 mM. The actual range of standard concentrations varied depending upon the enzyme system under study, with the lowest standard concentrations used for CYP1A2, CYP2A6, CYP2D6 and CYP2C9 in order to enhance accuracy at the low metabolite concentrations encountered for these enzymes. Higher concentrations were used for CYP2E1 and CYP3A4. All standards were centrifuged, filtered and stored as described by Sarver *et al.* (1998). Linear calibration curves were generated using the ratio of ethosuximide peak area to internal standard peak area versus concentration. The ethosuximide calibration curves were also used to quantify the metabolite concentration based on the ratio of peak area of metabolite versus the peak area of internal standard. As noted in the original description of this method (Sarver *et al.* 1998), since there was no source of metabolite to use as a standard, the metabolite was assumed to have the same peak area response per mole as ethosuximide. The analyte peak for the metabolite was previously identified as hydroxyethylethosuximide (II) by electrospray ionization-liquid chromatography-mass spectroscopy (EIS-LCMS) (Sarver *et al.* 1998).

NADPH-generating system

The NADPH-generating system was prepared by adding NADP⁺ (5.4 mg), glucose 6-phosphate (27.2 mg), and glucose 6-phosphate dehydrogenase (1 unit) to 1.0 ml 60 mM magnesium chloride. This was then followed by a pre-incubation at 37 °C for 5 min.

Preparation of Supersomes[®] for incubation with ethosuximide

Supersome[®] suspensions were stored at -80 °C. They were thawed at 37 °C and then placed on ice before the start of each experiment. Details of the incubations were described by Sarver (1998) and are outlined further below.

Estimates of K_m and V_{max} values from metabolite formation

To characterize the kinetics using CYP3A4 35 μ l of varying concentrations of ethosuximide (4, 8, 12, 16, 20, 30, 40 and 50 mM), 50 μ l of an NADPH-generating system and 350 μ l buffer (containing 0.1 M potassium phosphate and 0.01 M EDTA, pH 7.4) were added into 0.5 ml plastic culture tubes and pre-incubated at 37 °C for 5 min before use. A total of 50 μ l thawed Supersomes[®] were added into each culture tube and incubated for 30 min in a 37 °C shaking water bath. After the 30-min incubation, the reactions were quenched by adding 250 μ l ice-cold TCA. While sitting on ice, 100 μ l 3,3-dimethylglutarimide solution was added into each incubation system as an internal standard for the HPLC assay. Samples were centrifuged, filtered and quantitated by HPLC.

The kinetics of metabolite formation using CYP2E1 Supersomes[®] were quantified similarly using 25- μ l aliquots of varying concentrations of ethosuximide (4, 8, 12, 16 and 20 mM), 50 μ l of the NADPH-generating system and 260 μ l buffer (containing 0.1 M potassium phosphate and 0.01 M EDTA, pH 7.4) were added into 0.5-ml plastic culture tubes and pre-incubated at 37 °C for 5 min before use. A total of 50 μ l thawed Supersomes[®] were added into each culture tube and incubated for 60 min in a 37 °C shaking water bath. After the 60-min incubation, the reactions were quenched by adding 100 μ l ice-cold TCA. While sitting on ice, 15 μ l 3,3-dimethylglutarimide solution was added into

each incubation system as an internal standard for the HPLC assay. Samples were centrifuged, filtered and quantitated by HPLC.

$V_{\max}$ and K_m were derived from a non-linear regression fit of the Michaelis-Menten equation calculated using KaleidagraphTM software.

Antibody inhibition

Sets of 100- μ l aliquots of each microsomal system were pre-incubated at room temperature for 30 min with amounts of the corresponding specific anti-CYP antisera to give 0, 20, 30, 50, 100, and 200 μ g antisera protein. Sufficient normal rabbit serum was added to maintain a constant total serum volume of 50 μ l in each pre-incubation system. After pre-incubation, 50 μ l 0.5 M potassium phosphate buffer (pH 7.4, 0.5 M EDTA), 10 l 80 mM ethosuximide and 50 μ l of an NADPH-generating solution were added and the system was incubated at 37 °C in a shaking water bath for 120 min. Reactions were stopped by adding 50 μ l ice-cold 0.4% TCA and placing the tubes on ice, and 50 μ l 360 μ g ml⁻¹ 3,3-dimethylglutarimide solution were added as an HPLC internal standard. Samples were centrifuged, filtered and stored as described for the HPLC assay. Incubations were performed in duplicate for each microsomal system at each antisera addition level.

In vitro CL_{int}' estimates and scale-up assessment of the relative roles of CYP3A4 and CYP2E1 in the metabolism of ethosuximide

CL_{int} was calculated for a wide range of ethosuximide concentrations as:

$$CL'_{int} = \frac{V_{\max}}{K_m + [S]} \quad (1)$$

where $V_{\max}$ and K_m were experimentally derived and $[S]$ is the ethosuximide concentration.

Results

HPLC analysis

Retention times were approximately 6.8 min for the ethosuximide metabolite, 8.9 min for the internal standard and 9.7 min for ethosuximide. All calibration curves gave correlation coefficients of 0.99 or higher. Within-day variation measured by triplicate injections across the concentration range 0.02–1.0 mM was 1.2–9.2%, and between day variation for the same concentrations was 4.8–9.4%.

Metabolite formation over time for different CYPs

For CYP3A4, a linear relationship was maintained between time and the metabolite concentration within the first 30 min of incubation (figure 2), and a 30-min incubation was chosen in all subsequent experiments to determine the K_m and $V_{\max}$ values of metabolite formation by CYP3A4.

For CYP2E1 metabolite formation did not plateau until after 90 min (figure 3). We used a 60-min incubation time to determine the K_m and $V_{\max}$ values for metabolite formation by CYP2E1.

For CYP1A2 the metabolite concentrations found in samples incubated for as long as 120 min were below the detection limit of HPLC assay. For CYP2D6 metabolite concentrations after 90 min, incubations were below the detection limit of the HPLC assay. For CYP2A6 and CYP2C9 no metabolite could be detected at any time, even though the assay detection limit was 0.0005 mM (i.e. 40 times lower than for the CYP3A4 system and four times lower than for the CYP2E1 system).

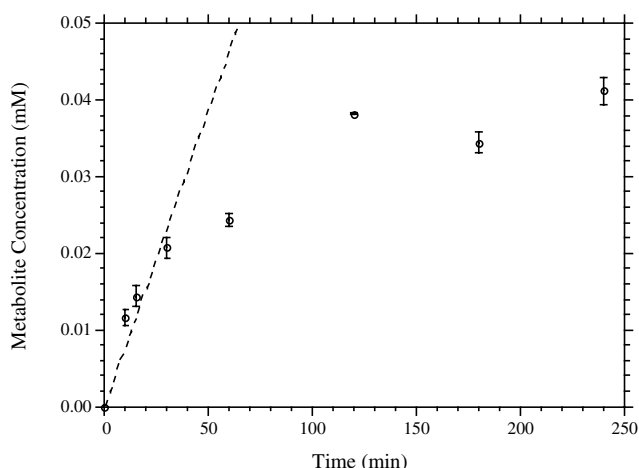


Figure 2. Metabolite formation of ethosuximide over time for CYP3A4 (0–240 min). The x-axis is the incubation period and the y-axis the concentration of metabolite. A linear relationship was maintained between time and the metabolite concentration within the first 30 min of incubation. Data are the mean $\pm$ SD of triplicate determinations.

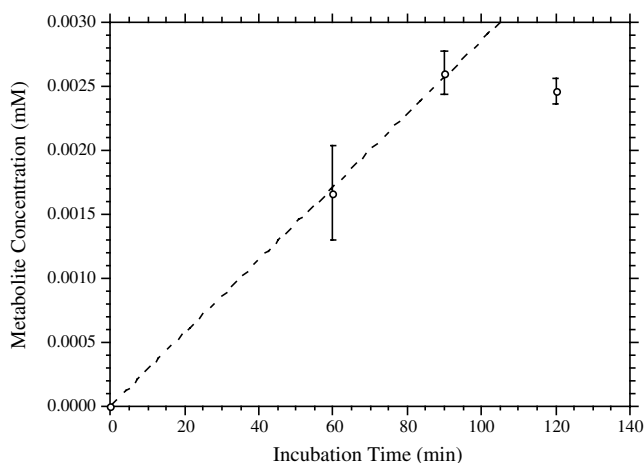


Figure 3. Metabolite formation of ethosuximide over time for CYP2E1. Data are the mean $\pm$ SD of triplicate determinations.

K_m and V_{max} values of metabolite formation by CYP3A4 and CYP2E1

The K_m and V_{max} values for CYP3A4 and CYP2E1 are shown in table 1. The rate of metabolite formation as a function of ethosuximide concentration is shown for CYP3A4 and CYP2E1 in figures 4 and 5, respectively. For all the other enzymes, the metabolite concentrations formed from those incubation systems were below the limits of HPLC detection even if a 2-h incubation was used, therefore precluding estimates of V_{max} and K_m .

CL'_{int} was scaled to whole liver values based on the following scaling factors:

- CYP content of $0.43 \text{ nmol mg}^{-1}$ protein (Iwatsubo *et al.* 1997).
- $52.5 \text{ mg protein g}^{-1}$ liver (Iwatsubo *et al.* 1997).

Table 1. K_m and V_{max} values of metabolite formation by CYP3A4 and CYP2E1.

CYP	K_m (mM)	V_{max} (nmol mg ⁻¹ protein min ⁻¹)	V_{max} (nmol protein ⁻¹ min ⁻¹)
3A4	1.40	0.65	0.0038
2E1	0.24	0.14	0.00031

Data are derived from triplicate determinations at each substrate concentration.

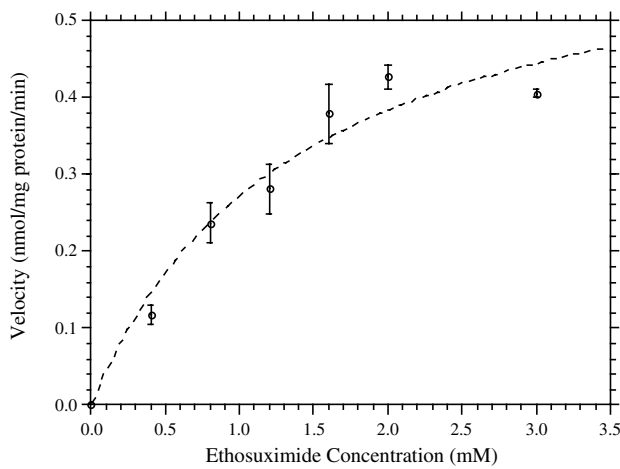


Figure 4. Determination of K_m and V_{max} values of metabolite formation of ethosuximide by CYP3A4 (Michaelis and Menten model). The curve shows a non-linear regression fit yielding parameter values in table 1 ($r^2 = 0.964$). Data are the mean $\pm$ SD of triplicate determinations.

- Liver mass of 1500 g (Diem and Lentner 1970).
- CY3A4 represents 30% of total hepatic CYP content (Shimada *et al.* 1994).
- CYP2E1 represents 7% of total hepatic CYP content (Shimada *et al.* 1994).

Applying the foregoing values, the CL'_{int} for ethosuximide clearance via formation of the hydroxyethyl metabolite was calculated as 27.3 ml min⁻¹ via CYP3A4 and 1.4 ml min⁻¹ for CYP2E1. Since these rates are much smaller than hepatic blood flow (1500 ml min⁻¹), they approximate hepatic clearance ($CL_{hepatic}$). A simulation of ethosuximide $CL_{hepatic}$ values based on the rate of metabolite formation and calculated for both CYP3A4 and CYP2E1 is shown in figure 6. It encompasses a range of ethosuximide concentrations that would be considered as therapeutic.

Immuno-inhibition studies

The inhibition of the CYP2E1 and CYP3A4-enriched microsomal systems with the corresponding specific anti-CYP antibodies is shown in figure 7. In both microsomal systems, the ethosuximide metabolite levels decreased as the amount of added antibody increased. The CYP3A4 system exhibited a visibly higher level of inhibition than the CYP2E1 system at all but the highest levels of added antisera. The differences from antisera free incubations (100% level) were sig-

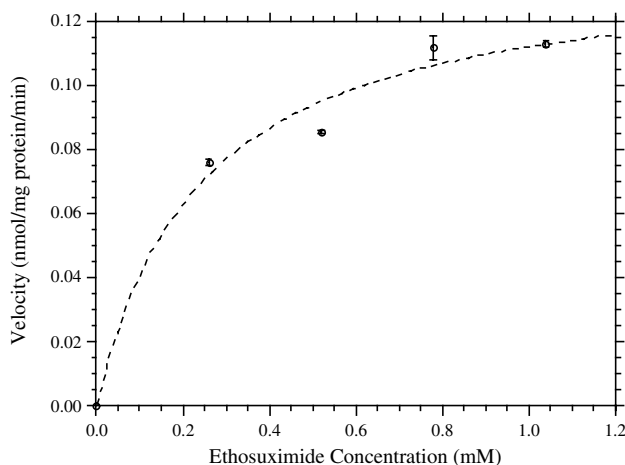


Figure 5. Determination of K_m and V_{max} values of metabolite formation of ethosuximide by CYP2E1 (Michaelis and Menton model). The curve shows a non-linear regression fit yielding parameter values in table 1 ($r^2 = 0.984$). Data are the mean $\pm$ SD of triplicate determinations.

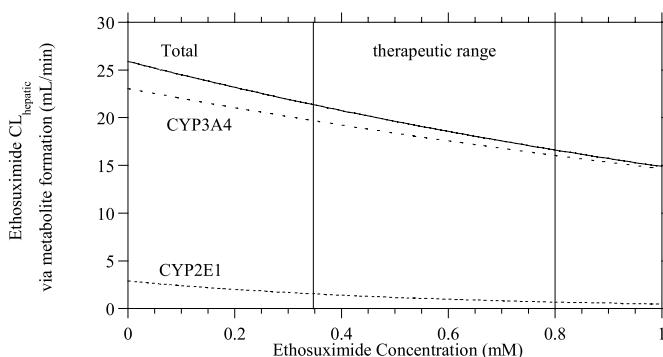


Figure 6. Simulated clearance of ethosuximide via formation of hydroxylated metabolite by CYP2E1 and CYP3A4, separately and combined (total).

nificantly different for all antisera levels in the CYP3A4-enriched/anti-CYP3A4 system, whereas the CYP2E1-enriched/anti-CYP2E1 system differences were significant for each antisera addition except for the lowest of 20 μ g protein.

Discussion

These findings have extended our earlier observations pertaining to the roles of CYP enzymes in the metabolism of ethosuximide in rats (Bachmann *et al.* 1992, Sarver *et al.* 1998) and humans (Bachmann and Jauregui 1993). From those studies, we inferred that CYP3A4 was largely responsible for the disposition kinetics of ethosuximide in humans. Based on our findings in rats, a role for CYP2E1 was also deemed probable. Our previous findings as well as those reported here are based on the formation of a hydroxylated metabolite of ethosuximide – the only metabolite we could detect in our *in vitro* microsomal systems.

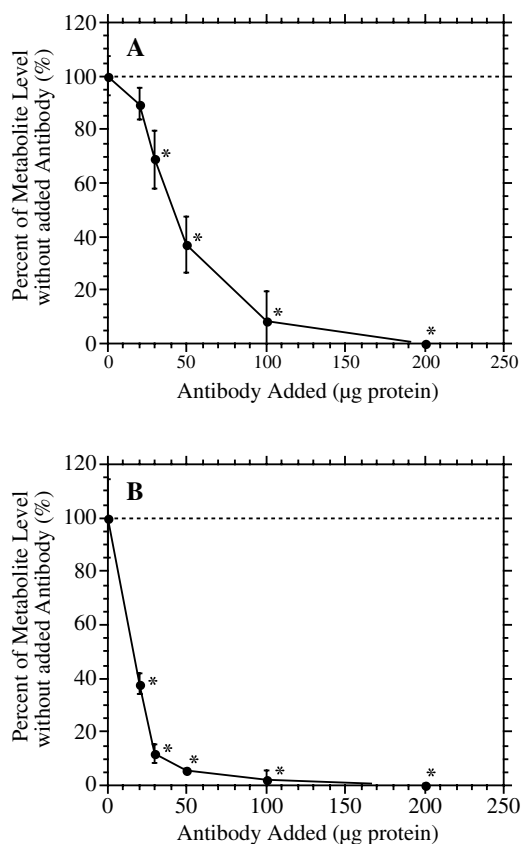


Figure 7. Inhibition of ethosuximide metabolite formation by antibodies to specific CYP isoforms. (Plot A) Results for the CYP2E1 expression system incubated with anti-CYP2E1 antibodies; (plot B) results for the CYP3A4 system incubated with anti-CYP3A4 antibodies. The initial metabolite concentration in Plot A was $32.2 \text{ nmol ml}^{-1}$, and in Plot B was 4.8 nmol ml^{-1} . *Significant difference ($p < 0.05$) versus the same system without added antibody. Data are the mean $\pm$ SD of triplicate determinations.

Specific antibodies directed against CYP3A4 and CYP2E1 inhibit ethosuximide metabolite formation, and the inhibition is more pronounced for the CYP3A4 system after addition of small amounts of anti-CYP3A4 antibody than that for the CYP2E1 system after the addition of small amounts of anti-CYP2E1 antibody. Neither anti-human CYP3A4 antibody nor anti-human CYP2E1 antibodies exhibit detectable cross-reactivities with other CYP enzymes (Gentest Corporation 1997).

The V_{max} and K_m values for metabolite formation by CYP3A4 and CYP2E1 reported here verify that CYP3A4 plays an important role in the metabolism of ethosuximide. CYP2E1 also participates in the metabolism of ethosuximide, but the role is relatively minor, even though the K_m value for CYP2E1 is smaller than for CYP3A4. However, the lower K_m value for CYP2E1 is very much in accord with expectations based on molecular volume (Lewis 2000) since ethosuximide is a relatively small molecule with a molecular weight of 141 Daltons. On the other hand, since no detectable amount of metabolite was formed when ethosuximide

was incubated with CYP1A2, CYP2A6, CYP2D6 or CYP2C9 *in vitro*, it was concluded that these enzymes do not play significant roles in the biotransformation of ethosuximide. We have not investigated a potential role for CYP2C19 in ethosuximide metabolism, although no existing pharmacogenetic or epidemiological data implicate this enzyme to our knowledge.

Estimating intrinsic *in vitro* clearances by each enzyme, and scaling to *in vivo* CL_{hepatic} values, we evaluated the relative roles of CYP3A4 and CYP2E1 in the metabolism of ethosuximide via formation of the hydroxylated metabolite. This scaling took account of the differences in hepatic content of CYP2E1 and CYP3A4, which represent about 7 and 30% of total hepatic CYP content, respectively (Shimada *et al.* 1994). Over the therapeutic plasma concentration range of ethosuximide, the estimated CL_{hepatic} projected exclusively from metabolite formation is approximately 28.4 ml min^{-1} . This compares favourably with reported *in vivo* total clearances of 13.3 ml min^{-1} (Thummel and Shen 2001). Moreover, more than 90% of the computed CL_{hepatic} was due to CYP3A4 (figure 6). The incorporation of relative activity factors (RAF) for scaling purposes was not performed. For CYP3A4, the ratio of RAF: immunoquantified CYP content has been shown to be close to unity (about 0.87) (Venkatakrishnan *et al.* 2000). Likewise, Shimada *et al.* (1999) have shown that values of $V/[S]$ predicted from a CYP2E1 expression system are virtually identical to those measured using human liver microsomes, suggesting that the RAF-immunoquantified CYP content for CYP2E1 is also close to unity.

In all studies of ethosuximide metabolism to date, biotransformation has been the main route of ethosuximide elimination. *In vivo* studies in rats encompassing the use of both selective inducers and inhibitors of the CYP enzymes suggested that CYP3A4 and CYP2E1 were the only CYP enzymes to play a significant role in ethosuximide clearance in that species (Bachmann *et al.* 1992), and *in vivo* studies in humans using CYP inducers also implicated primarily CYP3A4 in the human clearance of ethosuximide (Bachmann and Jauregui 1993). In rats, imidazole-induced increases in ethosuximide clearance correlated with the extent of imidazole-induced hepatic CYP3A4 mRNA content and with imidazole-induced increases in erythromycin demethylase activity (Slama *et al.* 1998). Giaccone *et al.* (1996) compared the oral clearances of ethosuximide in 10 patients receiving anticonvulsants that are known inducers of CYP3A4 with ethosuximide clearances in 12 control subjects and found a 66% faster oral clearance among the anticonvulsant-treated patients. They concluded that the effect probably occurred as a result of anticonvulsant-induced stimulation of CYP3A and that the phenomenon was likely to be clinically relevant *vis-à-vis* the ethosuximide dose requirements in epileptic patients taking other anticonvulsant drugs.

Our *in vitro* study of human hepatic microsomal metabolism of ethosuximide using specific antibodies and microsomal systems selectively expressing CYP3A4, CYP2E1, CYP2A6, CYP2D6, CYP1A2 and CYP2C9 revealed that CYP3A4 plays an important role in metabolizing ethosuximide and that CYP2E1 may play a minor role in the biotransformation of ethosuximide *in vitro*. These differences are amplified *in vivo* owing to the greater hepatic content of CYP3A4 than CYP2E1 such that over 90% of the hepatic clearance of ethosuximide to its hydroxylated metabolite can be accounted for by CYP3A4.

The current study agrees with the results of *in vivo* studies in humans and rats and an *in vitro* study that used rat microsomes enriched with individual CYP

enzymes in affirming the role of cytochrome P4503A as a key enzyme in ethosuximide metabolism. These results have both fundamental and clinical implications. Ethosuximide may have a role as a probe for phenotyping human CYP3A activity and for clinically evaluating the influences of drugs, chemicals and diseases on this important enzyme subfamily. Several probes already exist for phenotyping human CYP3A activity, however none satisfy all of the validating criteria proposed by Watkins (1994). Correlations between the erythromycin breath test and urinary ratios of 6- β -cortisol/free cortisol were quite poor (Hunt *et al.* 1992, Watkins *et al.* 1992). Likewise, alfentanil clearance and the erythromycin breath test correlated poorly (Krivoruk *et al.* 1994), and urinary dapsone recovery ratios failed to correlate with erythromycin breath test (Stein *et al.* 1996). In fact, urinary 6- β -hydroxycortisol/cortisol ratios, dapsone recovery ratios and the erythromycin breath test all failed to correlate with one another (Kinirons *et al.* 1993).

Midazolam has also been recommended as an *in vivo* probe of human CYP3A activity (Thummel *et al.* 1994a, b). However, a poor correlation between the erythromycin breath test and midazolam clearance has recently been demonstrated and ascribed to the differential role of P-glycoprotein in the processing of erythromycin versus midazolam (Kinirons *et al.* 1999). However, midazolam is subject to significant non-hepatic presystemic clearance (Paine *et al.* 1996, Thummel *et al.* 1996), which may limit its utility as an orally administered probe for evaluating the influence of drugs, chemical exposures or diseases on hepatic CYP3A activity in humans *in vivo*. Ethosuximide, on the other hand, is metabolized too slowly to exhibit significant presystemic clearance. It is virtually unbound to plasma proteins and its systemic clearance can be estimated from a single plasma concentration measurement (Bachmann and Jauregui 1993).

In conclusion, our current *in vitro* findings are consistent with those from several *in vitro* and *in vivo* studies in rats and humans implicating CYP3A4 as a key enzyme responsible for ethosuximide metabolism. Correlative studies in humans comparing ethosuximide clearance versus clearance or point estimates of the ratios of metabolite to parent of other *in vivo* CYP3A4 probes such as midazolam, triazolam, alfentanil or dapsone in subjects treated with selective CYP3A4 inducers and inhibitors will be required in order to establish a place for ethosuximide as an *in vivo* probe of CYP3A.

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Non-synonymous single nucleotide alterations in the microsomal epoxide hydrolase gene and their functional effects

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Received 1 August 2002

1. By sequencing genomic DNA from 72 established cell lines derived from Japanese individuals, we detected 25 single nucleotide alterations in the microsomal epoxide hydrolase (EPHX1) gene. Of them, five were exonic alterations resulting in amino acid alterations (77C > G, T26S; 128G > C, R43T; 337T > C, Y113H; 416A > G, H139R; 823A > G, T275A). The T26S, R43T, Y113H and H139R substitutions were found at relatively high frequencies and seemed to be polymorphic, and T26S and T275A were novel.

2. To examine the effects of these amino acid alterations on EPHX1 function, EPHX1 cDNA constructs of wild-type and five variants were transfected into COS-1 cells, and their hydrolytic activities for *cis*-stilbene oxide were determined *in vitro*. Although all of the transfectants expressed EPHX1 mRNA and protein at similar levels, the variant H139R protein was expressed at a significantly higher level (128% of the wild-type). K_m values were not significantly different between the wild-type and variants.

3. Increase (140%) in the enzymatic activity (V_{max}) of the variant H139R was accompanied by the increased EPHX1 protein level without any significant change in the intrinsic EPHX1 activity. On the other hand, the variant R43T showed increased values for V_{max} and clearance (V_{max}/K_m) (around 130%) both on a microsomal protein basis and on a EPHX1 protein basis.

4. These results suggest that R43T as well as H139R increase epoxide hydrolase activity.

Introduction

Microsomal epoxide hydrolase (EPHX1) is a member of an α/β -hydrolase family (Seidegard and DePierre 1983, Fretland and Omiecinski 2000). The enzyme catalyses the hydrolysis of a wide range of arene and aliphatic epoxides by the *trans*-addition of water to form water-soluble dihydrodiols (Watabe *et al.* 1981).

The human EPHX1 gene is assigned to chromosome 1q42.1 and consists of nine exons. This enzyme is a type-I membrane protein and is expressed at the cytosolic side of the endoplasmic reticulum membrane in all examined tissues with the highest activity in the liver (Oesch *et al.* 1977). Although EPHX1 may participate in the metabolism of endogeneous steroids (Vogel-Bindel *et al.* 1982), the enzyme has been characterized extensively with regard to exogeneous

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drugs and chemicals as substrates. In these cases, the majority of epoxides are thought to be formed by cytochrome P450-mediated oxidation (Jerina 1983, Seidegard and DePierre 1983). For example, the anti-epileptic drug carbamazepine is metabolized by CYP3A4 into its 10,11-epoxide form, which is still pharmacologically active. EPHX1 transforms this epoxide into the inactive 10,11-diol form, which is a major urinary metabolite of carbamazepine (Tybring *et al.* 1981). EPHX1 also plays important roles in the detoxication of metabolically formed hazardous epoxides of chemicals such as styrene 7,8-epoxide, benzo[a]pyrene 4,5-epoxide amongst others (Seidegard and DePierre 1983, Christou *et al.* 1989, Fretland and Omiecinski 2000). Thus, EPHX1 is an important factor when considering the efficacy of drugs and chemical-induced carcinogenesis.

Information on single nucleotide polymorphisms (SNPs) of drug-metabolizing enzymes has increased in recent years. As for EPHX1, the rates of EPHX1-catalysed benzo[a]pyrene-4,5-epoxide dihydration have been reported to differ by about 40-fold in the panels of human blood lymphocytes (Omiecinski *et al.* 1993), and this difference has been suggested to be attributed to genetic polymorphisms of EPHX1. Hassett *et al.* (1994) reported on two polymorphisms (Y113H and H139R) and analysed their effects on enzyme function using benzo[a]pyrene-4,5-epoxide as a substrate. Raaka *et al.* (1998) reported seven SNPs in the promoter region of the gene, three of which reduced the transcriptional activity by 30% in a reporter gene assay. However, there have been no other reports on the association between EPHX1 polymorphisms and the functional changes. Recently, Saito *et al.* (2001) reported on 33 polymorphisms of the EPHX1 gene including 16 novel ones using genomic DNA from 48 Japanese individuals. However, no further non-synonymous variation except for the above two (Y113H and H139R) were reported.

In the present study, we sequenced the entire exons and surrounding introns of the EPHX1 gene using 72 established cell lines derived from different Japanese individuals, and detected 25 single nucleotide alternations including 11 novel ones. For five non-synonymous SNPs, we assessed the changes in metabolic activities for an EPHX1-specific substrate, *cis*-stilbene oxide (Seidegard *et al.* 1984).

Materials and methods

Materials

cis-Stilbene oxide and its metabolite, dihydrobenzoin, were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Human EPHX1-expressed microsomes were obtained from Gentest Corporation (Woburn, MA, USA) through Daiichi Pure Chemicals (Tokyo, Japan). [α - ^{32} P] deoxy CTP, ^{125} I-labelled anti-rabbit Ig [F(ab')₂ fragment], Megaprime DNA Labelling Systems and ProbeQuant G-50 Micro Columns were from Amersham Pharmacia Biotech. (Amersham, UK). Human liver QUICK-Clone cDNA was obtained from Clontech (Palo Alto, CA, USA). LIPOFECTAMINE PLUS reagent and Eukaryotic TA Cloning Kit were from Invitrogen (Groningen, the Netherlands). Blood and Cell Culture DNA Kit, DyeEx96 kit, QIAquick Gel Extraction Kit and RNeasy Mini kit were purchased from Qiagen (Hilden, Germany). Rabbit anti-human EPHX1 peptide antibody was prepared by immunization of peptide (from the 54th to the 71st amino acids) coupled with bovine serum albumin. This antibody specifically reacts with the EPHX1 protein, giving a single band in Western blotting. Rabbit anti-calnexin (CNX) carboxyl terminus polyclonal antibody was from StressGen Biotechnologies Corp. (Victoria, BC, Canada). All other chemicals and reagents used were of the highest grade available.

DNA extraction

Established cell lines derived from 72 Japanese individuals were obtained from the Health Science Research Resources Bank (Osaka, Japan) or directly from the Japanese Collection of Research Bioresources, National Institute of Health Sciences (Tokyo, Japan). The cells were cultured according

to the banks' instructions. DNA extraction was carried out from approximately 5×10^7 cells using a Blood and Cell Culture DNA Kit. The concentrations of the extracted DNA were determined by UV absorbance and the DNA solutions were stored at 4°C until sequence analysis.

Sequencing of EPHX1 exons

At first, the entire EPHX1 gene was amplified from genomic DNA (100 ng) using 1.25 units Z-Taq (Takara Shuzo, Tokyo, Japan) with the EPHX1-specific primer set (0.2 µM) listed in table 1. The first PCR conditions were 30 cycles of 98°C for 5 s, 55°C for 5 s and 72°C for 190 s. Then, each exon was amplified by Ex-Taq (0.625 units) (Takara Shuzo) with the appropriate set of EPHX1-specific primers (0.5 µM) designed in intronic regions based on a genomic contig sequence (NT_004525) (table 1). The second PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min. Then the PCR products purified using a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) were directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The excess dye was removed with a DyeEx96 kit. The eluates were analysed on an ABI Prism 3700 DNA Analyser (Applied Biosystems).

Plasmid construction

A full-length wild-type EPHX1 fragment was amplified from human adult normal liver Quick-Clone cDNA. The nucleotide sequence used for the forward primer was 5'-TGAGGTACAGGAGC-CATGTG-3', and that for the reverse primer was 5'-GGGAGAGGGGTGGGTCATT-3'. The PCR products were cloned directly into the pCR3.1 vector using the Eukaryotic TA Cloning Kit. Five single EPHX1 variants (77C > G, T26S; 128G > C, R43T; 337T > C, Y113H; 416A > G, H139R; 823A > G, T275A) were constructed with a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the wild-type EPHX1/pCR3.1 plasmid as a template. All the sequences of the wild-type and its variants were confirmed by DNA sequencing.

Cell culture and transient transfection

COS-1 cells, an African green monkey kidney cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) with non-essential amino acids, penicillin, streptomycin and 10% foetal bovine serum under 5% CO₂ at 37°C. The cells (2×10^6) were seeded in a 10 cm culture dish 1 day before transfection. After 24 h of culture, the cells were rinsed with serum-free DMEM. Ten micrograms of wild-type EPHX1, its five single variant plasmids or vacant pCR 3.1 plasmid was transfected using the LIPOFECTAMINE PLUS reagent according to the manufacturer's instructions. After 30 h, the cells were harvested for microsomal preparation or total RNA extraction with a RNeasy kit.

Microsomal preparations

Microsomes from transfected COS-1 cells were prepared by sequential centrifugation according to a standard procedure described by Ekins *et al.* (1999). After centrifugation, the microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.4) and stored at -90°C until use. Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using IgG as a standard.

Table 1. Primer pairs for amplifying the entire gene and each exon of epoxide hydrolase.

Exon	Forward primer	Reverse primer	PCR product (bp)
1-9	5'-CACCAGGCTAATCAAGAACA-3'	5'-GTCCAGGCTCCTAGCACAACT-3'	
1	5'-TCCCAGATTCCCTTCTTTTA-3'	5'-AGGAGGCTGCAGCATAATA-3'	479
2	5'-AATAAAATCAGGGACAGGGT-3'	5'-AGGAAAAGCAGACTGAAACA-3'	456
3	5'-AGGGTTTGCTGTGTTTCTTG-3'	5'-AATTGGGTTCTGAATCTCTCC-3'	378
4	5'-TTACTGTCAATACCATGAAGGG-3'	5'-CCCTCTGGTGTTCTATGAAA-3'	642
5	5'-TTTCATAGAACACCAGAGGG-3'	5'-GCACACAGGTCCACTTCTTA-3'	288
6	5'-AGCAGAACTCCCCAGAACT-3'	5'-CTGGGAGGTCCTTACAAGA-3'	534
7	5'-ATGTGAGCACCCAGGAGTTA-3'	5'-CAGGGTGAGGATTTGTAAGA-3'	405
8	5'-ACACGAGGATACCACACAG-3'	5'-CCTTTAGTGTTCCCATCCAA-3'	420
9	5'-TGTCCTTTTCTTTATGGCTCC-3'	5'-CGTCATGTCACTGAGTTCC-3'	575

Northern blot analysis

As a template of the hybridization probe for EPHX1, wild-type EPHX1/pCR3.1 plasmid was cut with Apa I and Kpn I, and the fragment was extracted from agarose gel with a QIAquick Gel Extraction Kit. For a control probe, a 983-bp fragment of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was amplified with reverse-transcribed total RNA from COS-1 cells using a set of forward (5'-TGAAGGTCGGAGTCAACGGATTTGGT-3') and reverse (5'-CATGTGGGCCATGAGGTCCACCAC-3') primers (Clontech). These fragments were labelled with [α - ^{32} P] deoxy CTP using Megaprime DNA labelling systems and purified with ProbeQuant G-50 Micro Columns. Northern blotting was performed with glyoxal as a denaturant as described by Brown and Mackey (1997). Total RNAs (5 μ g) were fractionated by electrophoresis on a 1.0% agarose gel, and transferred to a nylon membrane (GeneScreen Plus, Du Pont, Boston, MA, USA) and baked at 80°C for 2 h. The membrane was prehybridized and then hybridized with the ^{32} P-labelled DNA probe for EPHX1 or G3PDH at 42°C overnight. The membrane was washed twice with $2 \times$ SSC/0.1% SDS for 5 min, then twice with $0.1 \times$ SSC/0.1% SDS for 30 min at 42°C, and analysed with Bioimage Analyser BAS-1500, a quantification system for autoradiography with a dynamic range of 10^4 , which is 100-fold better than X-ray film (Fuji Film, Tokyo, Japan).

Western blot analysis

To determine the transiently expressed levels of EPHX1 and its variants in COS-1 cells, microsomal fractions (30 μ g proteins) were dissolved in SDS-sample buffer, electrophoresed on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose sheets (Schleicher & Schuell, Inc., Dassel, Germany). For immunostaining, either rabbit anti-human EPHX1 polyclonal antibody or rabbit anti-calnexin (CNX) polyclonal antibody as the primary antibody, and ^{125} I-labelled anti-rabbit Ig [F(ab') $_2$ fragment; 2×10^6 cpm] as the second antibody were used, and data were analysed with BAS-1500. The quantified values for the wild-type and all the variant EPHX1 proteins were within the linear range that was obtained with the various amounts of the human EPHX1-expressed microsomes. The immunoreactivity of EPHX1 was normalized to that of CNX to assess the endoplasmic reticulum (ER) content in the microsomal fractions.

Assay for hydrolysis of *cis*-stilbene oxide

Hydrolysis of *cis*-stilbene oxide was determined by measurement of the formation rate of dihydrobenzoin, according to the method of Kitteringham *et al.* (1996) with some modifications. The incubation mixture contained *cis*-stilbene oxide (1.25–160 μ M) and the microsomal fraction (50 μ g ml $^{-1}$ protein) of COS-1 cells transfected with either wild-type EPHX1, its five single variant plasmids or vacant pCR 3.1 plasmid in 50 mM Tris-HCl buffer (pH 8.0) in a final volume of 200 μ l. *cis*-Stilbene oxide was dissolved in ethanol (final concentration in reaction medium, 1% v/v). After pre-incubation at 37°C for 2 min, the reaction was started by the addition of *cis*-stilbene oxide. The mixture was incubated at 37°C for 10 min and stopped with 200 μ l ice-cold acetonitrile with vortexing. The samples were centrifuged at 6000g for 20 min at 4°C. The supernatant was filtered with a polytetrafluoroethylene membrane filter of 0.2 μ m pore size (Millipore, Bedford, MA, USA), and a 40- μ l portion was subjected to high-performance liquid chromatography (HPLC). HPLC analysis was performed using an LC-VP system (Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-80A column (5 μ m, 150 $\times$ 4.6 mm i.d., GL Sciences, Tokyo, Japan). The elution was performed isocratically with water-acetonitrile (70:30 v/v) for 10 min, followed by a 5-min linear gradient water-acetonitrile (45:55 v/v), and held for 15 min at a flow rate of 1.2 ml min $^{-1}$. The UV wavelength was fixed at 210 nm, and the column was maintained at 40°C. The standard samples were treated in the same manner as the incubation samples. Under these conditions, the retention times of dihydrobenzoin and *cis*-stilbene oxide were 8.5 and 23.4 min, respectively. The detection limit of dihydrobenzoin was 10 pmol/assay. The intra- and interday variation coefficients did not exceed 6% in any of the assays. Kinetic parameters such as K_m and V_{max} were estimated using the computer program Prism v.3.0a (GraphPad Software, San Diego, CA, USA) designed for non-linear regression analysis of a hyperbolic Michaelis-Menten equation. Data are presented as mean $\pm$ SE of five to nine experiments derived from two to four separate transfections and were analysed with one-way ANOVA, after which multiple comparisons were made with the Scheffé test.

Results

EPHX1 gene polymorphisms

By sequencing all the nine exons and their flanking introns of the extracted genomic DNA from 72 established Japanese cell lines, we detected 25 single nucleotide alternations in the EPHX1 gene (table 2). Thirteen were located in the

Table 2. Summary of detected SNPs in the EPHX1 gene of 72 cell lines.

Location	Alterations (on NT_004525)	Position (from A in ATG)	Amino acid alterations	Allele frequency	Reference
Exon 1	1792781 G > T	-214		0.014	IMS-JST 083746
	1792777 G > A	-210		0.208	IMS-JST 083747
	1792712 A > G	-145		0.007	
	1792685 A > T	-118		0.007	
	1792656 T > C	-89		0.007	
Intron 1	1792616 T > C	-49		0.007	
	1789435 G > A	IVS1 -45		0.139	
	1789421 G > A	IVS1 -29		0.139	
Exon 2	1789311 C > G	+77	T 26 S	0.021	
	1789260 G > C	+128	R 43 T	0.035	Gaedigk <i>et al.</i> (1994)
Intron 2	1789185 G > A	IVS2 +20		0.229	IMS-JST 083754
Exon 3	1786291 G > A	+231	E 77 E	0.028	
	1786185 T > C	+337	Y 113 H	0.368	NCBI rs1051740 ^a
	1786165 G > A	+357	K 119 K	0.375	NCBI rs2292566 ^b
Intron 3	1786074 G > A	IVS3 +84		0.007	
Exon 4	1779412 A > G	+416	H 139 R	0.326	NCBI rs2234922 ^c
Intron 4	1779202 G > A	IVS4 +34		0.014	NCBI rs2292567 ^d
Exon 6	1778270 C > T	+741	H 247 H	0.007	Gaedigk <i>et al.</i> (1994)
	1778188 A > G	+823	T 275 A	0.014	
	1778159 C > T	+852	P 284 P	0.104	NCBI rs2292568 ^e
Exon 8	1773589 C > T	+1071	N 357 N	0.243	NCBI rs1051741 ^f
Exon 9	1772922 T > C	+1216	L 406 L	0.007	NCBI rs2234700
	1772890 G > A	+1248	K 416 K	0.056	IMS-JST 105433
	1772788 G > C	+1350	S 450 S	0.118	IMS-JST 105434
3'-Non-coding	1772735 A > C	+1403		0.056	

^{a-f} Refer to the IMS-JST database; 105421 (a), 105422 (b), 105424 (c), 105425 (d), 105429 (e) and 105430 (f).

coding exons, six were in the 5'-non-coding region (exon 1), five were in the introns and one was in the 3'-untranslated region (3'-UTR). Most of the alterations were found in more than two cell lines, suggesting they are single nucleotide polymorphisms (SNPs). There was no apparent linkage between the detected SNPs except that the SNPs at the positions of 1789435 and 1789421 (NT_004525) showed a perfect linkage.

Seven SNPs were reported earlier in the dbSNP database by the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/SNP/index.html>). Eleven SNPs including six SNPs publicized in the NCBI database have also been reported in the IMS-JST SNPs database of the University of Tokyo and the Japan Science and Technology Corporation (<http://snp.ims.u-tokyo.ac.jp>). The SNPs at 1789260 and 1778270 have only been reported by Gaedigk *et al.* (1994). The other 11 SNPs are novel.

Of the 13 SNPs in the coding exon, five resulted in altered amino acids (77C > G, T26S; 128G > C, R43T; 337T > C, Y113H; 416A > G, H139R; 823A > G, T275A). The SNPs at 1789311 (77C > G, T26S) and 1778188 (823A > G, T275A) on NT_004525 were novel.

Expression of EPHX1 mRNA

To compare the expression levels of EPHX1 mRNAs between the wild-type and the variants, Northern blot analysis was performed using total RNA isolated



Figure 1. Northern blot analysis of EPHX1 and G3PDH mRNAs. Total RNA (5 μ g) isolated from COS-1 cells transfected with either wild-type EPHX1, its five single variant plasmids or vacant plasmid were electrophoretically separated, transferred to a membrane and hybridized with ^{32} P-labelled DNA fragment of EPHX1 or G3PDH (as control). Representative data of three independent experiments are shown. Lanes 1, vacant plasmid; 2, wild-type; 3, T26S; 4, R43T; 5, Y113H; 6, H139R; 7, T275A.

from COS-1 cells transfected with either wild-type EPHX1, its five single variant plasmids, or the vacant pCR 3.1 plasmid. EPHX1 mRNA was not detected in the mock-transfected COS-1 cells (figure 1). When the levels of the EPHX1 mRNAs were normalized to the housekeeping gene G3PDH mRNA, there were no differences in the levels of EPHX1 mRNAs between the wild-type and the variants. This result suggested that all the transfected plasmids expressed their mRNAs at the same levels.

Expression of EPHX1 immunoreactive protein

To determine the effect of the amino acid substitution on the expression levels of EPHX1 proteins, Western blot analyses were performed with the microsomal fractions of COS-1 cells transfected with vacant, wild-type or its variant plasmids. Five independent experiments were performed, and representative data were shown in figure 2. The amounts of EPHX1 protein were normalized to an ER-resident protein, calnexin (CNX), and were shown as relative levels to wild-type EPHX1 protein set as 100%. The mean level ($n = 5$) of EPHX1 protein expressed in the variant H139R was significantly increased to 128% of the wild-type

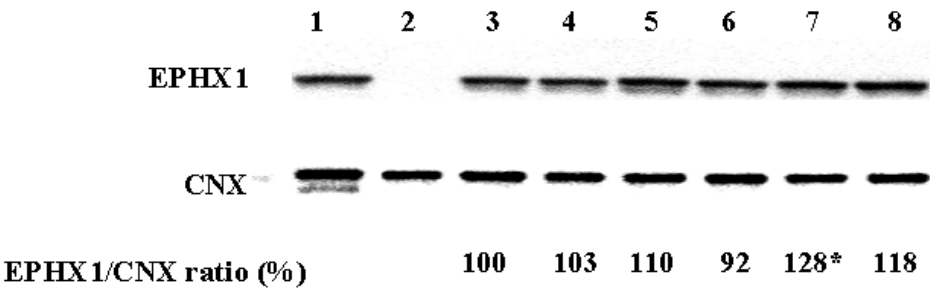


Figure 2. Western blot analysis of EPHX1 and CNX proteins. Microsomal protein (30 μ g) from COS-1 cells transfected with either wild-type EPHX1, its five single variant plasmids or vacant plasmid were electrophoretically separated, transferred to a membrane and immunoblotted with anti-EPHX1 or anti-CN (as control) antibody. Human EPHX1-expressed microsome obtained from Gentest was used as an EPHX1 standard. Representative data of five independent experiments are shown. Mean EPHX1/CNX signal ratios were calculated with the wild-type as 100%, and indicated below each lane. Lanes 1, standard EPHX1-expressed microsome; 2, vacant plasmid; 3, wild-type; 4, T26S; 5, R43T; 6, Y113H; 7, H139R; 8, T275A. * $p < 0.05$ versus wild-type. Student's t -test.

(Student's *t*-test, $p < 0.05$). The mean levels in the variant R43T and T275A were slightly increased to 110 and 118% (n.s.), respectively. On the other hand, the mean expression level of the variant Y113H was slightly reduced to 92% (n.s.). The level of the variant T26S was almost the same as that of the wild-type.

Hydrolysing activity of recombinant EPHX1s for cis-stilbene oxide

EPHX1-catalysed hydrolysis of *cis*-stilbene oxide was assessed using the microsomal preparation of COS-1 cells transfected with either wild-type EPHX1 or its five single variant plasmids. The kinetic parameters are summarized in table 3. The hydrolysis of *cis*-stilbene oxide was not observed in the microsomal preparation derived from mock-transfected COS-1 cells. As for the wild-type EPHX1, its K_m and V_{max} values were comparable with those of human EPHX1-expressed microsome obtained from Gentest Corporation ($K_m = 21.9 \mu\text{M}$, $V_{max} = 13.4 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$). K_m values were not significantly different among the wild-type and variants. On a milligram microsomal protein basis, the values for V_{max} and hence intrinsic clearance (V_{max}/K_m) of the two variants, R43T and H139R, were significantly increased compared with the wild-type. Even after normalizing to the corresponding EPHX1 protein contents determined by Western blotting, the values for V_{max} and clearance of R43T were still significantly increased. On the other hand, the values for V_{max} and clearance of H139R were not significantly different from the wild-type due to its increased level of protein expression. Since the expression levels of the variant T275A protein were slightly increased (118%), the values for V_{max} and clearance per EPHX1 protein were decreased by 11.2 and 12.6%, respectively.

These results suggested that the R43T substitution increased the intrinsic hydrolysis activity against *cis*-stilbene oxide. On the other hand, the H139R substitution increased the expression level of the protein, resulting in an augmented activity of the hydrolysis on a milligram protein basis.

Discussion

EPHX1 is a single-gene enzyme responsible for the hydrolysis of epoxides derived from the oxidative metabolism of xenobiotics (Seidegard and DePierre 1983, Fretland and Omiecinski 2000). The polymorphism in EPHX1, therefore, is thought to be an important determinant of drug and chemical toxicity. In the present study, we detected 25 single nucleotide alternations in the EPHX1 gene using 72 established cell lines derived from the Japanese population. Most of the alterations in the coding region were found in more than two cell lines, suggesting that they are polymorphisms, i.e. SNPs, but not mutations. Of the 13 SNPs in the coding exon, five SNPs resulted in altered amino acids (77C > G, T26S; 128G > C, R43T; 337T > C, Y113H; 416A > G, H139R; 823A > G, T275A). These included two common variations previously reported by others, i.e. 337T > C (Y113H) and 416A > G (H139R) (Hassett *et al.* 1994). In our studies, the apparent allele frequencies for 337T > C and 416A > G were 0.368 and 0.326, respectively. Our frequency of 337T > C was comparable with that reported in Japanese individuals (0.44) by Yoshikawa *et al.* (2000), while our frequency of 416A > G was higher than theirs (0.14). The reason for this discrepancy at 416 is not clear at present. The SNP for R43T previously reported by Gaedigk *et al.* (1994) was also detected

Table 3. Kinetic parameters for hydrolytic activities of wild-type and variant EPHX1 with *cis*-stilbene oxide as substrate.

Amino acid alteration	K_m (μ M)	V_{max} ($\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$)	$V_{max}/\text{EPHX1 protein}$ (%)	Intrinsic clearance (V_{max}/K_m) ($\text{ml min}^{-1} \text{mg}^{-1} \text{protein}$)	Intrinsic clearance/EPHX1 protein (%)
Wild-type					
T26S	22.2 $\pm$ 0.8	12.6 $\pm$ 0.3 (100%)	100.0	0.57 $\pm$ 0.01 (100%)	100.0
R43T	22.9 $\pm$ 1.5	13.7 $\pm$ 0.3 (109%)	108.4 $\pm$ 3.3	0.61 $\pm$ 0.04 (107%)	102.7 $\pm$ 5.7
Y113H	22.2 $\pm$ 0.8	16.8 $\pm$ 0.2*** (133%)	125.9 $\pm$ 6.4**	0.76 $\pm$ 0.03* (134%)	121.5 $\pm$ 6.2*
H139R	22.6 $\pm$ 0.9	12.4 $\pm$ 0.1 (98%)	110.8 $\pm$ 4.1	0.55 $\pm$ 0.02 (97%)	104.9 $\pm$ 4.2
T275A	22.8 $\pm$ 1.4	17.6 $\pm$ 0.4*** (140%)	112.3 $\pm$ 3.9	0.78 $\pm$ 0.05* (138%)	106.1 $\pm$ 4.7
	23.3 $\pm$ 1.1	12.9 $\pm$ 0.6 (102%)	88.8 $\pm$ 2.3	0.56 $\pm$ 0.05 (99%)	87.4 $\pm$ 3.9

Data are mean $\pm$ SE of five to nine experiments derived from two to four separate transfections.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ versus wild type. One-way ANOVA, post-hoc test; Scheffé.

in a Japanese epileptic patient (unpublished data). T26S and T275A are novel SNPs.

For five of these coding SNPs that produced amino acid alterations, we assessed the variants for their expression and hydrolytic activities. Examination of EPHX1 mRNAs expressed in the transfected COS-1 cells indicated there was no difference in the mRNA levels between the wild-type and the variants. Despite equivalent levels of expressed mRNA, the amounts of EPHX1 proteins in the microsomal fractions were different for each construct. The expression level of EPHX1 protein in the variant H139R was significantly increased to 128%, whereas that for Y113H was slightly reduced (92% of wild-type). Previous studies showed an increase (154%) and decrease (56%) in expression of EPHX1 protein in the H139R and Y113H variants transfected in COS-1 cells (Hassett *et al.* 1994), and these differences seemed to be caused by the altered protein stability from protein half-life experiments (Laurenzana *et al.* 1998). Our present results for the protein levels in the H139R and Y113H variants agree with the latter observations, although the changes observed in our study were smaller.

We determined the values for K_m (22.2 μM) and $V_{\max}$ (12.6 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein) for the hydrolysis of *cis*-stilbene oxide in the microsomal fraction of COS-1 cells transfected with wild-type EPHX1. These values were almost comparable with those of Kitteringham *et al.* (1996) (K_m and $V_{\max}$ ranged from 2.5 to 18 μM and from 7.5 to 25.6 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, respectively) in human liver microsomes using *cis*-stilbene oxide as a substrate.

As for the two common mutations (Y113H and H139R) frequently observed in Japanese and Caucasian populations, Hassett *et al.* (1994) demonstrated using benzo[a]pyrene-4,5-epoxide as a substrate that the net increase (25% for H139R) or decrease (40% for Y113H) in the enzymatic activity normalized to total S9 proteins from COS-1 cells transfected with these variants paralleled the respective increase or decrease in EPHX1 immunoreactive protein levels, and that there were no significant changes in the intrinsic EPHX1 activities between the wild-type and the variants. Our results, at least for H139R, confirmed this latter report, i.e. the changes in the enzymatic activities (on a microsomal protein basis) were due to a change in the EPHX1 protein level. These results suggest that these variants affect the hydrolytic activity similarly, regardless of the difference in the substrate.

The values for $V_{\max}$ and intrinsic clearance ($V_{\max}/K_m$) of the R43T variant were significantly increased compared with the wild-type both on a microsomal protein basis and on an EPHX1 immunoreactive protein basis. Thus, this R43T substitution increases its intrinsic hydrolytic activity against *cis*-stilbene oxide. Although the crystal structure of human EPHX1 has not yet been elucidated, the residues that play important roles in the hydrolysis activity of EPHX1 have been determined by mutation experiments, e.g. Trp150, Glu376, His126, His129, His148, His247 and His431 (Bell and Kasper 1993, Tzeng *et al.* 1998). Moreover, it has been reported that Asp226, Glu404 and His431 constitute the EPHX1 catalytic triad. The Asp226 residue attacks the oxirane ring to yield an alkyl-enzyme intermediate, which is followed by subsequent hydrolysis with water activated through proton abstraction by Glu404 and His431 residues (Arand *et al.* 1999). Together with these latter residues, the non-synonymous change at the residue 43 might exert an influence on the catalytic activity or the conformational structure of the active site.

Many human epidemiological studies have been performed to explore the contribution of the EPHX1 genotype to altered susceptibility to various diseases. The Y113H variant has been suggested to increase the risk of pulmonary disorders (Smith and Harrison 1997, Yoshikawa *et al.* 2000) and colon cancer (Harrison *et al.* 1999). On the other hand, the H139R variant, which showed increased enzymatic activity by 40% in our study, was reported to be associated with an increased risk of alcoholic liver disease (Wong *et al.* 2000), a type of acute myeloid leukaemia (Lebailly *et al.* 2002), and lung cancer in Caucasian smokers (Benhamou *et al.* 1998) and in Taiwanese (Lin *et al.* 2000). Although the hydrolytic products by EPHX1 are generally less reactive than the parent epoxide, some resulting diols, such as the 7,8-dihydrodiol of benzo(a)pyrene, are precursors of highly reactive and carcinogenic compounds (Sims *et al.* 1974, Seidegard and DePierre 1983). We revealed first that the R43T substitution also significantly increased the intrinsic hydrolytic activity. Therefore, this substitution (R43T) might be related with increased risks of several types of cancers. Our present findings would be helpful in elucidating the potential association between EPHX1 polymorphisms and the risk of disease development.

Acknowledgements

This work was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences (MPJ-6) of the Organization for Pharmaceutical Safety and Research.

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Metabolism of a novel nucleoside analogue, OGT 719, in the isolated perfused rat liver model, in rats, in tumour models and in patients

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Received 19 June 2002

1. The metabolic pathway(s) of OGT 719, a novel nucleoside analogue in which galactose is covalently attached to the N1 of 5-fluorouracil (FU), have been investigated with ¹⁹F-NMR spectroscopy in (1) the isolated perfused rat liver (IPRL) model, (2) normal rats, (3) rats bearing the HSN LV10 sarcoma, (4) nude mice xenografted with the human hepatoma HepG2 and (5) urine from patients.

2. The administration of OGT 719 results in the formation of small amounts of FU. IPRL experiments with OGT 719 in combination with asialofetuin, a natural asialoglycoprotein receptor (ASGP-r), suggest competitive binding of OGT 719 to the ASGP-r.

3. The data obtained in non-tumour rats also demonstrated an extremely low metabolism of OGT 719 into FU and α -fluoro- β -alanine, the well-known major metabolite of FU.

4. A comparison of tumour extracts from rats bearing the HSN LV10 sarcoma treated with FU or OGT 719 showed the incorporation of FU into RNA in rats treated with FU but not in rats treated with OGT 719; nevertheless, the incorporation of FU into RNA was observed in the liver from rats treated with OGT 719.

5. In a human hepatoma xenografted to nude mice, both the OGT 719 and FU contents of the tumour were markedly higher than in the corresponding liver, suggesting a tumour-specific trapping of OGT 719 in hepatoma.

6. The metabolism of OGT 719 was also extremely low in patients.

7. In conclusion, the present study shows the value of ¹⁹F-NMR for demonstrating for the first time that OGT 719 is a prodrug of FU although very poorly metabolized.

Introduction

Carbohydrate-binding proteins represent one important class of cell-surface receptors. One such receptor, the asialoglycoprotein receptor (ASGP-r), is found in the liver on the surface of hepatocytes (Weiss and Ashwell 1989, Lodish 1991), hepatoma cells (Schwartz *et al.* 1981, Nakabayashi *et al.* 1982), and some colorectal tumour cells (Mu *et al.* 1997). The ASGP-r functions to clear desialylated glycoproteins from the circulation and accumulate them in the liver. The receptor recognizes terminal galactose and *N*-acetylgalactosamine residues, and the resulting receptor–ligand complex is rapidly internalized into endosomes. This approach

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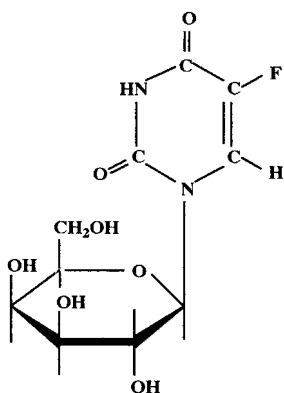


Figure 1. Structure of OGT 719 (N1-galactopyranosyl-5-fluorouracil).

has been used to target 5-fluorouracil (FU) to cells, which express the ASGP-r. OGT 719 (N1-galactopyranosyl-5-fluorouracil) is a novel nucleoside analogue in which galactose is covalently attached to the N1 of FU (figure 1). OGT 719 has been designed to reduce the systemic toxicity of FU while retaining activity against primary hepatocellular carcinoma or colorectal cancer metastases in the liver, in which it may be preferentially localized via binding to the ASGP-r, thereby triggering receptor-mediated endocytosis.

The activity of OGT 719 in inhibiting the growth of metastatic human colorectal tumours in the livers of nude mice has been reported (Rohlf *et al.* 1999). Oral administration of $1.5 \text{ g kg}^{-1} \text{ b.w. day}^{-1}$ OGT 719 for 30 days inhibited liver tumour burden by 95% compared with vehicle control, without any observable signs of toxicity. A comparison between this OGT 719 treatment and a standard clinical dose regimen of $25 \text{ mg kg}^{-1} \text{ day}^{-1}$ of FU + leucovorin given intravenously (i.v.) showed that both treatments were equally efficacious. The first clinical trial of oral and i.v. administration of OGT 719 in 16 patients with primary or secondary liver malignancies has been recently reported (Sharma *et al.* 2001). OGT 719 was well tolerated but no objective tumour responses could be demonstrated.

However, the metabolism and mechanism of action of OGT 719 are unknown. HPLC analysis of plasma and urine from patients treated with OGT 719 failed to demonstrate the presence of quantifiable amount of FU (Sharma *et al.* 2001). In an abstract only, it was reported that HPLC profiles of radiolabelled OGT 719-treated human hepatoma implanted in nude mice displayed metabolic FU intermediates similar to those observed when the same tumours were treated with radiolabelled FU (Rohlf *et al.* 1997). Even if the metabolites were not identified, it was suggested from this study that OGT 719 and FU followed the same metabolic pathway.

Since fluorine-19 nuclear magnetic resonance (^{19}F -NMR) spectroscopy gives an overview of all fluorinated compounds present without any prior separation process, we employed this method tentatively to clarify the metabolism of OGT 719. For this purpose, the isolated perfused rat liver (IPRL) model treated with OGT 719 alone or in combination with asialofetuin (ASF), a natural ASGP-r ligand, was used. Both normal and tumour tissues from rats and nude mice and patients' urine were also analysed after treatment with OGT 719.

Materials and methods

Materials

FU, fluorocytosine, β -D-glucuronidase (EC 3.2.1.1.31) from bovine liver (type B-3), ASF (type I, batch 86H9502; salt free and containing less than 0.5% of *N*-acetylneuraminic acid) were purchased from Sigma, and chromium (III) acetylacetonate ($\text{Cr}(\text{acac})_3$), sodium fluoride, trifluoroacetic acid and 4-fluorobenzoic acid were from Aldrich (all from Sigma-Aldrich Chimie, Saint Quentin Fallavier, France). α -Fluoro- β -alanine (FBAL) hydrochloride and fluoroacetamide were provided by Tokyo Kasei Chemicals (Tokyo, Japan). 5,6-Dihydro-5-fluorouracil (5FUH₂) was generously supplied by Hoffmann-La Roche (Basel, Switzerland). α -Fluoro- β -ureidopropionic acid (FUPA) was prepared by chemical opening of 5FUH₂ pyrimidine ring in 1 M NaOH (Malet-Martino *et al.* 1986). Sodium 4-fluorobenzoate (FBEN) was prepared by titrating 4-fluorobenzoic acid with an NaOH solution. The ¹⁹F-NMR characteristics of 5-fluorouridine-2'-monophosphate (2'FUMP) and 5-fluorouridine-3'-monophosphate (3'FUMP) were obtained from chemical and enzymatic hydrolyses of FU incorporated into RNA from *Nectria haematococca* strains as already described (Parisot *et al.* 1991). All other chemicals were reagent grade and obtained from standard commercial sources. OGT 719 (Oxford GlycoSciences Ltd (UK); purity 99.9%) and ASF were made up in distilled water.

IPRL experiments

Male Wistar rats (Iffa Credo, Lyon, France; 399 ± 36 g) were used and IPRL experiments were described by Arellano *et al.* (1997). The experiments were carried out with solutions of OGT 719 prepared immediately before use at 1 mg ml^{-1} perfusate, i.e. a dose of about 375 mg kg^{-1} b.w.. For the experiments with OGT 719 alone ($n = 3$) or with added ASF ($n = 4$), the drug was injected into the perfusate after 1 h of liver equilibration and the experiments were continued for 3 h. For the experiments with OGT 719 + ASF, ASF (1 mg ml^{-1} perfusate, i.e. 150 mg) was injected 30 min before OGT 719. At the end of the experiments, an aliquot of the perfusate was immediately frozen and kept at -80°C until ¹⁹F-NMR analysis. For qualitative purposes, the remaining perfusate was freeze-dried and then resuspended in about 3 ml H₂O immediately before ¹⁹F-NMR analysis. Bile samples were gathered and stored at -80°C until analysis. Liver was weighed, immersed in liquid nitrogen, powdered and sequentially extracted with cold and hot 1 M perchloric acid (PCA) by using the method of Wain and Staatz (1973). The acid-soluble (AS) and acid-insoluble (AI) fractions thus obtained were lyophilized to dryness and stored at -80°C until analysis. The lyophilized materials were resuspended in a known volume of H₂O containing 30 mM EDTA (approximately 3.5 ml), and centrifuged immediately before ¹⁹F-NMR analysis. The pH of the supernatant was adjusted to 5.5 ± 0.1 .

Non-tumour rat experiments

Five experiments were carried out by giving per os 1 g kg^{-1} b.w. OGT 719 to rats weighing 440 ± 37 g. After 4 h, the rat was bled and a plasma sample was prepared. Urine collected directly in the bladder was added to urine excreted over 4 h ($n = 4$). Urine and plasma samples were immediately frozen and stored at -80°C until ¹⁹F-NMR analysis. The liver and kidneys were extracted with PCA as reported above, giving AS and AI fractions ($n = 4$ for liver AI extracts).

Treatment of rat urine with β -D-glucuronidase

A rat urine sample (2.5 ml) was incubated at 37°C for 2 h with 12 500 Fishman units of β -D-glucuronidase. A control incubation was performed simultaneously without enzyme. The pH of all the samples was adjusted to 5.5. Following incubations, the samples were transferred to NMR tubes and their ¹⁹F-NMR spectra were recorded.

Rat tumours

Forty male CBH/cbi rats (252 ± 9 g) were injected with HSN LV10 sarcoma cells (3×10^3 cells in 0.2 ml). Three days after the inoculation, they were divided into four groups of 10 rats. Group 1 was the control and did not receive any treatment. Groups 2 and 3 were orally treated with OGT 719 at 3 and 0.75 g kg^{-1} b.w. day⁻¹ respectively in two doses for 20 days. Group 4 was treated with FU at a dose of 20 mg kg^{-1} day⁻¹ through implanted mini-pumps over 20 days. On day 21, rats were killed by CO₂ asphyxia 3–4 h after the morning oral dose of OGT 719. The tumours were dissected out and weighed. The total tumour number, median tumour mass and total tumour burden were determined. The tumours were then snap-frozen, pooled into four categories (control, OGT 719 3000, OGT 719 750, FU) and extracted with PCA as reported above yielding AS and AI fractions. Liver and kidney extracts from the group of rats treated with OGT 719 at 3 g kg^{-1} b.w. for 20 days were also analysed.

Nude mice xenografted with the human hepatic tumour HepG2

HepG2 human hepatoma tumour cells were passaged four times before subcutaneous injection (approximately 7×10^6 cells in 0.1 ml) into athymic nude mice. The mice were examined for tumours before randomized division into a control group of eight mice and four treatment groups of eight mice each, 32 days following tumour cell implantation. Treatment was initiated on the day of randomization. OGT 719 was administered by oral gavage twice daily for 28 days (at doses of 0.75, 1.5 and $2 \text{ g kg}^{-1} \text{ day}^{-1}$) and FU by a single daily i.v. injection ($25 \text{ mg kg}^{-1} \text{ day}^{-1}$) until day 10. The animals were weighed, and the two major diameters of tumours were measured every 3–4 days by using a caliper to estimate the tumour volume by standard calculations. This experiment was terminated after 28 days. This study was performed on behalf of Oxford GlycoSciences by Quintiles Scotland Ltd. By the end of the treatment period with OGT 719 at $1.5 \text{ g kg}^{-1} \text{ b.w. day}^{-1}$, livers and hepatoma from the eight mice were removed, pooled by category and extracted with PCA as described above leading to AS and AI fractions.

Patients' urine

Urine samples were collected over 12 h in two fractions (0–4, 4–12 h) from two patients (body surface areas: 1.86 m^2 for patient 1, 1.69 m^2 for patient 2) receiving OGT 719 at a dose of 16 g m^{-2} . The urine collection started after the 3-h infusion of OGT 719. The urine samples were analysed without being concentrated.

Experiments to test the purity and stability of OGT 719

The ^{19}F -NMR spectrum of a 0.115 M solution of OGT 719 recorded for 18 h at pHs 5.1 or 7.9 showed that the purity of OGT 719 was 99.9%. The solution contained fluoride ion (F^-), which represented 0.05% with respect to the signal of OGT 719, and three unknown fluorinated impurities, one resonating at -87.7 ppm (0.03%), the second at -88.1 ppm (0.03%) and the last one at -131.8 ppm (0.007%). No FU could be detected. The FU contamination of OGT 719 measured by HPLC amounted to 0.0003%, which corresponds to a concentration of $3.5 \times 10^{-7} \text{ M}$, not detectable with ^{19}F -NMR.

In order to check the stability of OGT 719 in the harsh conditions in which the AS and AI PCA extracts were prepared, we also analysed 'control AS and AI' preparations of OGT 719. Pure OGT 719 was treated with PCA in the same conditions as those used for preparing tissue AS and AI extracts. The control AS and AI preparations of OGT 719 at pH 5.5 were recorded for 18 h. The control AS fraction contained F^- (0.06% with respect to the signal of OGT 719), and two impurities found in OGT 719 (-87.7 ppm (0.009%) and -88.1 ppm (0.009%)). The control AI preparation contained F^- (0.08%) and the impurity resonating at -88.1 ppm (0.01%). Control AS and AI showed that only impurities of OGT 719 were recovered. There was no interference with the signals observed in the tissue AS and AI from livers, tumours or kidneys, except F^- observed in two AI of tumours.

 ^{19}F -NMR spectroscopy

^{19}F -NMR spectra were recorded at 282.4 MHz with ^1H -decoupling on a Bruker WB-AM 300 spectrometer using 10-mm diameter NMR tubes. The recording conditions were: probe temperature, 25°C ; sweep width, 41 667 Hz; 32 768 data points zero-filled to 65 536; pulse width, $7 \mu\text{s}$ (flip angle about 40° in non-concentrated perfusate, bile and urine, about 30° in PCA extracts and about 20° in concentrated perfusate); pulse interval, 1.4 s for quantification of AS and AI extracts or 3.4 s for quantification of non-concentrated perfusates, bile and urine samples; number of scans, 10 000–50 000; line broadening caused by exponential multiplication, 5 Hz. The chemical shifts (δ) were reported relative to the resonance peak of trifluoroacetic acid (5% w/v aqueous solution) used as external chemical shift reference.

The concentrations of the fluorinated compounds were measured by comparing the expanded areas of their respective NMR signals with that of the external standard for quantification placed in a coaxial capillary, namely a solution of FBEN in D_2O doped at saturation with $\text{Cr}(\text{acac})_3$ to shorten the longitudinal relaxation time (T_1) of FBEN. The apparent concentration of the FBEN peak was previously calibrated against solutions of fluorocytosine and fluoroacetamide at known concentrations. $\text{Cr}(\text{acac})_3$ (about 2.5 mg) was also added to non-concentrated perfusates, bile and urine samples. The areas were determined after the different signals were cut out and weighed.

Fully relaxed spectra were obtained for all media analysed even when spectra were recorded with a pulse interval as short as 1.4 s and without $\text{Cr}(\text{acac})_3$. This was demonstrated for FU and FBAL in AS extracts containing EDTA recorded with a pulse interval of 1.4 s without $\text{Cr}(\text{acac})_3$ or 3.4 s with $\text{Cr}(\text{acac})_3$. The differences between the values of concentrations thus determined were not more than 10%, which corresponds to the precision of the method (5–10% depending on the concentration) (Martino *et al.* 2000). The high ionic strength of concentrated perfusates and perchloric acid extracts and the high viscosity of the former medium induced a decrease of the flip angle for a given value of the pulse width (see above) and probably of the T_1 , leading to an accurate quantification of PCA extracts

even with a short pulse interval and without $\text{Cr}(\text{acac})_3$. The detection threshold depends on the spectrometer magnetic field and the recording duration. The detection limit for our spectrometer after 15–18 h of recording was about 2 μM .

Statistics

Results are expressed as means $\pm$ SD except for ^{19}F -NMR data of tumour, liver and kidney PCA extracts from rats with HSN LV10 sarcoma and nude mice xenografted with the human hepatoma HepG2. Indeed, the sensitivity limit of the NMR method constrained us to pool samples from several animals. When possible, statistical significance was determined using Student's *t*-test. $p < 0.05$ was considered as statistically significant.

Concerning the total tumour number and median tumour mass of rats with HSN LV10 sarcoma and tumour volume of nude mice xenografted with the human hepatoma HepG2 untreated or treated with OGT 719 or FU, statistical analysis was performed using the Mann–Whitney *U*-test (two-tailed). $p < 0.05$ was considered as statistically significant.

Results

For clarity, the chemical shifts (δ) of OGT 719 and its metabolites are summarized in table 1. The δ of OGT 719 is sensitive to pH. It is deshielded when pH increases ($\Delta\delta = +2.4$ ppm from pH 5.1 to 9.0), giving a pK_A of 7.3.

IPRL experiments

Unchanged OGT 719 (checked by spiking with authentic standard) was the sole compound detected in perfusates (non-concentrated and concentrated) and in bile (table 2). In liver AS fractions (containing the low molecular weight and PCA soluble metabolites), OGT 719, FU (checked by spiking with authentic standard) and an unknown compound resonating at -93.5 ppm (U1) were found. U1, which resonates as a singlet signal in the ^1H -coupled ^{19}F -NMR spectrum, was also found in AS extracts from livers treated with FU or FBAL; it therefore arises from FBAL metabolism as FBAL is not degraded under the conditions of PCA treatment (unpublished data). OGT 719 represented $3.8 \pm 1.1\%$ of dose, FU $0.004 \pm 0.002\%$, and U1 $0.02 \pm 0.006\%$ in the experiments with OGT 719 alone, and $2.4 \pm 0.3\%$ for OGT 719, $0.005 \pm 0.002\%$ for FU, and $0.02 \pm 0.005\%$ for U1 in the experiments with OGT 719 and ASF. AI fractions from livers treated with FU normally contain the metabolites incorporated into macromolecules, especially

Table 1. Chemical shifts (δ) of OGT 719 and metabolites.

Compound	Chemical shift
OGT 719	$-89.4/-87.0$ pH 5.1/9.0
FU	-93.4
Unknown U1	-93.5
FBAL	-112.4
Unknown -88 in rat urine	$-88.0/-86.3$ pH 5.9/8.3
Unknown -129.9 in rat urine	-129.8_5
Unknown -110 in tumour rats	-110.0
2'FUMP	-89.6 pH 5.5
3'FUMP	-89.9 pH 5.5

RNA, which are recovered as 2’FUMP and 3’FUMP (Parisot *et al.* 1991). In AI extracts from livers treated with OGT 719, OGT 719 and the unknown compound U1 were observed, except that only OGT 719 could be detected in experiments with OGT 719 + ASF. OGT 719 amounted to $0.03 \pm 0.01\%$ of dose, and U1 $0.001 \pm 0.001\%$ in the experiments with OGT 719 alone, and $0.03 \pm 0.01\%$ for OGT 719 in the experiments with OGT 719 and ASF. All these compounds are very probably ‘residues’ of OGT 719 and U1 not completely extracted during the preparation of the AS fraction. Therefore, the AS and AI fractions were counted together, and the data are reported in table 2.

In both kinds of experiments, only a very low fraction of OGT 719 was metabolized by the IPRL (about 0.02% of dose). Regarding the uptake of OGT 719 from the perfusate as assessed by the levels of OGT 719 in the bile and liver tissue, a moderate decrease was noted in the ASF treated IPRL from 4.3% of dose in the untreated IPRL to 2.7% in the ASF treated IPRL.

Non-tumour rat experiments

Plasma only contained OGT 719 whose ¹⁹F-NMR signal was broadened probably due to non-covalent binding with plasma proteins. The concentration measured after 4 h was variable due to the variable absorption after an oral dose (Sharma *et al.* 2001). The mean concentration was $1.6 \times 10^{-4} \pm 8.1 \times 10^{-5}$ M ($46.8 \pm 23.7 \mu\text{g ml}^{-1}$).

Urine contained mostly OGT 719 (table 3). FBAL was detected in all experiments. The amount of F⁻ was lower than the level of F⁻ measured in the administered OGT 719 (0.05%). The chemical shift of the signal referred to as ‘-88’ was, like that of OGT 719, dependent of the urinary pH (table 1), in contrast to signals at -87.7 and -88.1 ppm detected in OGT 719 solutions. It does not thus correspond to any of these impurities. As this signal resonates close to that of OGT 719 and is deshielded when pH increases ($\Delta\delta = +1.7$ ppm between pHs 5.9 to 8.3), the structural modification is distant from the fluorine atom and occurs at the sugar moiety. This signal is still present after treatment with β -glucuronidase (data not shown). As some O-glucuronides are resistant to β -glucuronidase (Castle 1993, Roberts *et al.* 2000, Wood *et al.* 2000), it cannot be definitively excluded that the -88 compound does not correspond to a glucuroconjugate of OGT 719. The level

Table 2. Amounts of OGT 719 and metabolites ($\mu\text{mol g}^{-1}$ liver and percentage of dose) recovered in perfusate, bile and liver from isolated perfused rat livers treated with OGT 719 alone or in combination with asialofetuin (ASF) injected 30 min before OGT 719.

		OGT 719 1 mg ml ⁻¹ Injected dose $38.9 \pm 2.8 \mu\text{mol g}^{-1}$ liver <i>n</i> = 3		OGT 719 1 mg ml ⁻¹ + ASF 1 mg ml ⁻¹ Injected dose $40.3 \pm 7.7 \mu\text{mol g}^{-1}$ liver <i>n</i> = 4	
		$\mu\text{mol g}^{-1}$	% of dose	$\mu\text{mol g}^{-1}$	% of dose
Perfusate	OGT 719	31.9 ± 0.9	82.6 ± 6.5	42.6 ± 6.7	106.1 ± 4.6
Bile	OGT 719	0.18 ± 0.04	0.5 ± 0.1	0.08 ± 0.01	0.2 ± 0.06
Liver	OGT 719	1.5 ± 0.5	3.8 ± 1.1	1.0 ± 0.2	2.5 ± 0.3
	FU	0.0015 ± 0.0008	0.004 ± 0.002	0.002 ± 0.0009	0.005 ± 0.002
	U1(?)	0.007 ± 0.003	0.02 ± 0.007	0.007 ± 0.002	0.02 ± 0.005

Table 3. Amounts of OGT 719 and metabolites (μmol in urine, $\mu\text{mol g}^{-1}$ liver or kidney, and percentage of dose) in urine, liver and kidneys from non-tumour rats treated with 1 g kg^{-1} b.w. OGT 719.

		Administered dose of OGT 719 $1003.7 \pm 4.3 \text{ mg kg}^{-1}$ $103.0 \pm 3.2 \mu\text{moles g}^{-1}$ liver ^a $617.7 \pm 70.8 \mu\text{mol g}^{-1}$ kidney ^a $n = 5^b$	
		$\mu\text{mol in urine}$ $\mu\text{mol g}^{-1}$ liver or kidney	% of dose
Urine	OGT 719	73.2 ± 64.3	3.9 ± 3.2
	F ⁻	0.20 ± 0.10	0.01 ± 0.005
	FBAL	0.02 ± 0.01	0.001 ± 0.0005
	-88 (?)	0.25 ± 0.23	0.013 ± 0.01
	-129.9 (?)	0.01 ± 0.01	0.0007 ± 0.0007
Liver	OGT 719	0.09 ± 0.04	0.09 ± 0.04
	U1 (?)	0.0004 ± 0.0005	0.0004 ± 0.0005
	FBAL	0.0005 ± 0.0003	0.0005 ± 0.0003
Kidneys	OGT 719	0.94 ± 0.75	0.17 ± 0.17
	FU	0.004 ± 0.002	0.0007 ± 0.0004
	FBAL	0.0009 ± 0.001	0.0001 ± 0.0002

^a $\mu\text{mol g}^{-1}$ liver or kidney correspond to the ratio of administered OGT 719 (in μmol) relative to the liver or kidney weight (g).

^b $n = 4$ for urine and liver acid-insoluble extracts.

of the unknown signal at -129.9 ppm, present in three out of four experiments, was very low.

In liver AS extracts, OGT 719 amounted to $0.09 \pm 0.03\%$ of dose, and U1, detected in only two experiments out of five, $0.0004 \pm 0.0005\%$. The level of FBAL was $0.0005 \pm 0.0003\%$. In liver AI extracts, OGT 719 was detected in three experiments out of four; its level was very low ($0.001 \pm 0.001\%$). If compounds in liver AS and AI extracts are summed, the data are identical (table 3).

In kidney AS extracts, OGT 719 ($0.17 \pm 0.17\%$ of dose), FU ($0.0005 \pm 0.0002\%$) and FBAL ($0.0001 \pm 0.0002\%$) were detected. OGT 719 ($0.0002 \pm 0.0002\%$), and FU ($0.0002 \pm 0.0003\%$) were observed in kidney AI extracts. The compounds detected in AI fractions are 'residues' of the AS extracts. When the compounds are regrouped (table 3), the amounts of OGT 719 and FBAL are unchanged but FU represented $0.0007 \pm 0.0004\%$.

Four hours after the administration of an oral dose of $1 \text{ g OGT 719 kg}^{-1}$ b.w. given to rats, only 4.2% of dose is recovered as OGT 719 in urine, liver and kidneys. F⁻ cannot be considered as a metabolite as it represents less than 0.05%, which is the percentage measured in pure OGT 719. Unknown compounds (-88, -129.9, U1) cumulatively represent 0.014%. FBAL and FU amount to 0.0023%; the level of FBAL (0.0016%) is higher than the contaminant FU in OGT 719 determined by Oxford GlycoSciences ($< 0.0003\%$). Overall, the metabolism of OGT 719 is extremely low (0.018% of dose).

Rat sarcoma

Tumour growth inhibitions were 40 and 59% with increasing doses of OGT 719, and 54% with FU (data not shown). The total number of tumours was not

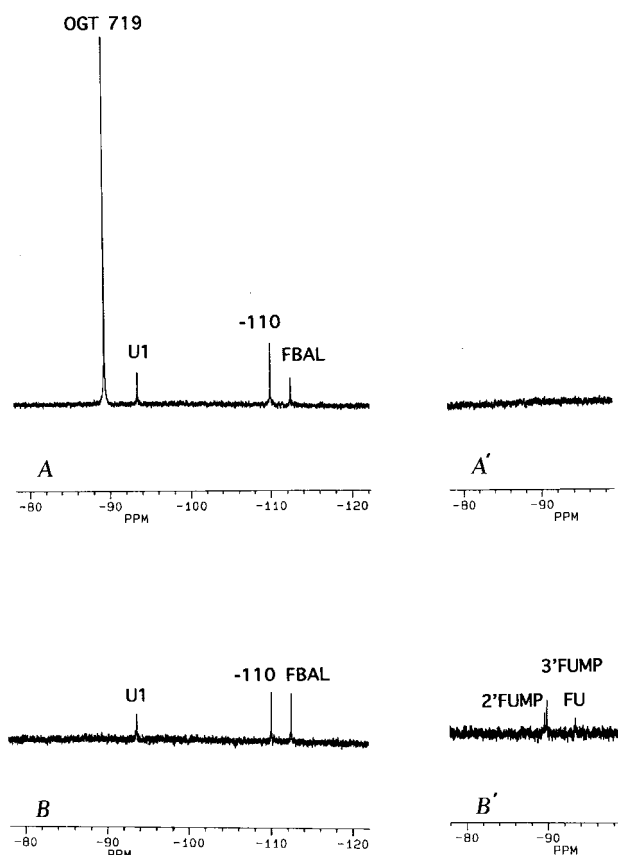


Figure 2. ^1H -decoupled ^{19}F -NMR spectra of acid-soluble and acid-insoluble extracts (pH 5.5) from sarcoma of rats treated with oral OGT 719 ($3\text{ g kg}^{-1}\text{ b.w. day}^{-1}$) (A, A') or i.v. FU ($20\text{ mg kg}^{-1}\text{ b.w. day}^{-1}$) (B, B') over 20 days.

significantly different between control rats (41) and animals treated with the two doses of OGT 719 (34 and 35), but it was significantly different between control and animals treated with FU (25). The median mass of individual tumours was significantly reduced between OGT 719 groups and the control group, but not between the FU group and the control group (data not shown).

In AS fractions of tumours from control animals, no signal could be detected. On the other hand, a very low amount of F^- ($2.7\text{ nanomol g}^{-1}$ of tumour) was quantified in AI fractions of tumours from control.

In AS fractions of tumours from animals treated with OGT 719 or FU (figure 2A and B), FBAL, U1 and an unknown compound resonating at -110.0 ppm as a doublet of doublet of doublet in the ^1H -coupled ^{19}F -NMR spectrum, which is not FUPA as shown by spiking with authentic standard, were detected. The signal at -110.0 ppm is likely to represent a FBAL derivative. Its multiplicity and H-F coupling constants ($^2J_{\text{HF}} = 49.2\text{ Hz}$, $^3J_{\text{HF}} = 23.0$ and 26.2 Hz) support the $\text{N-CH}_2\text{-CHF-C=O}$ sequence. Its chemical shift is characteristic of a $\text{R-CO-NH-CH}_2\text{-CHF-COOH}$ structure by comparison with that of *N*-acetylFBAL $\text{CH}_3\text{-CO-NH-CH}_2\text{-CHF-COOH}$ (-110.3 ppm relative to the signal of FBAL at -112.4 ppm) and FBAL methyl ester $\text{NH}_2\text{-CH}_2\text{-CHF-COOCH}_3$ at -122.1 ppm

Table 4. Amounts of OGT 719 and metabolites (nanomol g⁻¹ tumour) in acid-soluble (AS) and acid-insoluble (AI) extracts of tumours from rats bearing HSN LV10 sarcoma and treated with oral OGT 719 or i.v. injected fluorouracil over 20 days.

		OGT 719 oral ^a		FU i.v. ^a
		3 g kg ⁻¹ day ⁻¹	0.75 g kg ⁻¹ day ⁻¹	20 mg kg ⁻¹ day ⁻¹
AS	OGT 719	171	32.5	
	U1 (?)	11.7	εεε ^b	4.8
	-110 (?)	28.3	8.4	7.1
	FBAL	5.0	8.9	6.4
	sum	216	50	18
AI	metabolism	45	17	18
	2'FUMP	< LD ^c	< LD ^c	1.2
	3'FUMP	< LD ^c	< LD ^c	1.7
	FU	< LD ^c	1.7	0.83
	OGT 719	< LD ^c	< LD ^c	
	F ⁻	εεε ^b	< LD ^c	< LD ^c
	sum	0	1.7	3.7

^a To increase sensitivity, tumours from five animals were pooled for the 3 g kg⁻¹ day⁻¹ dose of OGT 719, 10 for the 0.75 g kg⁻¹ day⁻¹ dose, and nine for FU.

^b εεε means that the ¹⁹F-NMR signal is observable but too low to be accurately quantified.

^c LD, limit of detection.

(Martino *et al.* 1987). OGT 719 was observed in all experiments with this compound, and its level was proportional to the dose injected (table 4). The metabolites detected (FBAL and FBAL derivatives) were the same in the experiments with OGT 719 or FU. The levels of metabolites measured in the experiment with OGT 719 at 0.75 g kg⁻¹ b.w. day⁻¹ or FU are very similar. On the other hand, the amounts of metabolites in the experiments with OGT 719 increase proportionately with increased dose (table 4).

Analysis of the respective AI fractions reveals clear differences between the FU and OGT 719 treated animals (table 4). In tumours from animals treated with FU, the incorporation of FU into RNA could be measured by the formation of 2'FUMP and 3'FUMP (figure 2B'). These compounds were not observed in the experiments with OGT 719 whatever the dose administered (figure 2A'). However, a small amount of FU was detected in the AI fraction from the experiment with OGT 719 at 0.75 g kg⁻¹ b.w.

Liver and kidney extracts of rats treated with 3 g kg⁻¹ b.w. day⁻¹ OGT 719 over 20 days were analysed (table 5). The amounts of OGT 719 are very similar in tumour and liver, but about three-fold higher in kidney. The metabolites found in liver and kidney are mostly FU catabolites (FBAL and its derivative at -110 ppm). The amount of catabolites is much higher in liver and kidney than in the tumour. This is in agreement with the fact that the liver is the main organ of FU catabolism and the excretion of FU is essentially urinary. The incorporation of FU into RNA measured by the formation of 2'FUMP and 3'FUMP was observed in the AI fraction from liver.

Human hepatic tumour (HepG2) xenograft in nude mice

Figure 3 shows the mean relative tumour volumes in nude mice bearing HepG2 tumours and treated either with FU or OGT 719. Both FU and OGT

Table 5. Amounts of OGT 719 and metabolites (nanomol g⁻¹) in rats bearing HSN LV10 sarcoma treated with OGT 719 at a dose of 3 g kg⁻¹ b.w. day⁻¹ for 20 days.

	Liver ^a	Kidney ^a	Tumour ^a
OGT 719	195	538	171
FU	< LD ^b	9.5	< LD ^b
U1 (?)	< LD ^b	< LD ^b	11.7
-110 (?)	260	36.3	28.3
FBAL	73.2	152	5.0
2'FUMP	1.0	< LD ^b	< LD ^b
3'FUMP	3.0	< LD ^b	< LD ^b
Sum	532	736	216
Metabolism	337	198	45

^a To increase sensitivity, tumours and organs from five animals were pooled.
^b LD, limit of detection.

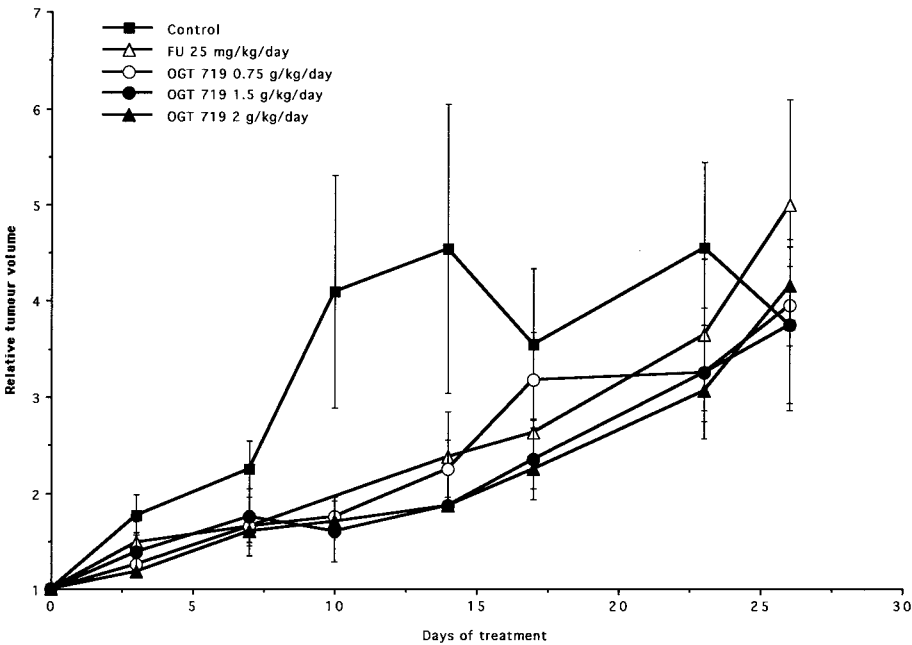


Figure 3. Effect of OGT 719 on the growth of the human hepatic tumour HepG2 xenografted to nude mice ($n = 8$ in the control group as well as in each of the four treatment groups). Tumours were measured at regular intervals throughout and relative tumour volumes were calculated.

719 treated groups showed a clear delay in tumour growth relative to the control animals. The observed reduction in tumour volume was found to be statistically significant on day 10. After 28 days of treatment with OGT 719 at 0.75, 1.5 or 2 g kg⁻¹ b.w. day⁻¹, the tumour volumes were similar to those of the control group.

In AS fractions of liver and tumours, OGT 719, FU and U1 were observed, whereas in AI fractions, only FU and U1 were detected. No incorporation of FU into RNA could be found. The OGT 719 content of the tumour was four times that in the liver. Similarly, the FU content was five-fold higher in the tumour. The metabolite U1 was also observed at a higher level (2.6-fold) in the tumour (table 6).

Table 6. Amounts of OGT 719 and metabolites (nanomol g⁻¹ tumour) in acid-soluble (AS) and acid-insoluble (AI) extracts of tumour and liver from nude mice xenografted with HepG2 and treated with oral OGT 719 at 1.5 g kg⁻¹ b.w. day⁻¹ over 28 days.

		Tumour ^a	Liver ^b
AS	OGT 719	25.5	6.3
	FU	14.5	2.3
	U1 (?)	16.5	4.6
	sum	56.5	13.2
	metabolism	31	6.9
AI	FU	2.5	0.9
	U1 (?)	6.1	4.1
	sum	8.6	5.0
	metabolism	8.6	5.0

^aTo increase sensitivity, tumours from eight animals were pooled.

^bTo increase sensitivity, livers from nine animals were pooled.

Table 7. Amounts of OGT 719 and metabolites expressed in percentage of dose in urine from two patients treated with OGT 719.

Fraction (h)	Compound	Patient 1 % of dose	Patient 2 % of dose
0–4	OGT 719	22.9	75.7
	F ⁻	0.006	0.02
	FBAL	0.002	0.01
	FU	< LD ^a	0.002
4–12	OGT 719	20.2	38.8
	F ⁻	0.01	0.008
	FBAL	0.004	0.01
Total 0–12	OGT 719	43.1	114.5
	F ⁻	0.02	0.03
	FBAL	0.006	0.02
	FU	< LD ^a	0.002

^a LD, limit of detection.

Patients' urine

Unchanged OGT 719, FBAL, FU (in one sample) and F⁻ were observed in the ¹⁹F-NMR spectra of the four fractions from the two patients (table 7).

Despite the huge variation in OGT 719 percentage of dose recovered between the two patients, it can be pointed out that the metabolism of OGT was extremely low: 0.03% of dose for patient 1 and 0.05% for patient 2 (table 7). The small conversion of OGT 719 into FBAL seems real as, in both patients, FBAL formed is > 0.0003% of FU contaminating OGT 719. On the other hand, the data do not support metabolism into F⁻ as the F⁻ formed is < 0.05% of F⁻ contaminating OGT 719. In patient 2, FU observed is > 0.0003% of FU contaminating OGT.

Discussion

Despite substantial progress in the mode of administration of FU alone or in combination with modulators and in the design of FU prodrugs, there remains a genuine need for tumour-specific, orally available prodrugs that can maintain the efficacy of the parent drug while substantially reducing its systemic toxicity. In an

attempt to achieve this, OGT 719, a novel nucleoside FU analogue containing a galactose moiety chosen for its affinity to the ASGP-r, had been designed, the rationale being that this compound could show greater activity against cancer cells carrying the ASGP-r and correspondingly less activity and thus reduced toxicity in cells lacking the receptor. It has been reported that OGT 719 inhibits the growth of a metastatic human colorectal tumour in the liver of nude mice (Rohlf *et al.* 1999).

It is thought that OGT 719 binds to the ASGP-r. The receptor ligand complex is then rapidly internalized into lysosomes. However, the subsequent events, especially the metabolism and mechanism of action of OGT 719, are not clearly understood. It is well known that the two principal FU metabolites 5-fluorouridine-5'-triphosphate (FUTP) and 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) contribute to the cytotoxic effect of FU via incorporation into RNA and inhibition of thymidylate synthase (TS), respectively, to a varying extent in different cell types (Pinedo and Peters 1988). Rohlf *et al.* (1999) demonstrated that OGT 719 was inactive against isolated TS partially purified from mouse L1210:C15 cells. On the other hand, the same authors showed that OGT 719 at a concentration of 80 mM (approximately two times the 50% inhibitory concentration (IC_{50})) or a metabolite thereof inhibits about 40% of TS activity in C170HM2 cells (a human colon carcinoma cell line) whereas FU at a concentration of 6 mM (also approximately two times the IC_{50}) has no effect.

From these preliminary data and the fact that (1) the liver-specific carbohydrate-binding ASGP-r is known to be present almost exclusively on hepatocytes (Ashwell and Morell 1974) and (2) the liver is the main organ of FU catabolism (Malet-Martino and Martino 1992), we decided to use the IPRL model for a better understanding of OGT 719 metabolism. The disadvantage of such a model is that it is disconnected from the numerous interactions existing in the whole body. For this reason, we analysed (1) plasma, urine, liver and kidney extracts from normal rats, (2) tumour, liver and kidney extracts from rats bearing HSN LV10 sarcoma, (3) tumour and liver extracts from nude mice xenografted with the human hepatoma HepG2 and (4) urine from patients treated with OGT 719.

^{19}F -NMR is the method of choice for this kind of study. It permits a direct study of any biological medium without prior treatment, avoiding the problems encountered in extraction recovery and chemical derivatization. Moreover, all fluorine-containing compounds can be detected simultaneously and quantified in a single run with a detection threshold of about $2\text{ }\mu\text{M}$ using our analytical conditions. Besides, ^{19}F -NMR has been recently employed to study the urinary metabolic fate of 2-chloro-4-trifluoromethylaniline (Scarfe *et al.* 1999) and to quantify the urinary metabolites of a novel quinoxaline non-nucleoside reverse transcriptase inhibitor (Dear *et al.* 2000) and of two novel anticonvulsant drug candidates (Ismail *et al.* 2002).

It is well known that less than 5% of a FU dose administered to patients contributes to the cytotoxicity of this drug. About 95% of the dose is urinary excreted, FBAL being by far the major metabolite accounting for about 75% of the excreted dose, the other metabolites being unchanged FU (about 10%), 5FUH_2 (about 4%), FUPA (about 8%) and F^- (about 2%) (Martino *et al.* 2000). From the current study, it is obvious that the administration of OGT 719 results in the formation of small amounts of FU. In IPRL treated with about $375\text{ mg kg}^{-1}\text{ b.w.}$ OGT 719 alone or in combination with ASF, FU was only found in the liver and

represented about 0.005% of dose (table 2). This result is quite surprising as the liver is the main organ of FU catabolism leading almost exclusively to FBAL. This could suggest that in IPRL, FU generated from OGT 719 remains in the lysosomes and does not reach the cytosol where the enzymes of the catabolic pathway of FU are localized. ASF, a natural ASGP-r ligand, had no effect on the formation of FU. The only significant (Student's *t*-test $p < 0.05$) differences in the experiments with or without ASF are that the amounts of OGT 719 are higher in perfusate and lower in bile and liver when IPRL are treated with OGT 719 and ASF (table 2). This observation is suggestive of a competitive binding of OGT 719 on ASGP-r, and that OGT 719 uptake can be decreased by natural ASGP-r ligands.

In livers from rats treated with 1 g kg^{-1} b.w. of oral OGT 719 and dissected out after 4 h, FU is not observed but an extremely low amount of FBAL can be quantified (table 3). The discrepancy between the IPRL and *in vivo* experiments might be explained by (1) an extrahepatic origin for FBAL or (2) a higher penetration of OGT 719 in the cytosol of hepatocytes. If we consider globally the *in vivo* data, it appears that an extremely low metabolism of OGT 719 into FU and FBAL occurs in 4 h ($0.002 \pm 0.0005\%$ of dose). This amount is greater than the acknowledged amount of FU contaminant in OGT 719 ($< 0.0003\%$) as stated by the manufacturer. If the levels of unknown metabolites (-88 , -129.9 and $U1$) are added to the FU + FBAL level, the total metabolism reaches 0.016% i.d. If we compare the data between livers and kidneys sampled after 4 h from rats treated with 1 g kg^{-1} b.w. OGT 719 with those obtained after 20 days of treatment at $3 \text{ g OGT 719 kg}^{-1} \text{ day}^{-1}$ of rats bearing sarcoma (0.9 versus $337 \text{ nanomol g}^{-1}$ in liver and 4.9 versus $198 \text{ nanomol g}^{-1}$ in kidney; tables 3 and 5), there is an increase in the amount of hepatic FU metabolites much greater than could be accounted for by the increased dose. The same observation can be made in kidneys where the level of FU and FU metabolites is higher after 20 days. This strongly suggests an accumulation of OGT 719 metabolites in liver and kidneys when the treatment is over a long period.

The incorporation of FU into RNA, one of the two major mechanisms of action of this anticancer drug, was observed in HSN LV10 sarcoma tumours when rats were injected i.v. for 20 days with FU at a dose of 20 mg kg^{-1} b.w. day^{-1} (table 4). On the other hand, $2'$ - and $3'$ FUMP were undetected in the AI fractions of tumours from rats bearing sarcoma treated with OGT 719 whatever the dose, but were detected in the AI fractions of livers from rats treated at a dose of 3 g kg^{-1} b.w. day^{-1} (tables 4 and 5). This suggests that the incorporation of FU into RNA occurs after a treatment with OGT 719, which demonstrates a low metabolism into FU. A probable difference in blood flow between the implanted sarcoma and the liver can be evoked to explain why this incorporation was not detected in the sarcoma.

OGT 719 is clearly effective in retarding the tumour growth of the human hepatoma HepG2 xenografted in nude mice (figure 3). The tumours and livers from animals treated with oral OGT 719 at a dose of 1.5 g kg^{-1} b.w. day^{-1} for 28 days were analysed with ^{19}F -NMR. Even if the cytotoxic FU metabolites ($2'$ - and $3'$ FUMP indicative of the incorporation of FU into RNA, and/or FdUMP) were not detected, it is interesting to note that both the OGT 719 and FU contents of the HepG2 tumour are markedly higher than in the corresponding liver (table 6). This was not the case in sarcoma where the amounts of OGT 719 were very similar

in the tumour and the corresponding liver and the metabolism was much lower in the tumour than in the liver or the kidney (table 5). This may suggest tumour-specific trapping of OGT 719 in hepatoma. HepG2 cells are known to express the ASGP-r (Schwartz *et al.* 1981). However, this cannot be the sole explanation for a tumour-specific trapping since ASGP-r is also highly expressed in hepatocytes.

Although only four urine samples from two patients treated with OGT 719 were studied, the conclusion that can be drawn is that, as already observed in the IPRM model (table 2) and in non-tumour rats (table 3), the metabolism of OGT is extremely low as it represents 0.03–0.05% of i.d. (table 7). Our data are in agreement with those of a recent clinical study that showed by HPLC analysis that approximately 85% of the dose of OGT 719 is eliminated unchanged in the urine after i.v. dosing (Sharma *et al.* 2001). However, no quantifiable amount of FU could be detected, whereas our ^{19}F -NMR study demonstrated the presence of FU and its main catabolite, FBAL.

In conclusion, the present study shows the value of ^{19}F -NMR for demonstrating for the first time that OGT 719 is metabolized, although very poorly. All the fluorinated metabolites detected in rat and patient urine as well as in PCA extracts of liver, kidney or tumour tissues are FU, its main catabolite FBAL, and FBAL derivatives. OGT 719 is therefore a prodrug of FU. The very low extent of OGT 719 metabolism explains its lack of toxicity at doses as high as 2 g kg^{-1} b.w. in mice and rats and a maximum tolerated dose of 250 mg kg^{-1} b.w. day^{-1} for 14 days in dogs (Sharma *et al.* 2001).

Of the two principal mechanisms contributing to FU cytotoxicity, the incorporation of FU into RNA and the inhibition of TS by FdUMP, only the first one is accessible to ^{19}F -NMR as the level of FdUMP formed is too low for the sensitivity of this analytical technique. Owing to the low amount of FU liberated by OGT 719, the hydrolysis compounds of FU incorporated into RNA, i.e. 2' and 3'-FUMP, were only detected in PCA extracts of liver (the main organ of FU metabolism) from tumour rats treated with the highest dose of OGT 719 (3 g kg^{-1} b.w. day^{-1} over 20 days). This finding suggests that the cytotoxic effect of OGT 719 is probably related to the FU activation pathway. However, even if ^{19}F -NMR is a very efficient technique when studying the metabolism of fluorinated drugs in animals or humans, its lack of sensitivity does not permit observations of subtle mechanisms within the cell. Given that OGT 719 bears a fluorine atom, the biodistribution of this drug and its metabolites within the cell should be readily achieved with techniques such as scanning ion microscopy or confocal spectral imaging which give images representing the distribution of a drug within cells (Berry *et al.* 1994, Feofanov *et al.* 1999).

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Single- and repeated-dose pharmacokinetics of eplerenone, a selective aldosterone receptor blocker, in rats

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Received 12 July 2002

1. The pharmacokinetics of eplerenone (EP) were examined in rats following single or repeated dosing with ^{14}C -labelled or unlabelled EP to characterize absorption, metabolism and excretion. Rates of EP metabolism and cytochrome P450 activities were determined *in vitro* after repeated dose administration of EP.

2. Following a single i.v. dose (15 mg kg^{-1}), the elimination half-life of EP was 0.80 and 1.14 h in male and female rats, respectively. Plasma clearances (CL) of EP were 1.62 and $1.20\text{ l kg}^{-1}\text{ h}^{-1}$ in males and females, respectively. Following a single oral dose (15 mg kg^{-1}), C_{max} and T_{max} of EP were $1.71\text{ }\mu\text{g ml}^{-1}$ and 0.5 h in male rats. The corresponding values in female rats were $3.54\text{ }\mu\text{g ml}^{-1}$ and 1.0 h. The systemic availability of EP was 25.6% in male rats and 66.4% in female rats, demonstrating sex differences in the pharmacokinetics of EP.

3. In the 8-day study, the $\text{AUC}_{0-24\text{h}}$'s of total EP (closed lactone ring form plus open form) following 100 and 200 mg kg^{-1} oral doses were approximately half those on day 1 in male rats. After repeated dosing for 13 weeks, the pharmacokinetics of total EP did not change with study duration at the 20 mg kg^{-1} dose in both males and females. However, at the 100 mg kg^{-1} dose, $\text{AUC}_{0-24\text{h}}$'s were notably reduced on day 24 but progressively increased on subsequent days to approximate day 1 levels by day 86 in both sexes. At the 500 mg kg^{-1} dose, the AUCs on day 86 remained lower than those on day 1. Reductions in AUCs on days 8 and 24 appeared to be the result of metabolism induction.

4. EP was extensively metabolized in male rats and most faecal and urinary radioactivity was in the form of metabolites. In female rats, the vast majority of urine and faecal radioactivity was associated with total EP. Thus, the sex difference in the pharmacokinetics of EP was due to more extensive metabolism in male rats.

5. The major metabolite in the rat was $6\beta\text{-OH EP}$. EP 6β -hydroxylase activity was well correlated with testosterone 6β -hydroxylase activity, indicating that EP metabolism to $6\beta\text{-OH EP}$ was mediated primarily by CYP3A in the rat.

6. After repeated dose administration, EP increased 6β -hydroxylase activities of testosterone and EP itself in a dose-dependent manner in both male and female rats, indicating that EP was a CYP3A inducer in the rat. There appeared to be no effects on activities of CYP1A1, 2B and 2E1.

Introduction

Effector hormones of the renin-angiotensin-aldosterone system (RAAS), angiotensin II and aldosterone are important contributors to the pathophysiology of heart failure (Packer and Cohn 1999). Elevated levels of angiotensin II stimulate adrenal release of aldosterone, a potent mineralocorticoid that promotes sodium retention, causes potassium and magnesium loss, potentiates sympathetic activity,

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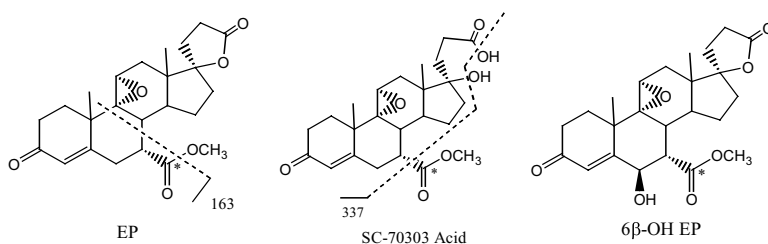


Figure 1. Structures of EP, SC-70303 (ring-opened form) acid, 6β-OH EP, and product ions of m/z 163 for EP and m/z 337 for SC-70303 acid. *Position of the labelled carbon atoms.

induces ventricular arrhythmias, stimulates myocardial fibrosis, and alters baroreceptor function (Weber and Villareal 1993, Katayama *et al.* 1994, Struthers 1995b, MacFadyen *et al.* 1997). It has been assumed that inhibition of the RAAS will suppress the formation of aldosterone, and several large clinical trials have demonstrated improvement in mortality rates and hospitalization following therapy with angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers in patients with heart failure (CONSENSUS Trial Study Group 1987, SOLVD Investigators 1991, McKelvie *et al.* 1999).

However, several studies (Staessen *et al.* 1981, Pitt 1995, Struthers 1995a) have shown an 'escape' of aldosterone production during long-term ACE inhibitor treatment, a phenomenon that allows residual aldosterone to exert adverse cardiac effects in heart failure patients being treated with standard therapies. Aldosterone receptor blockers offer an alternative strategy for reducing neurohormonal activation in heart failure. In a randomized, controlled clinical study in patients with severe heart failure, the addition of the aldosterone receptor blocker spironolactone to a treatment regimen of an ACE inhibitor, loop diuretic and digoxin reduced the risk rates of both morbidity and mortality (Pitt *et al.* 1999). Spironolactone, however, is a non-specific aldosterone receptor blocker that also inhibits androgen and progesterone receptors, leading to adverse effects, including gynecomastia, breast tenderness, menstrual dysfunction, and impotence (De Gasparo *et al.* 1986).

Eplerenone (EP; SC-66110) is a selective aldosterone receptor blocker that shows efficacy for the treatment of hypertension and heart failure. It is a closed-lactone ring molecule, which is in chemical and enzymatic equilibrium with the pharmacologically inactive open-ring form (figure 1). SC-70303 is the potassium salt of the open-ring form.

Phase III clinical studies have been completed and EP has recently been approved in the USA. Because EP selectively blocks the aldosterone receptor, adverse effects (e.g. gynecomastia) associated with its non-selective predecessor, spironolactone, may be reduced (De Gasparo *et al.* 1986). The current studies were conducted to determine the pharmacokinetics of EP after administration of single or repeated doses to rats, one of the animal species used in toxicity studies.

Materials and methods

Materials

EP (Lot No. GDS6050-025) with chemical purity greater than 95% (determined by HPLC) and [^{14}C]EP (Lot No. GDS2016-85) with radiochemical purity greater than 97% (by HPLC) were obtained from Pharmacia Corporation (Skokie, IL, USA). EP and [^{14}C]EP were protected from light during

storage at room temperature and at -20°C , respectively. All other chemicals were commercially available.

Single-dose studies

A single oral or intravenous (i.v.) dose of 15 mg kg^{-1} ^{14}C -labelled or unlabeled EP was administered to male and female rats (CrI:CD(SD)BR) from Charles River Laboratories, Inc. (Wilmington, MA, USA). The animals were approximately 6–8 weeks old and weighed between approximately 200 and 260 g. Environmental controls for the animal rooms were set to maintain $19\text{--}25^{\circ}\text{C}$, $50 \pm 20\%$ relative humidity, and a 12-h light/12-h dark cycle except for those periods required for dosing and sample collection. Animals were housed individually, fasted overnight and for 4 h after dosing. Water was available *ad libitum* throughout the study except for those periods required for dosing and collection of samples. In this pharmacokinetic study, blood samples were collected in heparinized vacutainers from four animals/dose route/sex at 0.033 (i.v. dose only), 0.25, 1, 2, 3, 5, 8, 24 and 48 h. Urine and faecal samples were collected from another four animals/dose route/sex every 24 h for 7 days. Extra blood samples were collected from a separate group of three animals/dose route/sex at 1 h to obtain metabolic profiles from plasma. For the biliary excretion study, the i.v. and oral doses of ^{14}C EP (15 mg kg^{-1}) were administered to three bile duct-cannulated male rats approximately 24 h after completion of surgery. In the pharmacokinetic study, the plasma, urine and faecal samples were analysed for concentrations of total radioactivity, EP, SC-70303 acid and total EP (EP plus SC-70303 acid). In the bile-duct-cannulated rat study, urine and bile samples were collected for 48 h and analysed for total radioactivity.

Repeated-dose studies

Two *in vivo* repeated-dose studies were conducted to determine whether the pharmacokinetics of EP change after repeated dose administration. In the first study, EP was administered orally to groups of male rats for a period of 8 days at single daily doses of 100 and 200 mg kg^{-1} . On days 1 and 8 ^{14}C EP was administered instead of unlabeled EP. In the second study, male and female rats in the 13-week toxicity study received daily doses of 20, 100 or 500 mg kg^{-1} . In the 20 and 100 mg kg^{-1} dose groups, ^{14}C EP was administered in place of non-labelled EP on days 1, 37 and 86 of the study. Blood samples were collected in heparinized vacutainers at 0.5, 1, 3, 5, 8 and 24 h after dosing on days 1 and 8 in the 8-day study, and on days 1, 24, 37, and 86 in the 13-week study. Each animal was bled twice on each day of sampling and three animals were bled at each time point. All animals were killed within 30 min after the last samples were collected. Plasma concentrations of total EP were determined using the liquid chromatographic/tandem mass spectrometric (LC-MS/MS) procedure. Urine and faecal samples were collected from separate groups of animals (four males in the 8-day study and three animals/sex in the 13-week study) daily for 7 days after each radioactive dose in both studies and analysed by HPLC with a radioactivity detector.

Determination of CYP activities

Male and female rats were administered EP at daily doses of 0, 20, 100 and 500 mg kg^{-1} for 36 days in order to examine changes in cytochrome P450 (CYP) activities after repeated dose administration of EP. Five rats per sex were killed on days 7, 24 and 36. In addition, in the 13-week study described above, the male and female rats were killed on day 92. The S9 and microsomal fractions were prepared from the liver samples obtained in the 36-day and 13-week studies using a method reported by Mazel (1972). Concentrations of total protein were determined by the Lowry method (Peterson 1979) using Kits obtained from Sigma-Aldrich Co. (St Louis, MO, USA). The hepatic microsomal fractions were also characterized for ethoxyresorufin *O*-deethylase (CYP1A), pentoxyresorufin *O*-dealkylase (CYP2B), testosterone 6β -hydroxylase (CYP3A) and lauric acid 11-hydroxylase (CYP2E) activities.

Ethoxyresorufin *O*-deethylase and pentoxyresorufin *O*-dealkylase activities were assayed using a method based on that of Plopper *et al.* (1993). Briefly, a 1.0 ml reaction mixture contained 0.5 mg microsomal protein, 0.1 M potassium phosphate buffer (pH 7.4) with 1 mM EDTA, a glucose 6-phosphate dehydrogenase (1 unit ml^{-1})-based NADPH-generating system containing 1 mM NADP, 5 mM glucose 6-phosphate and 3 mM MgCl_2 , and $2\text{ }\mu\text{M}$ ethoxyresorufin or pentoxyresorufin. Following incubation for 6 min at approximately 37°C , the reaction was terminated with the addition of 2 ml methanol. The samples were centrifuged and an aliquot (0.5 ml) of the supernatant was combined with 0.5 ml 25 mM phosphate buffer (pH 6.8). A 100- μl aliquot of the diluted supernatant was analysed in triplicate for resorufin by reverse-phase high-performance liquid chromatography (HPLC) with a fluorescence detector ($\lambda_{\text{ex}} = 535\text{ nm}$, $\lambda_{\text{em}} = 585\text{ nm}$).

Testosterone hydroxylase activity was determined using a method based on that of Sonderfan *et al.* (1987). A 1.0 ml reaction mixture contained 1 mg microsomal protein, 0.1 M potassium phosphate buffer (pH 7.4) with 1 mM EDTA, the glucose 6-phosphate dehydrogenase (1 unit ml^{-1})-based NADPH-generating system containing 1 mM NADP, 5 mM glucose 6-phosphate and 3 mM MgCl_2 , and 0.26 mM ^{14}C testosterone in 0.5% acetonitrile. Following incubation for 20 min at 37°C , the

reaction was stopped by addition of 6 ml ice-cold dichloromethane. The [^{14}C]testosterone and [^{14}C]testosterone metabolites were extracted into dichloromethane and analysed using an HPLC procedure.

Lauric acid hydroxylase activity was measured using a method based on that of Okita *et al.* (1991). The 1.0-ml reaction mixture consisted of 0.5 mg microsomal protein, 0.05 M Tris-HCl buffer (pH 7.4), an NADPH-generating system and 0.11 mM [^{14}C]lauric acid. Following incubation for 20 min, the reaction was terminated by addition of 0.2 ml ice cold 0.3 M HCl. The reaction mixture was extracted with ethyl acetate and the extracts were analysed for 11-hydroxy lauric acid by HPLC using a flow through radioactivity detector.

In vitro [^{14}C]EP metabolism

In vitro metabolism of [^{14}C]EP to 6 β -hydroxy [^{14}C]EP was determined using the rat hepatic S9 fractions prepared from the 36-day and 13-week studies. [^{14}C]EP was incubated for 30 min at 37°C with the glucose 6-phosphate dehydrogenase (1 unit ml $^{-1}$)-based NADPH-generating system containing 1 mM NADP, 5 mM glucose 6-phosphate and 3 mM MgCl $_2$, and 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA at a concentration of 100 μg [^{14}C]EP ml $^{-1}$ at a protein concentration of 4.2 mg ml $^{-1}$. The total volume of incubation mixture was 0.2 ml. The concentrations of [^{14}C]EP and the 6 β -OH metabolite were determined using an HPLC procedure with a radioactivity detector.

Plasma assay

Plasma concentrations of EP and SC-70303 acid in unacidified plasma were determined by a validated LC-MS/MS procedure using [D $_3$, ^{13}C]EP and [D $_3$, ^{13}C]SC-70303 acid, respectively, as internal standards as follows: internal standard solution containing 0.50 μg [D $_3$, ^{13}C]EP and 0.50 μg [D $_3$, ^{13}C]SC-70303 was added to 0.5 ml heparinized rat plasma containing EP and SC-70303. The plasma sample was extracted with a C18 Bond Elut (Varian, Inc., Harbor City, CA, USA) solid-phase extraction cartridge (SPEC) that was previously conditioned with acetonitrile and water. The contents of the cartridge were washed with water and the sample was eluted from the SPEC with 0.5 ml acetonitrile and evaporated to dryness. The residue was reconstituted in 0.125 ml mobile phase (10 mM ammonium acetate in acetonitrile:water, 40:60 by vol.) and injected into the LC of LC-MS/MS system which was comprised of an ISS 200 LC autosampler (Perkin-Elmer Norwalk, CT, USA), a 1050 HPLC pump (Hewlett Packard, Wilmington, DE, USA) and API-III Plus quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada). The separation was carried out on a reversed-phase Zorbax column (Hewlett Packard). Peak area to the m/z 415 (parent molecule) $\rightarrow$ 163 (figure 1) product ion of EP was measured against the m/z 419 $\rightarrow$ 163 product ion of its internal standard [D $_3$, ^{13}C]EP in the positive-ion multiple reaction monitoring (MRM) mode. Peak areas of the m/z 431 (parent molecule) $\rightarrow$ 337 (figure 1) production of SC-70303 were measured against the m/z 435 $\rightarrow$ 337 product ion of its internal standard [D $_3$, ^{13}C]SC-70303 in the negative-ion MRM mode. The lower limit of quantification of the rat plasma assay using the calibration lines was 0.01 μg EP ml $^{-1}$ of plasma with a precision (expressed as coefficient of variation) of 10.5% and an accuracy (expressed as analytical recovery) of 103% for the between-batch calculations. For the within-batch calculation, the precision was 8.56% and the accuracy was 97.7%. For SC-70303, the lower limit of quantification was also 0.01 μg ml $^{-1}$ with a precision of 15.0% and an accuracy of 104% for the between-batch calculations. For the within-batch calculations, the precision was 10.3% and the accuracy was 94.0%. Calibration lines were linear over a concentration range of 0.01–2.50 μg ml $^{-1}$ for both EP and SC-70303 acid.

To determine concentrations of total EP, pooled plasma samples were acidified to convert SC-70303 acid to EP. Concentrations of total EP were analysed by the same LC-MS/MS procedure described for EP. The lower limit of quantification was 0.05 μg ml $^{-1}$ for total EP in the single dose study. For the repeated-dose studies, the lower limit of quantification for total EP was 0.20 μg ml $^{-1}$. Concentrations below the assay sensitivities were set to zero for calculations. Radioactivity in plasma and urine was analysed using a liquid scintillation counting (LSC) procedure after addition of Ultima-Gold scintillation cocktail to duplicate weighed aliquots. Faecal samples were mixed thoroughly with a tissue homogenizer. Duplicate 0.1-g aliquots were taken and radioactivity was determined by LSC after combustion in a sample oxidizer (model 306 or 307; Packard Instrument Co., Meriden, CT, USA).

Metabolic profiles of plasma, urine and faecal samples

The distribution of total radioactivity was determined in samples of plasma, urine and faeces. For urine and faeces, proportional aliquots by weight of sample were pooled from 0–24 and 24–48 h collections from each animal, and for plasma, 1 h samples pooled in equal amount from a metabolic-profile group of three animals were analysed. Aliquots of plasma and faecal samples were extracted with acetonitrile and dried under nitrogen. Dried samples were reconstituted in acetonitrile and water without acidification and analysed by HPLC. To convert the open lactone ring forms of EP and its metabolites to corresponding closed forms, acidified samples were prepared using the same procedure except for the addition of 1 N HCl to the reconstitution mixture. Urine samples were injected directly

onto the HPLC system after centrifugation with and without acidification. The HPLC system employed a linear gradient of 10:90 acetonitrile:water over 40 min at a flow rate of 1.0 ml min⁻¹. The radioactivity in the HPLC column eluates was monitored using a Flo-One radioactivity detector (Packard).

Pharmacokinetic and statistical analysis

Plasma concentration–time curves of EP, SC-70303 acid and total EP after i.v. and oral administration were analysed according to a non-compartment model using the WinNonlin computer program (Version 3.1, Pharsight Corporation, Mountain View, CA, USA). Statistical analyses in the *in vitro* studies were performed using an unpaired *t*-test.

Results

Single-dose pharmacokinetics

The mean plasma concentration of total radioactivity, EP, SC-70303 free acid and total EP after administration of a single i.v. or oral dose (15 mg kg⁻¹) of [¹⁴C]EP are shown in figure 2. After i.v. administration, plasma concentrations of total radioactivity were similar in the male and female rat and there were no substantial gender differences. The elimination half-lives of total radioactivity (mean ± SEM) were 1.90 ± 0.31 and 1.51 ± 0.30 h, respectively. The AUC_{0–∞} in males and females were 31.5 ± 3.5 and 33.7 ± 1.5 µg eq. h ml⁻¹, respectively. After oral administration of [¹⁴C]EP, mean peak plasma concentrations of total radioactivity of 7.64 ± 1.91 and 7.67 ± 0.99 µg eq. ml⁻¹ were reached at 1.1 ± 0.6 and 0.8 ± 0.3 h post-dose in male and female rats, respectively, indicating rapid absorption of the radioactive dose. The AUC_{0–∞} of total radioactivity after oral administration to male and female rats were 16.1 ± 2.3 and 21.3 ± 0.4 µg eq. h ml⁻¹, respectively, demonstrating only moderate gender differences. These values correspond to approximately 51 and 63% of the AUCs in the male and female rat, respectively, after i.v. administration.

The elimination half-life ($\beta t_{1/2}$) of EP estimated from EP concentrations in pooled plasma from four male and four female rats after i.v. administration was 0.80 and 1.14 h, respectively. The plasma clearance (CL) of EP was slightly higher in males than in females (table 1). Pooled plasma concentrations of SC-70303 acid were initially lower than those of EP after i.v. dosing but reached the same levels after 0.5 h. Maximum plasma concentrations ($C_{\max}$) of SC-70303 acid were reached within 0.25 h regardless of gender, indicating rapid conversion of EP to the open form of the lactone ring. The $C_{\max}$'s of SC-70303 acid were 7.79 and 8.49 µg ml⁻¹ in the male and female rat, respectively. As observed with EP concentrations, plasma concentrations of SC-70303 acid were higher in females than in males. Plasma concentrations of total EP were also higher and the plasma clearance of total EP was lower in females.

Plasma concentrations of EP after oral administration were considerably higher in female rats and this gender difference was more pronounced than with the i.v. dose (table 2). The $C_{\max}$ of EP in female rats was approximately twice that of male rats and was achieved within 1 h in both male and female rats, indicating rapid absorption of the drug. The systemic availabilities of EP were 25.6 and 66.4% in male and female rats, respectively. Plasma concentrations of SC-70303 acid after oral administration were similar to EP concentrations throughout the sampling period regardless of gender, resulting in similar AUC ratios of SC-70303 acid/EP between males and females although concentrations of SC-70303 acid were higher

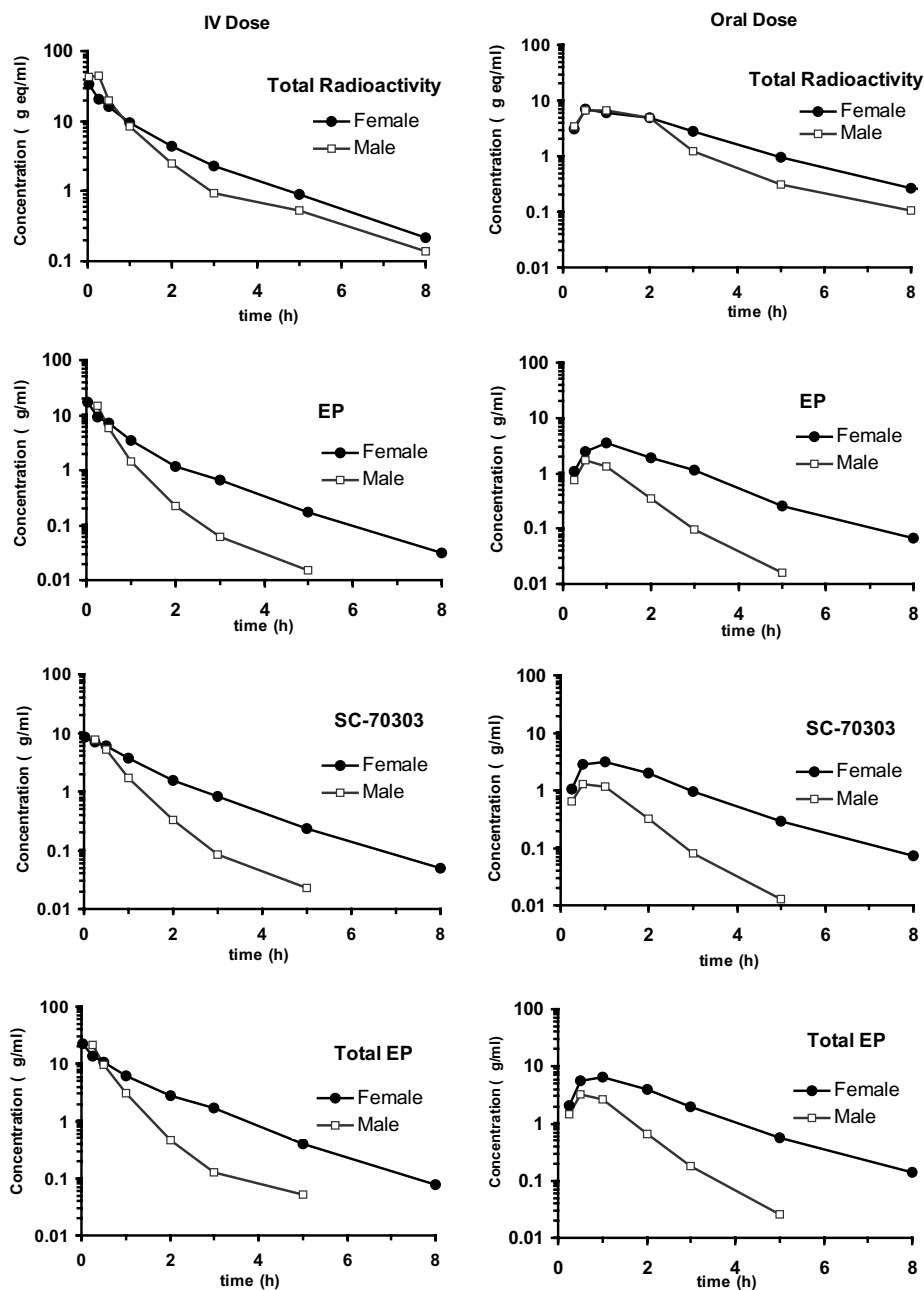


Figure 2. Plasma concentration-time curves of total radioactivity (C-14), EP, SC-70303 acid and total EP after i.v. (left) or oral (right) administration of 15 mg kg^{-1} [^{14}C]EP in male and female rats. Data for total radioactivity are the mean of four males and four females. Data for EP, SC-70303 acid, and total EP are the concentrations in plasma that were pooled in an equal volume from four males and four females.

in females than in males. The plasma concentrations of total EP were also higher in females after oral administration. The systemic availability of total EP after oral administration was 29.4 and 74.5% in male and female rats, respectively.

Table 1. Non-compartmental pharmacokinetic parameters for EP, SC-70303 acid and total EP^a after single i.v. administration of EP at a dose of 15 mg kg⁻¹.

Parameter	EP		SC-70303 Acid		Total EP ^a	
	Male	Female	Male	Female	Male	Female
β (h ⁻¹)	0.86	0.61	1.26	0.58	0.69	0.61
$\beta t_{1/2}$ (h)	0.80	1.14	0.55	1.21	1.01	1.14
V_d (l kg ⁻¹)	1.87	1.98	n.d.	n.d.	1.42	1.15
CL (l kg ⁻¹ h ⁻¹)	1.62	1.20	n.d.	n.d.	0.98	0.70
AUC (h μ g ml ⁻¹)	9.28	12.5	7.10	11.3	15.3	21.6

^aTotal EP = EP + SC-70303 acid.EP, eplerenone; β , elimination rate constant; $t_{1/2}$, half-life; V_d , volume of distribution; CL, clearance; n.d., not determined; AUC, area under the plasma concentration–time curve.Table 2. Non-compartmental pharmacokinetic parameters for EP, SC-70303 acid, and total EP^a after single oral administration of EP at a dose of 15 mg kg⁻¹.

Parameter	EP		SC-70303 Acid		Total EP ^a	
	Male	Female	Male	Female	Male	Female
$C_{\max}$ (μ g ml ⁻¹)	1.71	3.54	1.30	3.13	3.20	6.35
$t_{\max}$ (h)	0.5	1.0	0.5	1.0	0.5	1.0
K_e (h ⁻¹)	1.02	0.57	1.08	0.55	1.06	0.55
$t_{1/2}$ (h)	0.68	1.22	0.64	1.26	0.66	1.26
AUC (h μ g ml ⁻¹)	2.38	8.30	1.98	8.04	4.50	16.1
BA (%)	25.6	66.4	n.d.	n.d.	29.4	74.5

^aTotal EP = EP + SC-70303 acidEP, eplerenone; $C_{\max}$, maximum concentration; $t_{\max}$, time to reach peak plasma concentration; K_e , elimination rate constant; $t_{1/2}$, half-life; AUC, area under the plasma concentration–time curve; BA, bioavailability; n.d., not determined.

Repeated-dose pharmacokinetics

In a preliminary 8-day repeated-dose study in which daily oral doses of 100 or 200 mg kg⁻¹ were administered to male rats, plasma concentrations of total EP on day 8 were approximately 50% of the concentrations on day 1 regardless of dose (figure 3). In the 13-week study, male and female rats received daily doses of 20, 100 or 500 mg kg⁻¹ EP orally. As observed with the single-dose data, plasma concentrations of total EP were considerably higher in female rats and the differences in AUCs between male and female rats were nearly consistent across doses and days of dosing (figure 4). After repeated-dose administration, AUC_{0–24h}'s of total EP increased as the dose increased over the duration of the study and the increase in AUC_{0–24h}'s was approximately dose proportional between the 20 and 100 mg kg⁻¹ doses but generally less than dose proportional between the 100 and 500 mg kg⁻¹ doses during the treatment. The AUCs of total EP did not change with duration of dosing at the 20 mg kg⁻¹ dose. However, at the dose of 100 mg kg⁻¹, male and female AUC_{0–24h}'s decreased by 54 and 46%, respectively, from days 1 to 24 but progressively returned to day 1 levels by day 86 (figure 4). At the 500 mg kg⁻¹ dose, the AUCs of total EP also decreased between days 1 and 24 and increased at days 37 and 86, as observed at the 100 mg kg⁻¹ dose. However, the AUCs on day 86 remained lower than those on day 1.

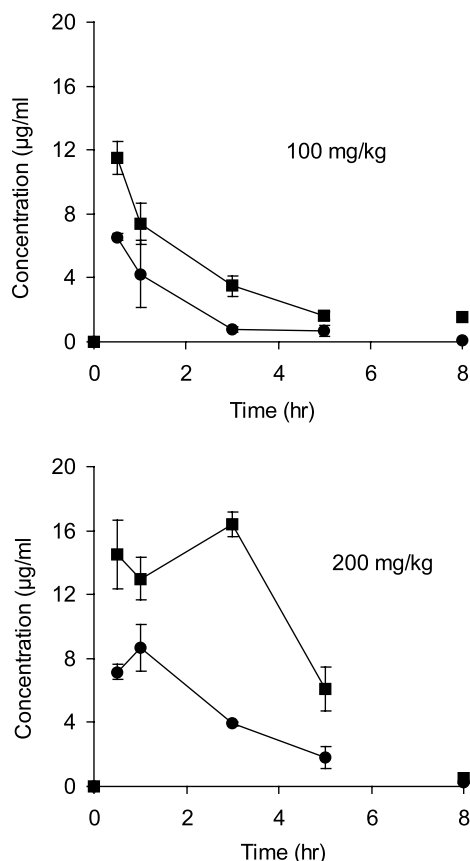


Figure 3. Plasma concentrations of total EP on days 1 (■) and 8 (●) in male rats during the 8-day study. Data are the means from three animals.

Excretion

The major route of excretion of total radioactivity after either i.v. or oral dosing was via the faeces, with approximately 75–84% of the dose excreted in faeces of both male and female rats (table 3). Urine accounted for 22.6 and 33.2% of the dosed radioactivity in males and females, respectively, after i.v. dosing and for 18.1 and 20.6%, respectively, after oral administration. After i.v. and oral administration to male bile duct-cannulated rats, the percentages of the dose excreted in 0–48 h urine were 18.0 and 17.1%, respectively, and the percentages of the total radioactive dose excreted in bile were 66.0 and 61.1%, respectively. The comparable urinary, faecal and biliary excretion results of total radioactivity between i.v. and oral routes suggest good absorption of [^{14}C]EP. The majority of the radioactive dose was eliminated in the first 24 h after dosing and no substantial gender differences were present for excretion of total radioactivity. In contrast to the excretion of total radioactivity, excretion of EP and total EP showed notable gender differences. The mean percentages of the dose excreted as EP and total EP in both faeces and urine were higher in female rats after single i.v. or oral administration (table 3). Approximately 76 and 25% of the dose were excreted as

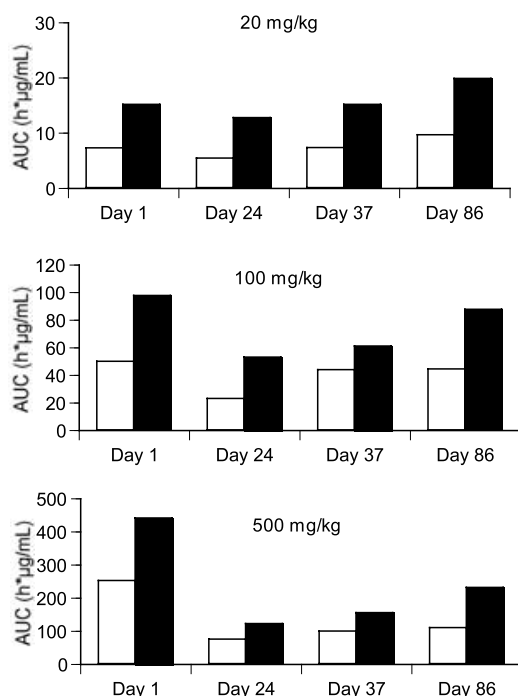


Figure 4. AUC_{0-24h} 's for total EP calculated from the mean plasma concentrations in male (white) and female (black) rats after repeated dosing with EP at 20, 100 and 500 mg kg^{-1} day $^{-1}$ in the 13-week study.

Table 3. Percentage of the dose excreted in urine and faeces following a single i.v. and oral dose (15 mg kg^{-1}) administration of EP to rats.

Dose route	Total				
	Matrix	Gender	Radioactivity	EP	Total EP ^a
Intravenous	urine	male	22.6 ± 4.5	6.9 ± 1.6	7.1 ± 1.6
		female	33.2 ± 1.4	14.3 ± 1.4	19.8 ± 1.8
	faeces	male	83.5 ± 0.9	6.7 ± 1.8	16.9 ± 0.8
		female	74.6 ± 3.6	9.2 ± 2.8	55.1 ± 3.3
Oral	urine	male	18.1 ± 0.5	< 1	< 1
		female	20.6 ± 0.6	1.8 ± 0.4	8.1 ± 1.4
	faeces	male	79.2 ± 0.8	4.9 ± 0.6	14.5 ± 0.5
		female	77.8 ± 1.3	18.9 ± 4.6	52.3 ± 3.4

^aTotal EP = EP + SC-70303 acid.

EP, eplerenone. Data are the mean ± SEM from four males and four females.

metabolites in urine and faeces of male and female rats, respectively, after i.v. administration. The corresponding values for the oral dose were approximately 85 and 40%, respectively.

The faecal route remained the major route of excretion after repeated oral dosing with EP (table 4, and figures 5 and 6). The mean percentages of the total radioactive dose excreted in faeces were in the range of 68.3–86.8% in the male rat in the 8-day study, and 66.4–83.7% regardless of gender, dose or day of dosing during 13-week study. Interestingly, the percentages of the dose excreted as total

Table 4. Percentage of dose of metabolites and parent compound excreted in urine and faeces of male rats in the 8-day study.

Dose (mg kg ⁻¹)	Matrix	Analyte	Day 1	Day 8
100	urine	C-14	14.7 ± 1.8	9.68 ± 1.2
		EP	0.463 ± 0.149	0.205 ± 0.032
		6β-OH	8.74 ± 1.18	6.10 ± 0.84
	faeces	C-14	68.3 ± 2.0	74.5 ± 2.3
		EP	12.0 ± 2.5	9.56 ± 1.0
		6β-OH	10.8 ± 0.5	13.7 ± 0.3
200	urine	C-14	20.6 ± 1.8	11.0 ± 1.0
		EP	0.490 ± 0.076	0.175 ± 0.035
		6β-OH	11.7 ± 1.2	6.67 ± 0.65
	faeces	C-14	86.8 ± 2.1	80.6 ± 1.5
		EP	15.3 ± 2.0	11.1 ± 0.2
		6β-OH	13.7 ± 0.3	14.8 ± 0.7

C-14, total radioactivity; EP, eplerenone; 6β-OH, 6β-hydroxy metabolite of eplerenone. Data are the mean ± SEM for four males and four females.

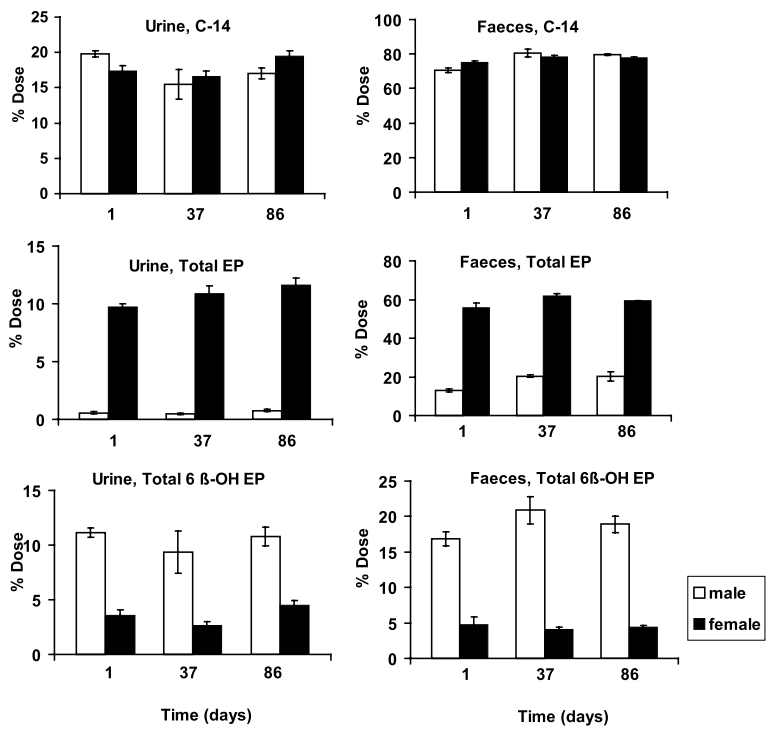


Figure 5. Percentage of dose excreted as C-14, total EP and total 6β-OH EP in urine (left) and faeces (right) of male (white) and female (black) rats at an EP dose of 20 mg kg⁻¹ day⁻¹ in the 13-week study. Data are the means of three males and three females.

EP in faeces on day 8 were similar to those on day 1, even though the AUCs of total EP were notably lower on day 8. Gender differences in urinary and faecal excretion of total EP continued to be observed in the 13-week study, with female rats excreting a higher percentage of the dose as total EP (figures 5 and 6).

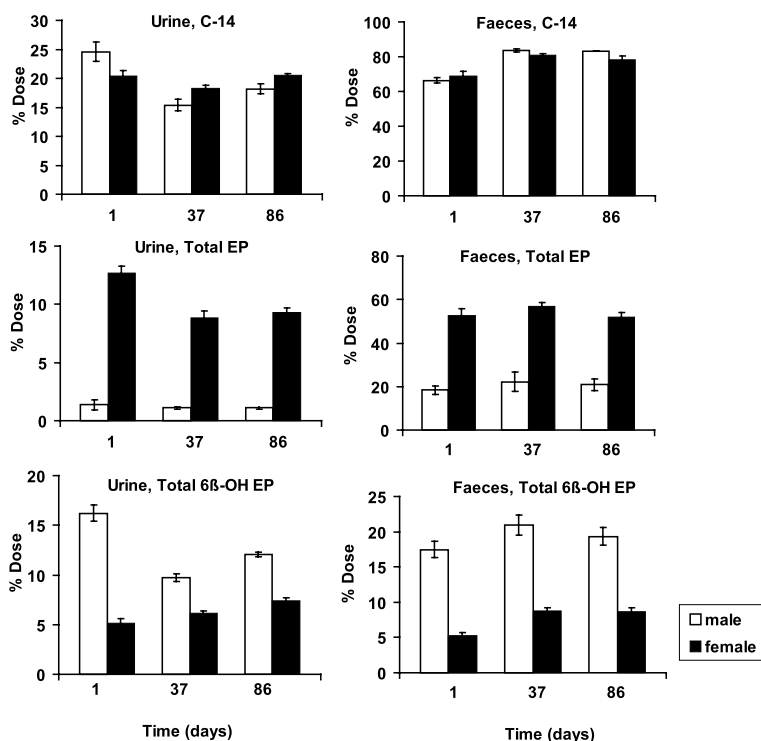


Figure 6. Percentage of dose excreted as C-14, total EP and total 6β-OH EP in urine (left) and faeces (right) of male (white) and female (black) rats at an EP dose of 100 mg kg⁻¹ day⁻¹ in the 13-week study. Data are the means of three males and three females.

Metabolism

As described above, the animals in the 20 and 100 mg kg⁻¹ dose groups received [¹⁴C]EP on days 1, 37 and 86 in the 13-week study in order to determine the metabolic profiles of [¹⁴C]EP. Representative HPLC profiles in acidified plasma, urine and faeces on day 1 after oral administration of [¹⁴C]EP at the 20 mg kg⁻¹ dose are shown in figure 7. Acidification of the samples converted open lactone-ring forms of the EP and its metabolites to closed forms. Substantial gender differences were noted for the metabolic profiles in plasma, urine and faeces. In male rats, most of the radioactivity in plasma was associated with total EP and the 6β-OH metabolite. However, in female rats, the vast majority of plasma radioactivity was due to total EP. Most of the radioactivity in urine and faeces was in the form of metabolites in male rats, and 6β-OH EP was the major metabolite in male rat urine. Faecal samples of male rats displayed 10–14 metabolite peaks in addition to [¹⁴C]EP. These results, coupled with urinary data, indicate extensive metabolism of EP in male rats. In the female rat, the vast majority of urine and faecal radioactivity was associated with total EP. The differences between male and female rats in the extent of plasma, urinary, and faecal metabolism persisted after continuous dosing for 13 weeks regardless of the dose (figures 5 and 6). After repetitive dose administration, the majority of radioactivity in plasma, urine, and faeces was due to total EP in female rats, as observed on day 1. In male rats, the contribution of total EP to total radioactivity

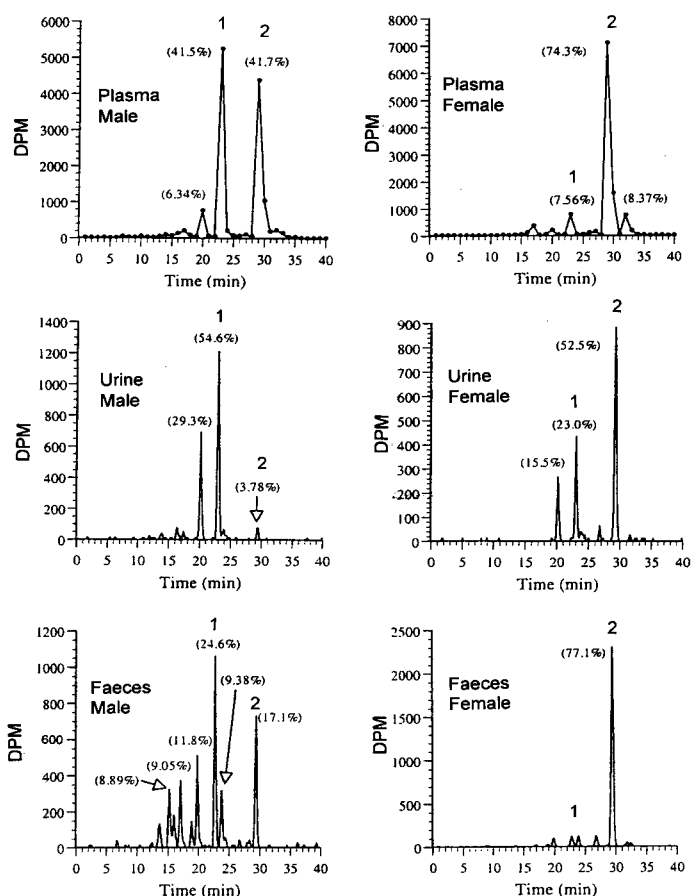


Figure 7. Representative HPLC chromatograms of acidified plasma (1 h), urine (0–48 h) and faeces (0–48 h) from male (left) and female (right) rats after oral administration of a single EP dose of 20 mg kg^{-1} . Peaks 1 and 2 are total 6β -OH EP and total EP, respectively.

was considerably smaller (1 h plasma: 68–75% for females versus 42–57% in males; urine: 41–72% for females versus < 10% for males; faeces: > 63% for females versus < 37% for males). Metabolic profiles in these three matrices after repetitive-dose administration were similar to those after single-dose administration (data not shown).

Effect of EP on CYP activities and EP metabolism

CYP3A activities in the liver after oral administration of EP at daily doses of 20, 100 and 500 mg kg^{-1} are shown in figure 8. EP increased testosterone 6β -hydroxylase activities over control levels in a dose dependent manner in both male and female rats. In male rats, mean testosterone 6β -hydroxylase activities increased significantly above control levels at the doses of 100 (1.6–2.1-fold) and 500 (1.7–3.1-fold) mg kg^{-1} up to 92 days of treatment. At the 20 mg kg^{-1} , the 6β -hydroxylase activities did not increase significantly in general. In female rats, mean testosterone 6β -hydroxylase activities increased significantly above control levels

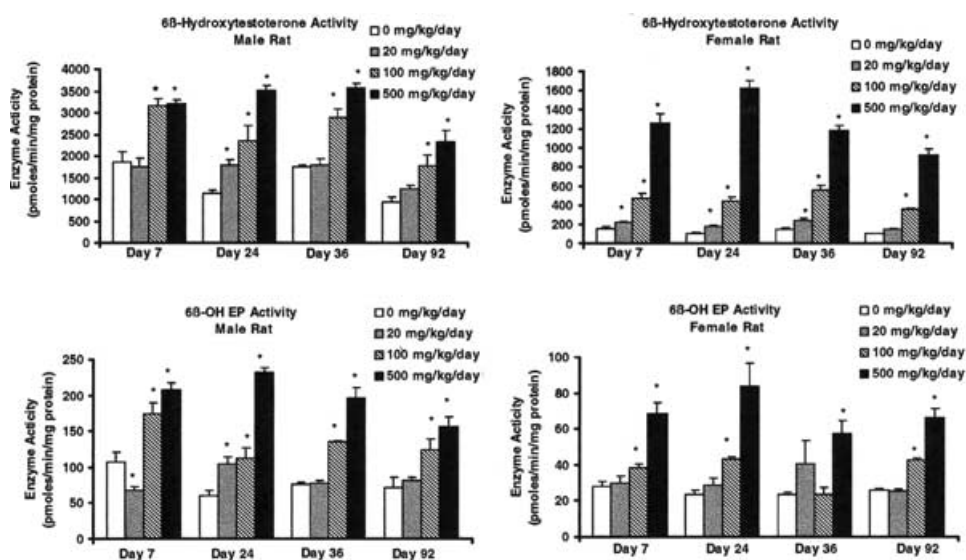


Figure 8. Activities of testosterone 6 β -hydroxylase (top) in liver microsomes and EP 6 β -hydroxylase (bottom) in liver S9 fractions obtained from male (left) and female (right) rats after repetitive oral administration of EP at different doses. Data are the means of four males and four females. *Statistical significance ($p > 0.05$) by *t*-test.

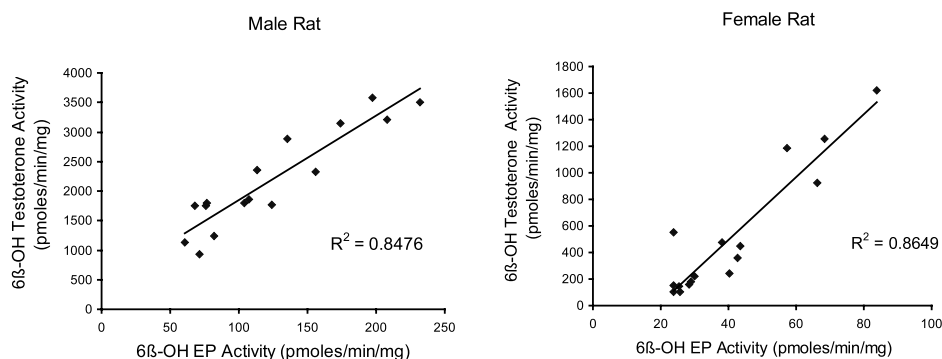


Figure 9. Correlation between microsomal testosterone 6 β -hydroxylase activities and S9 EP 6 β -hydroxylase activities in the male (left) and female (right) rat liver treated with EP at different doses.

at 20 (1.4–1.7-fold), 100 (3.0–4.3-fold) and 500 (7.8–15.6-fold) mg kg^{-1} doses at all periods except for day 92 at 20 mg kg^{-1} . When [^{14}C]EP was incubated with the liver S9 fractions, metabolism of EP to 6 β -hydroxy EP was increased at the doses of 100 and 500 mg kg^{-1} in male rats. In female rats, the metabolism of EP was also increased generally at the doses of 100 and 500 mg kg^{-1} . There was a good correlation between 6 β -hydroxylase activities of testosterone and EP (figure 9), indicating that metabolism of EP to 6 β -hydroxy EP was mediated by CYP3A.

In contrast to testosterone and EP 6 β -hydroxylase activities, there was no consistent effect of the administration of EP on ethoxyresorufin (CYP1A1) or pentoxyresorufin (CYP2B) *O*-deethylase activities regardless of the dose

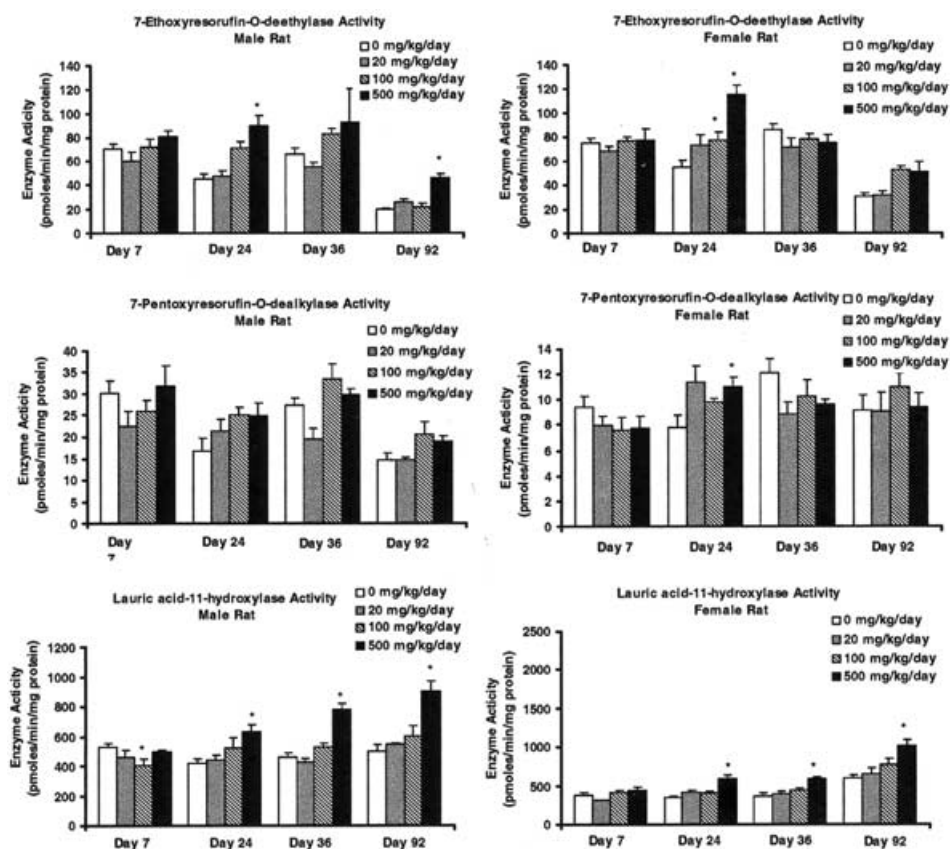


Figure 10. Activities of CYP1A (top), CYP 2B (middle) and CYP2E (bottom) in liver S9 fractions obtained from male (left) and female (right) rats after repetitive oral administration of EP. Data are from four males and four females. *Statistical significance ($p > 0.05$) by *t*-test.

(figure 10). When lauric acid 11-hydroxylase activities were measured for CYP2E1 activity, the 11-hydroxylase activities were significantly higher over control values on days 24, 36 and 91 in both males and females at the 500 mg kg⁻¹ dose only and there were no consistent increases at the other doses.

Discussion

The present study has demonstrated that EP is rapidly and well absorbed after oral administration to the rat. The *T*_{max} of EP was observed at 1 h or less following oral dose administration. The percentages of the radioactive dose excreted in urine are similar after single i.v. and oral dose (15 mg kg⁻¹) administration, suggesting good absorption of drug-related material. However, substantial gender differences are evident in the systemic availability of EP or total EP. Gender differences were also noted in SC-70303 acid concentrations.

The gender differences in the systemic availability of EP and total EP are due to more extensive metabolism of EP in male rats, which appeared to be mediated by CYP3As in the rat. EP is also metabolized by the CYP3A subfamily in the dog

and human (Cook *et al.* 2002). Steroid molecules such as testosterone and progesterone are also metabolized to 6 β -hydroxy metabolites by CYP3A (Yamazaki and Shimada 1997) in humans. Therefore, it is not surprising to find that 6 β -hydroxylation of EP is also catalysed by CYP3A in the rat. Gender differences in drug metabolism are more commonly observed in the rat than in other species and have been attributed in large part to the greater expression of CYP2C and CYP3A enzymes in males (Waxman *et al.* 1985).

After repeated oral administration at the 20 mg kg⁻¹ dose, the AUC did not change notably for the duration of the 13-week study. However, the AUCs of total EP were reduced by approximately 50% in male rats at the doses 100 and 200 mg kg⁻¹ in the 8-day study. In the 13-week study, the AUCs were also reduced notably on day 24 in both male and female rats at the 100 mg kg⁻¹ dose and on days of 24, 37 and 86 at 500 mg kg⁻¹. This was probably due to, at least in part, induction of oxidative metabolism. The *in vitro* metabolism study demonstrated that with repetitive treatment of EP at doses of 100 and 500 mg kg⁻¹, CYP3A activity in the liver was induced and EP metabolism increased. However, unlike plasma concentration data, the percentages of total EP in faecal radioactivity did not change considerably, although there was a trend toward lower percentages of the parent drug after repetitive dose administration. One possible explanation for these phenomena is that induced metabolism was counteracted by enhancement of biliary excretion of the drug, resulting in no remarkable changes in the ratio of the parent drug to metabolites in faeces. Enhancement of biliary excretion after repetitive dosing of EP is evidenced by the fact that ratios of the 6 β -OH metabolite excreted in faeces to that in urine increased on day 8 compared with day 1 in the 8-day study and at least up to day 37 in the 13-week study. In the 8-day study, excretion of the metabolite in urine and faeces was similar on day 1, whereas on day 8 excretion of the metabolite in faeces was approximately two times higher than that in urine at both 100 and 200 mg kg⁻¹ doses. In the 13-week study, the excretion ratios of the 6 β -OH metabolite in faeces to that in urine increased approximately 2-fold on day 37 in male rats and 40% in female rats at the dose of 100 kg⁻¹. Enhancement of biliary excretion has been reported after repeated-dose administration of another steroid drug, spironolactone (Smith and Gordon 1988), to the rat. Biliary excretion of ouabain is also increased after treatment with pregnelone-16 α -carbonitrile (Thomson and Klaassen 1995). Increased biliary excretion of EP coinciding with induced metabolism would reduce the AUC of the parent drug without altering the ratios of drug/metabolites excretion substantially. Alternatively, it is also possible that absorption is reduced after repeated dose administration, resulting in increase in excretion of unabsorbed EP in faeces. The increase in excretion of unabsorbed EP may have been counteracted by the enhanced metabolism of absorbed EP, thus maintaining similar ratios of EP/metabolites in faeces.

Interestingly, in the 13-week study, the AUC_{0-24h}'s at the 100 mg kg⁻¹ dose increased on day 86, reaching similar plasma concentrations as on day 1 (figure 4) whereas at the 500 mg kg⁻¹ dose, the AUCs on day 86 remained lower than those on day 1. Similar phenomena were observed in a 26-week study (data not shown). *In vitro* metabolism study demonstrated that induction of EP metabolism was sustained up to 92 days for both doses although induction was more pronounced at the 500 mg kg⁻¹ dose. Therefore, the exact reason for this rebounding phenomenon of AUCs at the 100 mg kg⁻¹ dose is not known. One possibility is that after

repeated dose administration longer than 8 days, the metabolites may have accumulated in the liver at high concentrations, and consequently both metabolism and possible active secretion in bile might be inhibited by these metabolites. At the 500 mg kg⁻¹ dose, drug-metabolizing enzymes were extensively induced and inhibition by the metabolites may not have fully counteracted the induction and/or further metabolism of these metabolites may have been induced. The terminal half-life of total radioactivity in the liver was approximately 130 h and it would take more than 22 days to attain steady-state levels. Another possibility is absorption that was initially reduced by repetitive dosing was gradually increased to day 1 level after repeated-dose administration for more than 8 days.

The major excretion route of total radioactivity following i.v. or oral dose administration was via the faeces, and no substantial gender differences were observed in excretion of total radioactivity. High faecal excretion of drug-related materials regardless of dose route was due to extensive biliary excretion. In contrast to the radioactivity data, excretion of the parent drug in urine and faeces was much greater in females due to more extensive metabolism in male rats.

In conclusion, EP was rapidly and well absorbed in the rat and was rapidly converted to its lactone-ring open form and to its 6 β -OH metabolites. Metabolism to 6 β -OH EP appears to be catalysed by CYP3A. A notable gender difference was seen in the systemic availability of the parent drug, which was due to more extensive metabolism in male rats. After repeated-dose administration, the reduction in the systemic exposure to the parent drug was due to induced CYP3A-related metabolism and possibly enhanced biliary excretion. The major route of excretion of total radioactivity after either i.v. or oral administration was via the faeces.

Acknowledgement

The authors thank Dr M. Berge and Ms K. Hoffmann at Covance Laboratories, Inc., for conducting the radioactive portion of the study, Drs M. McKeon, P. Adams and S. Thohan for conducting the *in vitro* experiments, and Mr L. Berry for helping with preparation of the manuscript.

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Disposition of a synthetic analogue of lipid A (E5564) in rats

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Received 2 September 2002

1. E5564, a lipid A analogue that potently antagonises lipopolysaccharide, is being developed to treat sepsis caused by Gram-negative bacterial infections. The pharmacokinetic profile of E5564 is independent of dose between 0.1 and 1 mg kg⁻¹. The distribution volume of E5564 is slightly larger than the total plasma volume, and the terminal elimination half-life is about 5 h.

2. Following ¹⁴C-E5564 administration (0.5 mg kg⁻¹), radioactivity rapidly accumulates in the liver and spleen. The half-life of E5564 in the liver is 5.1 h, which is similar to that in the plasma. At 48 weeks after dosing, 35.27% of the administered radioactivity was still present in the liver. Cumulative urinary and faecal excretion of radioactivity for up to 48 weeks after administration were 3.86 and 67.17% of the dose, respectively.

3. The results of mass spectroscopy and nuclear magnetic resonance analysis reveal that the main hepatic metabolite is di-dephosphorylated E5564. The half-life of di-dephosphorylated E5564 in the liver is 87.4 days, which is similar to that for the hepatic radioactivity.

4. The results indicate that E5564 is rapidly taken up by the liver, is metabolized via dephosphorylation pathways to form dephosphorylated E5564 and is mainly excreted in the faeces.

Introduction

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria that can stimulate the release of a number of pro-inflammatory mediators, such as interleukin (IL) 1, IL-6, IL-8 and tumour necrosis factor (TNF)- α by macrophages, monocytes and other cell types (Glauser *et al.* 1991). Structurally, LPS is composed of four distinct domains: the membrane-intercalated hydrophobic or lipid A domain, an inner core oligosaccharide (containing heptose and 3-deoxy-D-manno-2-octulosonic acid), an outer core oligosaccharide and the O-antigenic polysaccharide domain (Rietschel *et al.* 1994, Zähringer *et al.* 1994, Schletter *et al.* 1995). The lipid A portion of LPS consists of a backbone of two phosphorylated glucosamine molecules acylated with fatty acids and harbours the pro-inflammatory toxicity associated with LPS (Raetz 1990, Zähringer *et al.* 1999). It has been discovered that LPS or the lipid A portion from certain non-enterobacterial species, such as *Rhodobacter capsulatus* (Loppnow *et al.* 1990) or

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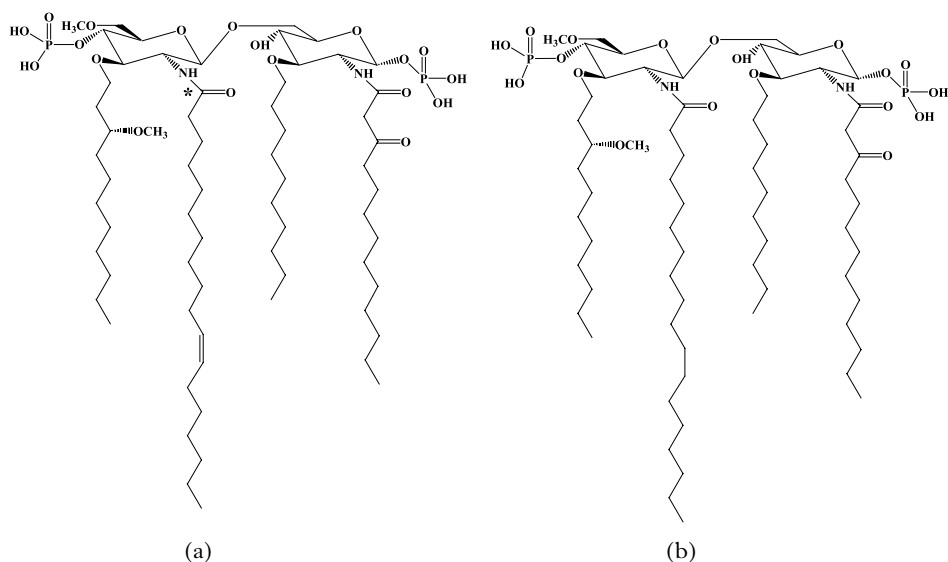


Figure 1. Chemical structures of E5564 (a) and IS (b). *Position radiolabelled with ^{14}C .

R. spaeroides (Takayama *et al.* 1989), has weak potential for cytokine release from monocytes and macrophages. Both *in vivo* and *in vitro*, these lipid A derivatives possess anti-endotoxin activity with a potency desirable of pharmaceutical antagonists. E5564, a synthetic analogue of *R. spaeroides* lipid A (figure 1), is one such compound. Results from *in vitro* studies with human blood show that E5564 potently antagonises LPS-mediated release of humoral mediators, and is devoid of agonistic activity (Rossignol *et al.* 1999).

This paper describes the results of a series of separate experiments conducted to determine the fate of E5564 in rats, the species used in the toxicological evaluation of the compound.

Materials and methods

Chemicals

^{14}C -E5564 and non-radiolabelled E5564 were synthesized by Eisai Research Institute (Wilmington, MA, USA) and Eisai Merrimack Valley Laboratories (Andover, MA, USA), respectively. The radiolabelled position of ^{14}C -E5564 is shown in figure 1a. The specific activity was 1.19 MBq mg^{-1} E5564, and the radiochemical purity was greater than 98%. The chemical purity of non-radiolabelled E5564 was greater than 98%. The structural analogue of E5564 was used for the internal standard (IS) in plasma assay, which was also synthesized by Eisai Merrimack Valley Laboratories (figure 1b). The E5564 metabolites M1 (mono-dephosphorylated at the 4'-carbon-E5564), M2 (mono-dephosphorylated at the 1-carbon-E5564) and M3 (di-dephosphorylated at the 4'- and 1-carbons E5564) used as chromatographic reference standards were also synthesized at the Eisai Research Institute. The chemical purities of these metabolites were 91, 84 and 89%, respectively. All other chemicals used were reagent grade or better and purchased from standard suppliers.

Animals

Separate groups (four animals per group) of male rats (Sprague-Dawley, 220–321 g) were used in these studies. For metabolite identification experiments, 10 male rats were used. The rats were obtained from SLC Japan (Shizuoka, Japan) and had free access to standard laboratory pelleted food (MF; Oriental Yeast; Tokyo, Japan) and water. The compound was administered to fed rats. During urine,

faeces and expired air collection, each rat was individually housed in its own glass cage. For other studies, rats were housed singly or in groups in stainless steel cages with raised wire mesh floors to prevent coprophagy. Standard conditions of temperature (20–26°C) and humidity (40–70%) were maintained in these studies. The animal room was lit for 12 h each day.

Preparation of dosing solution

Preparation and administration. For pharmacokinetic studies, a stock E5564 solution was made; it contained 6 mg ml⁻¹ non-radiolabelled E5564 in a vehicle consisting of 4.25 mM phosphate buffer (pH 7.4 ± 0.3) and 10% lactose (w/v; Mallinkrodt, Phillipsburg, NJ, USA). This stock solution was diluted with the vehicle solution to make administration solutions containing 0.1, 0.3 or 1 mg E5564 ml⁻¹. For the hepatic metabolite identification study, a stock E5564 solution was made; it contained 1.2 mg ml⁻¹ non-radiolabelled E5564 in a vehicle consisting of 4.25 mM phosphate buffer (pH 7.4 ± 0.3) and 10% lactose. For distribution, metabolism and excretion studies, the stock ¹⁴C-E5564 solution was mixed with equal volume of a vehicle consisting of 8.5 mM phosphate buffer (pH 7.4 ± 0.3) containing 20% lactose (w/v) to make a dosing solution that contained 0.5 mg ml⁻¹ E5564.

In pharmacokinetic experiments to determine the plasma concentration profile of E5564, non-radiolabelled E5564 at 0.1, 0.3 and 1 mg kg⁻¹ was administered intravenously to rats via the femoral vein. To identify the hepatic metabolite, non-radiolabelled E5564 was administered intravenously to rats via the femoral vein, jugular vein or both veins at 0.9 mg animal⁻¹ day⁻¹ for 5 days. For distribution, metabolism and excretion studies, a single intravenous administration of ¹⁴C-E5564 was given to each rat via the femoral vein at a dose of 0.5 mg kg⁻¹ (dose of radioactivity: 0.595 MBq kg⁻¹).

Sample collection

For pharmacokinetic studies, approximately 0.25 ml blood were drawn from jugular vein at pre-dose and at 5, 30 min, 1, 4, 8, 12 and 24 h after intravenous administration of E5564 to rats. The blood sample was centrifuged to separate the plasma, and 100 l plasma was stored at -20°C until analysis. Following the intravenous administration of ¹⁴C-E5564 to rats, approximately 50-mg blood samples for the determination of the blood level of radioactivity were collected at 2.5, 5, 15, 30 min, 1, 2, 4, 6, 8 and 24 h and every 24 h up to 720 h after administration.

For distribution studies, rats were sacrificed by exsanguination from the abdominal aorta under ether anaesthesia at 5 min, 1, 4, 8 h, 1, 2, 3 days, 1, 4, 12, 24, 36 and 48 weeks after administration, and the following tissues and organs were collected: blood, plasma, blood cells, cerebrum, cerebellum, spinal cord, pituitary gland, thyroid gland, lymph node, heart, lung, liver, kidney, adrenal gland, spleen, pancreas, adipose tissue around the kidney, muscle from the femoral region opposite side of injection, skin (latero-abdominal region), bone marrow, testes, aorta, vein, sciatic nerve, stomach, small intestine, large intestine, and the carcass.

Urine, faeces, and expired air ([¹⁴C]CO₂) were collected following intravenous administration of ¹⁴C-E5564 to rats. [¹⁴C]CO₂ in expired air was recovered by forcing a continuous flow of air through the metabolic cage and collecting the expired air in a 20% monoethanolamine solution. Urine and faeces were collected every 24 h up to 336 h after dosing, and then every 3, 6 and 7 days in a week for up to 336 days after administration. Cages were rinsed with water after each collection period and the washings were retained. In addition, cage debris was removed from cages before rinsing.

For the determination of metabolites, a portion of the faeces, lung, liver, kidney, adrenal gland, spleen and bone marrow were taken and stored in the dark at approximately -20°C.

To isolate metabolite from the liver, livers were collected 5 days after the last administration, perfused with 1.15% KCl solution then at -20°C until use.

Sample analysis

Plasma assay. Quantification of E5564 in the plasma was performed using a previously published method (Kaneko *et al.* 1999). Briefly, after the IS (an analogue of E5564) was added to each plasma sample, the samples were extracted and derivatized with the 9-anthryldiazomethane (ADAM; Funakoshi; Tokyo, Japan) reagent to add a fluorescent label. After solid-phase extraction, ADAM derivatives of E5564 and the IS were separated on a YMC-Pack AP-313 (250 × 6 mm i.d., Yamamura Chemicals Co., Ltd, Kyoto, Japan) using a gradient of methanol and ethanol that contained sodium acetate at a rate of 1.2 ml min⁻¹, and were detected by a fluorescence detector (excitation 254 nm, emission 415 nm). Quality control (QC) samples were separately prepared at 0.1, 1 and 10 µg ml⁻¹ in the rat plasma, and duplicate QC samples were concurrently measured to evaluate the reliability of the analytical run. The quantification range of this method validated for the E5564 concentration range from 0.03 to 20 µg ml⁻¹.

Determination of radioactivity

A Packard 2500TR (Packard Japan; Tokyo, Japan) liquid scintillation counter (LSC) was used to measure radioactivity. Before scintillation counting, all samples except urine samples were solubilized with 2 ml Soluene-350 (Packard), then mixed with 0.2 or 0.4 ml saturated benzoyl peroxide solution in benzene (if necessary) and 10 ml HIONIC-FLUOR (Packard). One gram of the urine sample was mixed with 10 ml HIONIC-FLUOR. For some tissues, a 20% (w/v) homogenate was prepared with water before they were solubilized. Dissected or homogenized tissue, blood, plasma and blood cells were further treated as described above. A portion of each faecal sample was mixed with distilled water to prepare a 20% (w/v) homogenate, then further treated as described above. The 20% monoethanolamine solutions containing [^{14}C]CO $_2$ in the expired air were mixed with 10 ml HIONIC-FLUOR. Cage wash water and debris were mixed with methanol (final concentration of methanol: about 50%) and homogenates were prepared. Aliquots of each homogenate were evaporated to remove the methanol, then reconstituted with water. The resulting solutions were individually mixed with 10 ml HIONIC-FLUOR. Each carcass was solubilized by heating under reflux in a mixture of 0.5 M sodium hydroxide and toluene for about 72 h. Each carcass solution was separately mixed with water, and aliquots of resulting mixtures were mixed with 10 ml HIONIC-FLUOR.

Determination of radioactive metabolites

Before analysis, about 0.5 g of each tissue was separately homogenized in about 2 ml distilled water. For the plasma and the homogenized liver, spleen, lung, kidney, adrenal gland, mandibular lymph node and bone marrow, 0.25 ml distilled water, 0.05 ml 1 M hydrochloric acid and 3 ml chloroform-methanol (1:2 v/v) were added to about 0.5 g of the samples, then mixed with a vortex mixer. For homogenized faeces, 3 ml chloroform-methanol (1:2 v/v) were added to about 0.5 g of the samples, then mixed with vortex mixer. The following procedures were then used to process all samples tested. First, after 2 ml chloroform were added and mixed, the samples were centrifuged (1500g 10 min) to separate the organic layer, which was collected. Then 2 ml chloroform-methanol (1:1) were again added to the water layer and mixed with a vortex mixer. Next, after 2 ml chloroform were again added to the water layer and mixed, the samples were centrifuged to separate the organic layer, which was collected. Finally, 2.5 ml chloroform-methanol (2:3) were added to the water layer and mixed. After 2 ml chloroform and 0.25 ml water were added and mixed, the samples were centrifuged to separate the organic layer, which was collected. The water layer was mixed with AQUASOL-2 (Packard) or HIONIC-FLUOR and counted in an LSC to determine the extraction efficiency. For each tissue sample, the three organic layers collected were combined and evaporated under a stream of nitrogen at 40°C. The residues were reconstituted in methanol, and an aliquot of each methanol solution was mixed with AQUASOL-2 or HIONIC-FLUOR. The radioactivity in these samples was measured to determine the extraction efficiency. The extraction efficiency was taken into account when calculating the concentration of each metabolite to the total radioactivity. The methanol solution was evaporated again, and the samples reconstituted with a small quantity of chloroform-methanol (1:1). An aliquot of each solution was applied onto a silica thin layer chromatography (TLC) plate (Kieselgel 60F254; 20 × 20 cm, 0.25 mm thick; Merck KGaA, Darmstadt, Germany) and separated with a chloroform-methanol-water-acetic acid (20:9:2:1 v/v/v/v) solution. After drying, the TLC plate was placed into contact with a BAS-III imaging plate (Fuji Photo Film Co., Ltd, Tokyo, Japan) in a shielded box for 1 week. A BAS-2000 (Fuji) Bio-imaging analyser was used to read the imaging plates, and the amount of radioactivity on each TLC plate was determined.

Isolation and identification of metabolites

Isolation. Crude radioactive metabolites extracted from the liver after administration of ^{14}C -E5564 were used as chromatographic markers, because the target metabolite was a major constituent of hepatic radioactivity. Each perfused liver was minced and homogenized with 1 M hydrochloric acid-water-chloroform-methanol (1:9:30:60 v/v/v/v), and each prepared solution was divided into 30-ml aliquots, which were added to separate test tubes. Each 30-ml aliquot was mixed with 15 ml chloroform, then centrifuged. After collection of the organic layer, 15 ml chloroform-methanol (1:1) were again added to the aqueous phase and the solutions were mixed well. After the addition of 15 ml chloroform to the resulting mixture, the organic layer was separated by centrifugation and collected. The organic phases collected were combined and evaporated under reduced pressure at 40°C. The residue was dissolved in a small amount of chloroform-methanol (5:3), and separated from biologic components on TLC plates with a chloroform-methanol-water-acetic acid solution (20:9:2:1 v/v/v/v) as the solvent. After separation, the silica gel area corresponding to the fraction containing the target metabolite was scraped off the plate, the silica gel scrapings were extracted with methanol and the silica was separated by centrifugation. After collection, each methanol solution was evaporated to dryness under reduced pressure at 40°C, then reconstituted with methanol before HPLC purification. The HPLC system consisted of a Waters automated gradient controller Model 600E (Waters Corporation, Milford, MA, USA) equipped with an SPD-10A UV detector and a Chromatopak CR-4A-X integrator (Shimadzu; Kyoto, Japan). Each concentrated methanol solution was loaded onto a YMC-pack ODS

AP-313 column (5 μm ; 25 cm $\times$ 6.0 mm i.d.) equipped with a YMC-Guard pack ODS-AP (5 μm ; 10 mm $\times$ 5.0 mm i.d.; Yamamura) by means of a mobile phase consisting of 0.2% (w/v) ammonium acetate in water-methanol (1:99) at ambient temperature. The flow rate of the mobile phase was 2 ml min⁻¹, the elution method was isocratic, and elution of the metabolite was detected by UV monitoring at 254 nm. The eluates containing the target metabolite were then loaded onto a YMC-pack Polymer C18 column (5 μm ; 25 cm $\times$ 4.6 mm i.d.) and separated using a gradient mobile phase consisting of a 1:1 water-methanol solution (A) and methanol (B) at ambient temperature. Elution proceeded at 1 ml min⁻¹ with a linear gradient of 0–100% B in 30 min. The eluates containing the target metabolite were collected and combined, then evaporated to dryness at 40°C. The dried samples were freeze-dried and stored at -20°C until analysis.

Identification. The authentic reference compound was dissolved in deuterium chloroform (approximately 5 mg ml⁻¹), and a JNM- α 600 (frequency 600 MHz) or a JNM- α 400 (400 MHz; JEOL, Tokyo, Japan) NMR spectrometer were used to measure the spectrum at 30°C. Similarly, purified metabolite was dissolved in deuterated chloroform and JNM- α 600 was used to measure their spectra. After NMR analysis, the authentic or purified metabolite samples were analysed by fast-atom bombardment (FAB) positive-mode mass-spectroscopy (JMS-SX102AQQ; JEOL). The acceleration voltage was 10 kV, and the FAB gas was Xe (6 kV). The resolution was 1000 s, scan range was m/z 100–1000 and the scan speed was 13.82 s, respectively.

Calculation of pharmacokinetic parameters

Pharmacokinetic parameters were calculated assuming a non-compartmental model and using WinNonlin, version 3.1 (Pharsight Corporation, Mountain View, CA, USA). The elimination half-lives were determined by linear regression analysis of data visually determined to lie in the terminal part of the log-transformed regression curve. A linear trapezoidal method was used to calculate the area under the plot of the plasma concentration against time to the last data point [$\text{AUC}_{(0-t)}$]. The extrapolated AUC from the time of the last quantifiable data point (t) to infinity [$\text{AUC}_{(t-\infty)}$] was estimated by dividing the concentration at t by the terminal elimination rate constant. $\text{AUC}_{(0-\infty)}$ was calculated as the sum of $\text{AUC}_{(0-t)}$ and $\text{AUC}_{(t-\infty)}$. Plasma clearance was calculated by dividing the dose by $\text{AUC}_{(0-\infty)}$. The volume of distribution at steady state (V_{dss}) was calculated as the plasma clearance multiplied by the mean residence time [$\text{AUMC}_{(0-\infty)}$] divided by $\text{AUC}_{(0-\infty)}$, where $\text{AUMC}_{(0-\infty)}$ is the area under the moment curve extrapolated to infinity.

Results

Dose-dependent pharmacokinetics

The plasma concentration–time profiles of E5564 in rats ($n = 4$) after a single intravenous bolus administration of 0.1, 0.3 or 1 mg E5564 kg⁻¹ are shown in figure 2, and the corresponding pharmacokinetic parameters are summarized in table 1. The plasma concentration of E5564 decreased time-dependently for all doses. For the 0.1 mg kg⁻¹ dose, half life ($t_{1/2}$) was 5.5 ± 0.1 h, and the distribution volume at steady state (V_{dss}) was 73.0 ± 4.4 ml kg⁻¹. The total plasma clearance and $\text{AUC}_{(0-\infty)}$ were independent of dose, which indicates that the pharmacokinetics of E5564 in rats is linear within the dose range from 0.1 to 1 mg kg⁻¹.

Blood level of radioactivity

The blood level of radioactivity–time profile in rats ($n = 4$) after a single intravenous bolus dose of 0.5 mg ¹⁴C-E5564 kg⁻¹ is shown in figure 3. The blood level of radioactivity decreased bi-exponentially. The $t_{1/2}$ of blood level of radioactivity was 5.0 ± 0.1 h calculated from 2.5 min to 24 h after dosing, which is comparable with that of the unchanged E5564 plasma concentration (table 2). The $t_{1/2}$ calculated for 2–20 (18 in some animals) days after administration was 5.8 ± 0.2 days, and the $\text{AUC}_{(0-\infty)}$ was 39.9 ± 0.7 ($\mu\text{g eq. h g}^{-1}$), respectively.

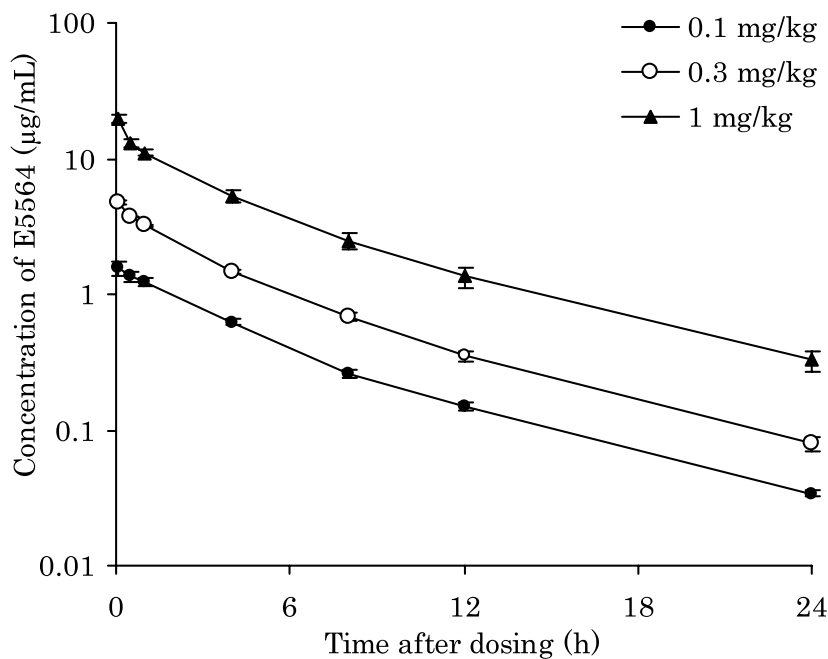


Figure 2. Plasma concentration–time profiles of E5564 after a single intravenous bolus administration of E5564 to rats: 0.1 mg kg^{-1} (closed circles), 0.3 mg kg^{-1} (open circles) and 1 mg kg^{-1} (closed triangles) Each point represents the mean $\pm$ SEM of four animals.

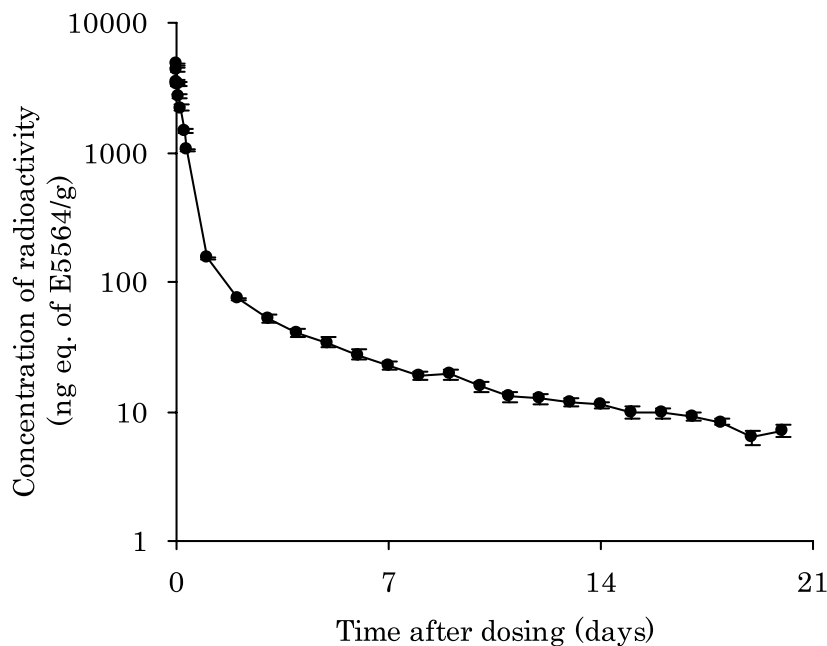


Figure 3. Blood level of radioactivity–time profile after a single intravenous bolus administration of $0.5\text{ mg }^{14}\text{C-E5564 kg}^{-1}$ to rats. Each point represents the mean $\pm$ SEM of four animals.

Table 1. Summary of pharmacokinetic values for E5564 in the plasma after a single intravenous bolus administration to rats at doses of 0.1, 0.3, or 1 mg kg⁻¹.

Pharmacokinetic values	Units	Administered dose		
		0.1 mg kg ⁻¹	0.3 mg kg ⁻¹	1 mg kg ⁻¹
AUC _(0-∞)	μg h ml ⁻¹	8.100 ± 0.535	20.403 ± 0.759	74.861 ± 6.950
<i>t</i> _{1/2}	h	5.5 ± 0.1	5.2 ± 0.2	5.6 ± 0.4
CL _{tot,p}	ml h ⁻¹ kg ⁻¹	12.5 ± 0.7	14.8 ± 0.5	13.7 ± 1.2
<i>V</i> _{dss}	ml kg ⁻¹	73.0 ± 4.4	81.1 ± 2.7	78.4 ± 5.1

Data are the mean ± SEM of four animals

Table 2. Summary of pharmacokinetic values for radioactivity in the blood and liver after a single intravenous bolus administration of 0.5 mg ¹⁴C-E5564 kg⁻¹ to rats.

Pharmacokinetic values	Units	Specimen	
		Blood	Liver
AUC _(0-∞)	μg eq. h ⁻¹ g ⁻¹	39.9 ± 0.7	n.a.
<i>t</i> _{1/2} (2.5 min–24 hr)	h	5.0 ± 0.1	n.a.
<i>t</i> _{1/2} (2–20(18) days)	days	5.8 ± 0.2	n.a.
<i>t</i> _{1/2} (4–48 weeks)	days	n.a.	111

n.a., not applicable

Data for blood represent the mean ± S.E.M. of four animals.

Data for liver is calculated with the mean of four animals.

Distribution

The tissue concentrations of radioactivity in rats (*n* = 4 for each group) after a single intravenous bolus administration 0.5 mg E5564 kg⁻¹ are shown in table 3. At 5 min after dosing, the plasma level of radioactivity (8895.3 ± 208.1 ng eq. g⁻¹) was the highest of all tissues examined, but relatively high concentrations were also found in the liver, adrenal gland, bone marrow and spleen, respectively. The concentration of radioactivity in blood cells was 703.0 ± 48.9 ng eq. g⁻¹, and corresponded to approximately 8% of the plasma value, indicating that radioactivity derived from ¹⁴C-E5564 is poorly absorbed by or adsorbed to blood cells. The concentration of radioactivity in other tissues examined was 9% of the plasma concentration or less. The distribution of radioactivity in the blood was 63.66% ± 2.01% of the administered radioactivity, and the amount in hepatic tissue was 31.82% ± 1.14% of the administered amount. By 24 h after dosing, the plasma level of radioactivity decreased to 156.0 ± 19.4 ng eq. g⁻¹, however, the concentrations of radioactivity in the liver, adrenal gland, bone marrow, spleen and mandibular lymph node increased. At 24 h after administration, the concentration of radioactivity in the liver, adrenal gland, bone marrow, spleen and mandibular lymph node were higher than those in other tissues including the plasma, and were comparable with the values at 1 h after dosing. The hepatic concentration of radioactivity was 8775.0 ± 253.9 ng eq. g⁻¹ at 24 h after dosing, and 67.81% ± 1.97% of administered radioactivity was found in this tissue. By 7 days after

Table 3. Concentrations of radioactivity in the tissues after a single intravenous bolus administration of 0.5 mg ¹⁴C-E5564 kg⁻¹ to rats.

Time	5 min	1 h	8 h	1 day	1 week	4 weeks	12 weeks	24 weeks	48 weeks
Blood	4935.4	3358.1	1001.3	87.0	21.2	n.d.	n.d.	n.d.	n.d.
Plasma	8895.3	5908.2	1729.8	156.0	33.1	6.5	n.d.	n.d.	n.d.
Blood cells	703.0	1618.2	387.8	19.9	12.1	n.d.	n.d.	n.d.	n.d.
Cerebrum	68.6	50.6	19.5	7.4	7.2	6.9	4.3	2.9	n.d.
Cerebellum	71.2	53.2	24.8	9.1	10.2	7.7	5.7	3.4	1.8
Lymph node	99.7	183.4	514.3	559.0	1133	971.9	593.5	289.6	135.3
Heart	445.2	437.5	207.9	124.0	121.1	72.7	36.7	24.7	11.8
Lung	653.1	577.0	354.0	257.9	223.4	141.5	64.1	36.2	13.4
Liver	3890.2	5613.7	8470.8	8775.0	5471.8	3609.3	2128.6	1262.5	323.5
Kidney	473.4	490.1	229.9	133.3	140.3	85.4	61.3	71.2	47.5
Adrenal gland	3465.6	5195.5	3132.0	3337.6	4766.5	3684.7	3049.4	3731.4	1824.6
Spleen	1464.1	2305.1	2545.3	2498.5	2425.3	1768.5	1319.0	893.1	269.9
Pancreas	220.7	155.2	85.3	80.1	95.4	68.4	45.1	30.2	14.3
Adipose tissue	37.2	31.7	28.7	27.7	44.8	37.6	34.5	24.6	11.8
Muscle	46.8	39.8	19.4	15.0	17.8	10.8	5.8	4.4	2.6
Skin	73.3	102.1	123.2	159.6	159.2	80.7	39.7	21.3	9.2
Bone marrow	1753.1	2356.6	2774.3	2657.6	2760.5	2744.4	1417.6	1838.6	400.5
Testes	60.1	170.6	264.5	232.0	236.7	183.7	153.3	80.6	71.4
Aorta	288.5	227.8	84.7	59.1	62.8	42.4	20.2	14.2	8.4
Vein	663.9	591.1	142.9	136.9	133.1	73.6	50.9	32.2	23.0
Sciatic nerve	31.2	67.5	47.7	57.9	42.5	34.8	19.7	15.7	9.1
Small intestine	239.4	283.9	758.5	609.2	810.2	477.6	313.1	226.1	76.4
Large intestine	164.6	123.4	220.3	217.6	160.4	61.3	28.4	35.8	12.4

n.d.; not detected.
Data are the means from four animals (ng eq. g⁻¹).

administration, the plasma level of radioactivity had decreased to 33.1 ± 2.0 ng eq. g⁻¹, however the levels of radioactivity in almost all other tissues were comparable with the values observed 24 h after dosing. The hepatic concentration of radioactivity was 5471.8 ± 293.6 ng eq. g⁻¹, which represent or $49.42\% \pm 3.35\%$ of the administered radioactivity.

At 12 weeks after administration, the plasma level of radioactivity was below the detection limit. However, relatively high concentrations of radioactivity were still found in the adrenal gland, liver, bone marrow, spleen and mandibular lymph node, respectively. The hepatic concentration of radioactivity was 2128.6 ± 130.6 ng eq. g⁻¹, which represent $23.03\% \pm 0.86\%$ of the administered radioactivity. By 48 weeks after dosing, the concentration of radioactivity in all tissues had decreased (table 3). At this time, 100 ng eq. g⁻¹ or more of radioactivity were still observed in the adrenal gland, bone marrow, liver, spleen and mandibular lymph node.

The modified Bligh–Dyer method yielded an extraction recovery of radioactivity from the plasma (up to 7 days after administration) that exceeded more than 80% of the radioactivity, and an efficiency for the liver (up to 48 weeks after dosing) that exceeded 90%.

The radioactive-concentration–time profiles of E5564 and its metabolites in the plasma (figure 4) and the liver (figure 5), determined by TLC analysis, are shown. In the plasma, unchanged E5564 accounted for the majority of radioactivity within 8 h after dosing. The concentration of E5564 in the plasma was 5309.9 ± 209.8 ng eq. g⁻¹ 5 min after dosing, which represents 59.7% of the radioactivity in plasma. The concentration of E5564 decreased almost monophasically, exhibiting a half-life of 5.0 h (table 4), a value almost identical to that determined from the pharmacokinetic studies. The di-dephosphorylated E5564 metabolite M3 accounted

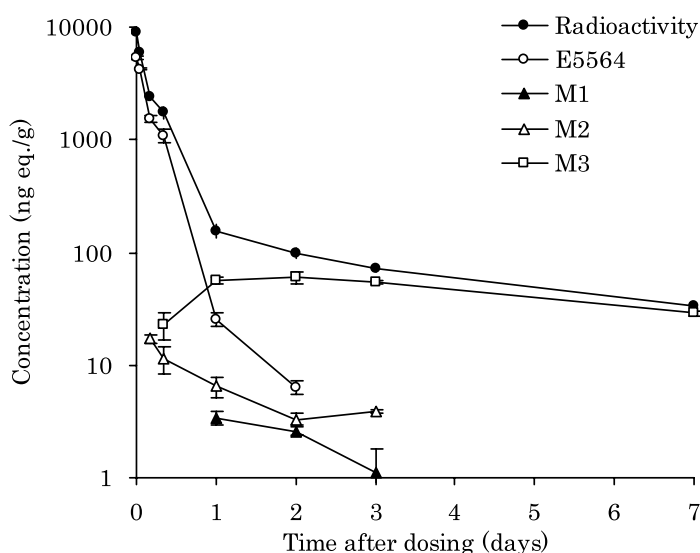


Figure 4. Concentrations of E5564 and its metabolites in the plasma–time profile after a single intravenous bolus administration of 0.5 mg ¹⁴C-E5564/kg⁻¹ to rats. Each point represents the mean $\pm$ SEM of four animals.

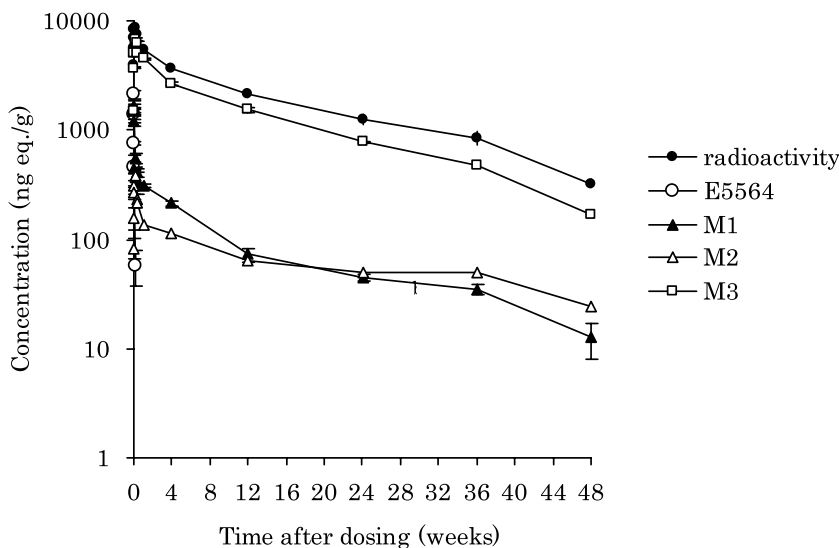


Figure 5. Concentrations of E5564 and its metabolites in the liver—time profile after a single intravenous bolus administration of 0.5 mg ^{14}C -E5564 kg^{-1} to rats. Each point represents the mean $\pm$ SEM of four animals.

Table 4. Half-lives of E5564 and its metabolites in the plasma and liver after a single intravenous bolus administration of 0.5 mg ^{14}C -E5564 kg^{-1} to rats.

Specimen	E5564 (h)	M1 (days)	M2 (days)	M3 (days)
Plasma	5.0	1.3	1.7	7.2
Liver	5.1	91.0	206.7	87.4

Data are the mean results from four animals.

for most of the radioactivity 24 h after dosing (table 4), and the maximum concentration of M3 occurred 2 days after dosing ($59.6 \pm 7.1 \text{ ng eq. g}^{-1}$). Additionally, low concentrations of the mono-dephosphorylated E5564 metabolites M1 and M2 were also found. In the liver, the highest concentration of E5564 occurred at 5 min after dosing ($2112.1 \pm 195.3 \text{ ng eq. g}^{-1}$ or $54.5\% \pm 5.3\%$ of administered radioactivity), and decreased to below the detection limit 2 days after dosing. The concentration of M3 reached its maximum 24 h after administration ($6789.2 \pm 82.6 \text{ ng eq. g}^{-1}$ or $77.5\% \pm 2.1\%$ of administered radioactivity), and M1 and M2 were also found. Forty-eight weeks after administration, $51.6\% \pm 2.2\%$ of the remaining hepatic radioactivity was present as M3 ($165.2 \pm 8.0 \text{ ng eq. g}^{-1}$), but small amounts of M1 and M2 were also present. The half-life for E5564 in the liver was 5.1 h, which was approximately the same as that of E5564 in the plasma. The elimination half-life of M3 in the liver was 87.4 days, approximately the same as the elimination half-life value for total hepatic radioactivity (111 days; table 2). In the spleen, lung, kidney, adrenal gland, mandibular lymph node, and bone marrow, similar metabolic profiles were observed (data not shown).

Table 5. Cumulative excretion of radioactivity into the urine, faeces and expired air after a single intravenous bolus administration of 0.5 mg ^{14}C -E5564 kg $^{-1}$ to rats.

Time (weeks)	Cumulative excretion (per cent of administered radioactivity)				
	Urine	Faeces	Expired air	Debris	Total excretion
0-1	0.17 $\pm$ 0.03	17.29 $\pm$ 2.39	0.40 $\pm$ 0.11	0.13 $\pm$ 0.05	17.98 $\pm$ 2.31
0-4	0.40 $\pm$ 0.06	33.07 $\pm$ 2.69	0.62 $\pm$ 0.21	0.19 $\pm$ 0.06	34.29 $\pm$ 2.42
0-13	1.33 $\pm$ 0.15	46.43 $\pm$ 2.29	0.76 $\pm$ 0.22	0.24 $\pm$ 0.07	48.77 $\pm$ 1.92
0-48	3.86 $\pm$ 0.26	67.17 $\pm$ 2.11	0.76 $\pm$ 0.22	0.28 $\pm$ 0.07	72.07 $\pm$ 1.68

Data are the mean $\pm$ SEM of four animals.

Table 6. Cumulative excretion of E5564 and its metabolites into the faeces after a single intravenous bolus administration of 0.5 mg ^{14}C -E5564 kg $^{-1}$ to rats.

Time (weeks)	Cumulative excretion (per cent of the administered radioactivity)			
	E5564	M1	M2	M3
0-1	0.317	1.649	1.151	11.128
0-4	0.323	1.804	3.762	19.409
0-13	0.323	1.804	8.060	23.719
0-48	0.323	1.804	8.803	23.719

Data are the mean of four animals.

Elimination

The cumulative excretion of radioactivity is shown in table 5. By 48 weeks after dosing, the cumulative excretion of radioactivity in urine was $3.86 \pm 0.26\%$ of the administered radioactivity, in the faeces was $67.17\% \pm 2.11\%$ and for all routes was $72.07\% \pm 1.68\%$. The amount of radioactivity remaining in the liver, bone marrow and carcass at 48 weeks after dosing was $4.50\% \pm 0.35\%$, $4.37\% \pm 0.54\%$ and $4.80\% \pm 0.51\%$ of the administered radioactivity, respectively.

Since only a trace amount of radioactivity was present in the urine, the metabolic profile of the urinary radioactivity was not determined. M3 accounted for the majority of the faecal radioactivity (23.7% of the dose) followed by M1 (1.8%), M2 (8.8%) and unchanged E5564 (0.3%), respectively (table 6). The metabolic pattern of faecal radioactivity is similar to that in the liver. The amount of radioactivity recovered in expired air was $0.76\% \pm 0.22\%$ of the administered radioactivity by 13 weeks after dosing. The radioactivity found in the expired air was presumably $[^{14}\text{C}]\text{CO}_2$. Although this radioactivity represents only a small portion of the total excretion, it suggests that the ^{14}C -labelled acyl chain of E5564 and its related metabolites is also degraded, resulting in the catabolic release of $[^{14}\text{C}]\text{CO}_2$.

Metabolite identification

Non-radiolabelled E5564 was administered intravenously to rats at a dose of 0.9 mg animal $^{-1}$ day $^{-1}$ for 5 days to obtain greater quantities of metabolites for structural characterization. The HPLC chromatographic retention time of the major metabolite isolated from the liver is consistent with that of

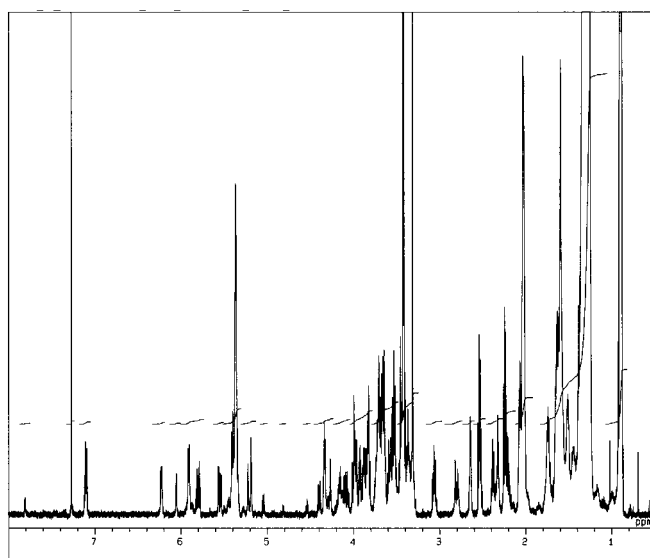


Figure 6. ^1H -NMR spectrum of metabolite isolated from rat liver after five repeated intravenous administrations of E5564 ($0.9\text{ mg E5564 day}^{-1}$).

di-dephosphorylated E5564 reference compound (data not shown). The ^1H -NMR spectrum of this metabolite isolated from rat liver is shown in figure 6. In the ^1H -NMR spectra for the di-dephosphorylated E5564 reference compound and the major metabolite isolated from the rat liver, the same signal shifts for aliphatic protons, olefin protons and glucopyranose ring protons were observed (figure 7). Two sets of proton signals, one at 5.19 and 5.40 ppm, the other at 4.13 and 4.54 ppm, were observed for the protons of carbons a1 and a2, respectively, in the glucopyranose ring. These signals result from an α,β -isomerization that occurs in solution at anomeric positions such as a1. The double signal at 5.40 ppm was assigned to a1 of the β configuration by the coupling constant ($J = 8.5\text{ Hz}$). The OH protons were not assigned, since it is thought that the OH signal was broadened because of an exchange with water. These results indicate that the proton signals of the major metabolite isolated from the rat liver is almost identical to those of the reference compound.

The FAB mass spectrum of the reference compound and its daughter ions is shown in figure 8. Peaks at m/z 1176 and 1192 were observed in purified metabolite; the m/z 1176 peak was assigned to the sodium ion adduct molecular ion $[\text{M} + \text{Na}^+]$, the m/z 1192 peak was assigned to the potassium ion adduct molecular ion $[\text{M} + \text{K}^+]$. The same mass spectrum was observed for the metabolite isolated from the rat liver (data not shown). Since the peak intensity of $[\text{M} + \text{Na}^+]$ adduct was much higher than that of the $[\text{M} + \text{K}^+]$ adduct, fragment ions from $[\text{M} + \text{Na}^+]$, were evaluated. In the daughter ion spectrum from the m/z 1176 ($[\text{M} + \text{Na}^+]$), ion, major m/z 1034, 1018, 978, 952, 693, 561, 594 and 158 were found for both materials, and the assignments of the major fragment ions are shown in figure 8. The results indicate that the fragment ion spectrum of metabolite purified from animals are the same as those for the reference compound. Consequently, the chemical structure of the major metabolite purified

Chemical shift (δ)				
Reference compound	Purified from the liver	Relative intensity	Multiplicity [Hz]	Assignment
7.10	7.10	1	d, J = 8.5 Hz	a2-NH
5.90	5.90		(—)	d1
5.40	5.40	1(β)	d, J = 8.5 Hz	a1 (β)
5.36	5.36	2	(—)	e11, e12
5.19	5.19		broad	a1 (α)
4.60–3.0	4.6–3.0		(—)	sugar ring proton, b2, c1
4.54	4.54	1(β)	(—)	a2 (β)
4.13	4.13	1(α)	(—)	a2 (α)
3.82	3.82	1	(—)	f1
3.70	3.70	1	(—)	f1
3.42	3.42	3	(—)	d6-OCH ₃
3.37	3.37	1	(—)	f3
3.30	3.30	3	(—)	f3-OCH ₃
2.54	2.54	2	(—)	e2
2.22	2.22	2	(—)	b4
2.03	2.03	4	(—)	e10, e13
1.74	1.74	2	(—)	f2
1.62	1.62	2	(—)	b5
1.58	1.58	2	(—)	e3
1.51	1.51	2	(—)	c2
1.45	1.45	2	(—)	f4
1.4–1.2	1.4–1.2	68	(—)	b6–b13, c3–c9, e4–e17, f5–f9
0.90	0.90	12	(—)	b14, c10, e18, f10

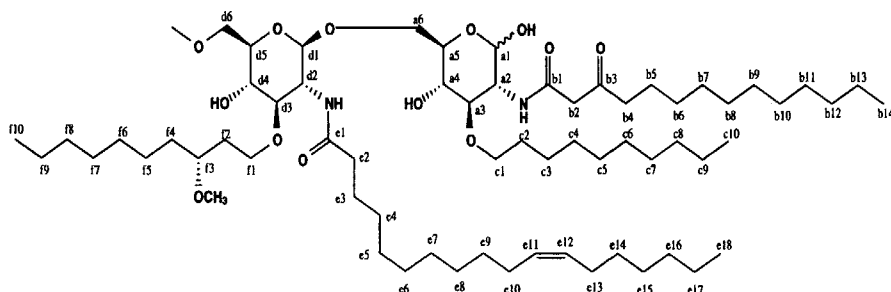


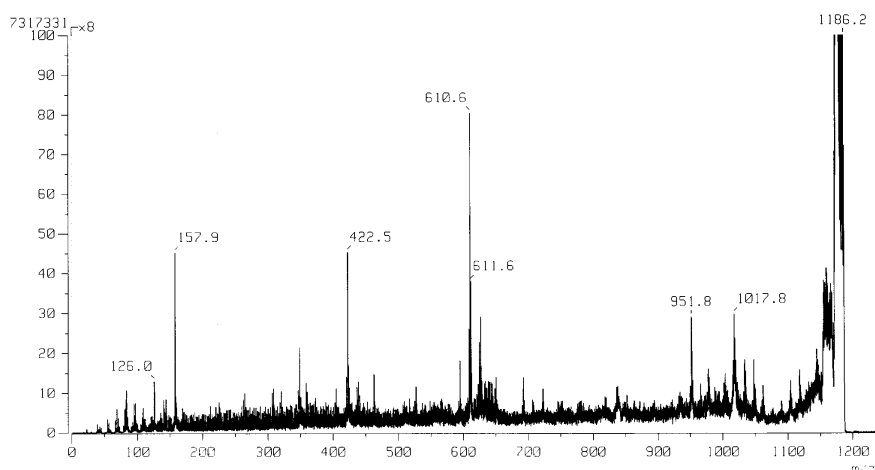
Figure 7. Assignment of proton signals for the metabolite isolated from rat liver after five repeated intravenous administrations of E5564 (0.9 mg E5564 day⁻¹).

from the rat liver is the structure of the di-dephosphorylated E5564 reference compound.

Discussion

Although the pharmacological effect of E5564 has been well studied (Rossignol *et al.* 1999), its disposition and metabolism have not been reported. Therefore, the pharmacokinetics and metabolic fate of E5564 were examined in male SD rats.

The V_{dss} of E5564 is about two times the theoretical plasma volume (31.3 ml kg⁻¹; Davies and Morris 1993), indicating that the distribution volume of this compound is very restricted. Indeed, high concentration of E5564 after dosing was found only in the liver, spleen, adrenal gland and bone marrow (data not shown). Both $\text{CL}_{\text{tot,p}}$ and V_{dss} are independent of the dose, indicating that



Fragmentation pattern

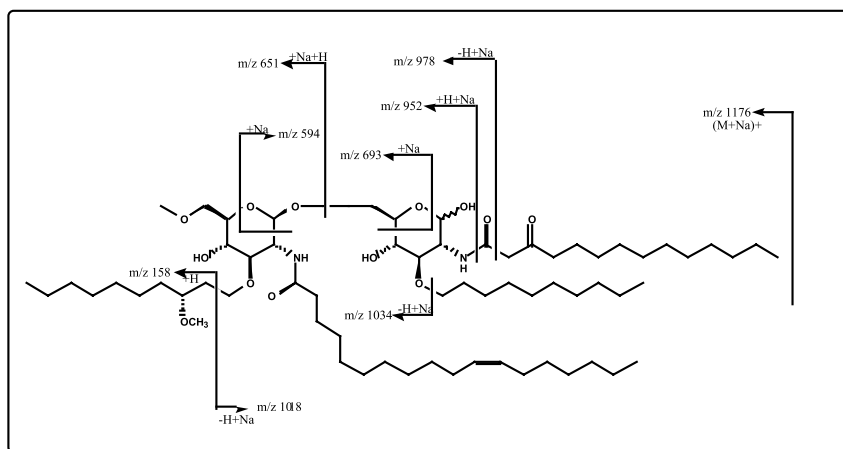


Figure 8. Positive fast-atom bombardment mass spectrum of the $[M + Na]^+$ ion of adduct of the major E5564 metabolite isolated from rat liver after five repeated intravenous administrations of E5564 ($0.9 \text{ mg E5564 day}^{-1}$).

E5564 has linear pharmacokinetics in rats within the dose range from 0.1 to 1 mg kg^{-1} . Following intravenous administration of ^{14}C -E5564 to rats, the majority of radioactivity is present in the plasma, not in the blood cells, at all times tested. If blood-to-plasma distribution ratio for this drug is equal to $(1 - \text{haematocrit level})$, the total blood clearance is equal to $CL_{\text{tot,p}}/0.65$ (no distribution to blood cells), which is much smaller than the hepatic blood flow ($4.8 \text{ l h}^{-1} \text{ kg}^{-1}$; Davies and Morris 1993); these results indicate that E5564 is not subjected to extensive hepatic extraction. The half-life of the blood level of radioactivity for 2.5 min to 24 h was 5.0 h, which is almost identical to the half-life of E5564 obtained from the pharmacokinetic study. This finding is supported by the fact that the majority of radioactivity in the plasma is accounted for E5564 for up to 8 h after dosing. In contrast, the terminal half-life of blood level of radioactivity is 5.8 days ± 0.2 days, which reflects the observation that metabolites

account for the majority of radioactivity in the plasma during the terminal elimination phase.

The tissue distribution of radioactivity can help explain the pharmacokinetic data. At 5 min after dosing, the concentrations of radioactivity in the liver, adrenal gland, bone marrow and spleen were markedly higher than in other tissues although not as high as the concentration in the plasma. The amount of radioactivity in the blood was 63.7% of the radioactivity administered, which is higher than the value observed for ^{125}I labelled LPS from *E. coli* (Harris *et al.* 1993). Hence, blood clearance of E5564 was lower than that of LPS in rats. After dosing of ^{14}C -E5564, the radioactivity was gradually and extensively distributed to the liver, spleen, bone marrow and mandibular lymph node, where it remained for more than 48 weeks after administration. These results suggest that E5564 and its metabolites are taken up by the reticuloendothelial system and retained for a long time, similar to the fate of lipid A (Mathison and Ulevitch 1979). Since these tissues contain macrophage-like cells, incorporation of radioactivity derived from ^{14}C -E5564 might be mediated by these cells, which is well known mechanism for LPS (Hampton and Raetz 1991, Morrison *et al.* 1994). However, the incorporation rate for E5564 is slower than that for LPS.

As shown in figure 1, E5564 has four aliphatic chains connected to glucosamine rings, which give this compound a lipid-like character. The Bligh-Dyer method (Bligh and Dyer 1959) is a known method to extract lipids from other biological compounds. For ^{14}C -E5564, a modified Bligh-Dyer method was used to extract the radioactivity in the plasma, liver, urine and faeces because the ratio of chloroform, methanol and water strongly influences the extraction efficiency. In an attempt to determine the solvent ratio for E5564 extraction, that would yield a recovery rate of 99% or higher, plasma samples from untreated rats were spiked with ^{14}C -E5564 (data not shown).

The main metabolites that account for hepatic radioactivity are M3 which is diphosphorylated E5564, and two mono-dephosphorylated forms of E5564, M1 and M2. Consequently, the main metabolic pathway of E5564 is dephosphorylation, the same route by which lipid A is metabolized (Peterson and Munford 1987, Hampton and Raetz 1991). Dephosphorylation of E5564 causes the loss of antagonistic activity (data not shown), which agrees with the observation that dephosphorylation of LPS and lipid A causes the loss of their biological activities (Loppnow *et al.* 1989). Hence, it is unlikely that the distributed metabolites are responsible for the biological action *in vivo*, even though they are retained at high concentrations for a long time. Additionally, between 4 and 48 weeks after dosing, the amount of radioactivity in the liver decreased with an apparent half-life of 111 days, which corresponds to half-life of M3 (table 2), suggesting that the slow elimination of radioactivity from the liver results from the slow elimination of M3.

High concentrations of radioactivity were found in the adrenal gland after dosing of ^{14}C -E5564 to rats in this study; this corresponds to a similar distribution of radioactivity observed after dosing of LPS (Mathison and Ulevitch 1979, Mathison and Ulevitch 1985, Munford and Dietschy 1985). The uptake of LPS by the adrenal gland is thought to occur as follows: intravenously administered LPS binds to HDL in the blood (Ulevitch *et al.* 1981, Wurfel *et al.* 1995), then the LPS-HDL complex is incorporated into the adrenal gland though the SR-B1 scavenger receptor, an HDL receptor found in the adrenal glands (Acton *et al.* 1996). Because a high level of E5564 is found in the lipoprotein fraction of the

plasma *in vitro* (data not shown), it is likely that E5564-HDL complexes are incorporated into the adrenal gland in rats similar to LPS.

After dosing of ^{14}C -E5564, less than 1% of the administered radioactivity was found in expired air. This result suggests that ^{14}C -vaccinic acid is liberated after the hydrolysis of the amide bond of ^{14}C -E5564 or its metabolites, and is metabolized in the beta oxidation cycle. Thus, the incorporation of ^{14}C into endogenous compounds as a result of beta-oxidation might also partially contribute to the slow elimination of radioactivity from the body after dosing of ^{14}C -E5564.

In conclusion, this paper describes the disposition and metabolic profile of E5564, a lipid A analogue, in rat. The distribution profile of E5564 is similar to that of LPS, however distribution of radioactivity to macrophage containing tissues (i.e. liver, spleen, bone marrow) is slower than that of LPS, which may reflect the slower binding of E5564 and/or its metabolites with macrophage like cells. Dephosphorylation is the main metabolic route for E5564, which is the same route for LPS and lipid A. This paper reveals that mono-dephosphorylated metabolites are also formed and excreted into the faeces, which has not been reported for LPS.

Acknowledgements

The authors acknowledge the help of Dr Y. Sano and K. Kikuchi in establishing the quantification method for plasma concentration of E5564, Y. Nagai and M. Hatanaka in measuring the NMR and mass spectra of the metabolite, Dr L. Hawkins for the synthesis of radiolabelled compound, Dr W. J. Christ and his group members for supply the metabolites, and Mr S. Johnson for help with the manuscript.

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Determination of the human cytochrome P450s involved in the metabolism of 2*n*-propylquinoline

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Received 27 April 2002

1 2*n*-Propylquinoline (2*n*PQ) is a newly developed drug for visceral antileishmaniasis and its activity has been previously evaluated in mice following oral administration. The study was carried out to investigate the kinetic formation of 2*n*PQ metabolites and to characterize the human liver CYP forms involved in its oxidative metabolism.

2. The inhibition of 2*n*PQ metabolite formation by specific substrates or inhibitors of CYP forms and correlation studies were performed in human liver microsomes. 2*n*PQ biotransformation was then studied in human lymphoblasts expressing specific CYPs and microsomal epoxide hydrolase.

3. Three major metabolites were produced by human liver microsomes and their structures were identified by ESI-LC/MS: dihydroxy-2*n*-propylquinoline, 3'-hydroxy-2*n*-propylquinoline and 1'-hydroxy-2*n*-propylquinoline. An intermediary metabolite, epoxy-2*n*-propylquinoline, formed by CYP was also biotransformed by microsomal epoxide hydrolase into dihydroxy-2*n*-propylquinoline.

4. 2*n*PQ oxidation follows Michaelis–Menten kinetics. In human liver microsomes, its metabolism was extremely inhibited by pilocarpine, coumarin and diethyldithiocarbamate. From a panel of 12 human liver microsome samples, the rate of 2*n*PQ oxidation was highly correlated with the activities of CYP2A6 and CYP2E1. Human lymphoblasts expressing specific CYPs showed the involvement of CYP2A6, CYP2E1 and CYP2C19.

5. The results indicate that 2*n*PQ metabolites are 3'- and 1'-hydroxylated by human liver microsomes and an epoxy-2*n*-propylquinoline is biotransformed into a dihydroxy-2*n*-propylquinoline by microsomal epoxide hydrolase.

Introduction

Visceral leishmaniasis (kala-azar) is a fatal human disease caused by *Leishmania donovani* and is endemic in many parts of tropical and subtropical regions of the world. The estimated global prevalence is 12 million infected cases, with 400 000–2 million new cases reported each year (Fournet *et al.* 1994b). Leishmaniasis is contracted through sand fly bites. Drugs currently available for treatment of kala-azar are potentially toxic, inconvenient to administer by the parenteral route and frequently give rise to clinical resistance (Fournet *et al.* 1994b, 1998). First choice drugs are pentavalent antimonials such as sodium stibogluconate and meglumine antimonate. In spite of their activity in kala-azar, diamidines (pentamidine) and amphotericin B are second-line drugs because of their toxicity (Fournet *et al.* 1996).

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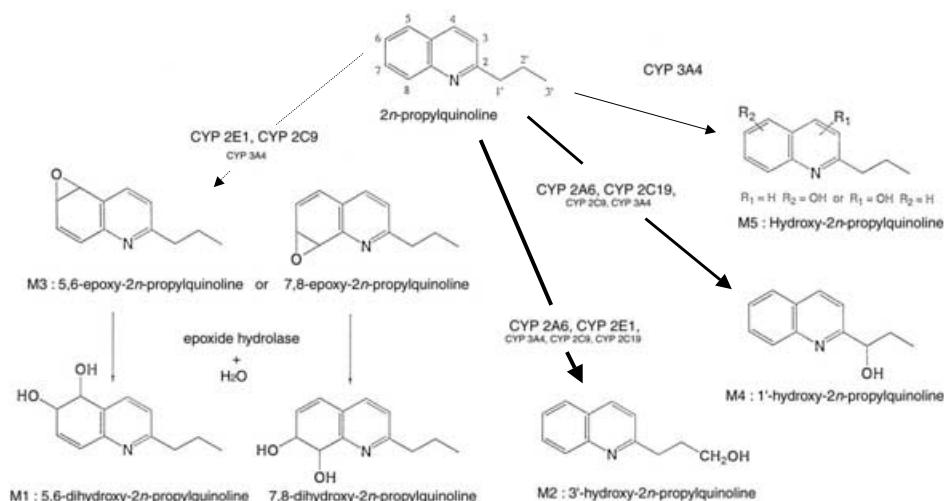


Figure 1. Proposed pathways for the hepatic metabolism of 2n-PQ.

In Bolivia, the Instituto Boliviano de Biología de Altura and the French Institute of Scientific Research for Development in Cooperation (ORSTOM) have developed alternative compounds for the treatment of leishmaniasis. One of these is 2*n*-propylquinoline (2*n*PQ) (figure 1), a new drug that has been shown to be orally active in treating visceral leishmaniasis in BALB/c mice (Fournet *et al.* 1993). The effectiveness of this treatment is equal to or superior than that of reference treatments such as meglumine antimonate administered by the parenteral route (Fournet *et al.* 1993, 1994a). The lack of any toxic effect in animals ($LD_{50} > 400 \text{ mg kg}^{-1}$) has encouraged the development of 2*n*PQ (Fournet *et al.* 1993). The first pharmacokinetic results in mice show an hepatic distribution that coincides with parasite localization ($T_{\text{max}} = 90 \text{ min}$, time to reach the maximal concentration of 2*n*PQ) and a plasma elimination half-life of about 100 min. Moreover, the presence of compounds more polar than 2*n*PQ in liver homogenates of treated mice suggests it is primarily metabolized in the liver (Iglarz *et al.* 1998).

The aim of the present study is to characterize the human oxidative hepatic pathways of 2*n*PQ metabolism and to identify the specific CYP forms involved in 2*n*PQ oxidation in human liver microsomes (hlm). Moreover, these results will be useful in predicting the effects of 2*n*PQ on the metabolism of other concomitantly administered drugs.

Materials and methods

Drugs and chemicals

2*n*-propylquinoline (2*n*PQ) (figure 1), 1'-hydroxy-2*n*-propylquinoline (1'-OH-PQ), 2'-hydroxy-2*n*-propylquinoline (2'-OH-PQ) and 3'-hydroxy-2*n*-propylquinoline (3'-OH-PQ) were synthesized in Pr. Hocquemiller's laboratory (Chatenay-Malabry, France). (There was 100% purity for each, which was checked by mass spectrometry.)

Drug substrates and inhibitors were obtained as follows: NADPH, alpha-naphthoflavone, coumarin, hydroxycoumarin, tolbutamide, quinidine, diethyldithiocarbamate (DETC), pilocarpine, ketoconazole, chlorzoxazone, midazolam and mephénytoin were obtained from Sigma-Aldrich Chemical Co. (Saint Quentin, France); hydroxychlorzoxazone and hydroxytolbutamide were from Bioblock Scientific (Illkirch, France). Sulphaphenazole was a gift from Dr Gasser (Hoffmann-La Roche, Bâle, Switzerland); 1-hydroxymidazolam was a gift from Dr Taburet (Pharmacie, Hôpital du Kremlin-Bicêtre, Kremlin-

Bicêtre, France). All other chemicals and reagents used were of the highest quality. Human microsomal epoxide hydrolase microsomes and microsomes prepared from B-lymphoblastoid cell line expressing CYP forms were obtained from Gentest Corporation (Woburn, MA, USA).

Analysis of 2nPQ and its metabolites

2nPQ and its metabolites were separated by HPLC using a Spherisorb S50D2-15F column (150×4.5 mm; particle size 5 μ m; Interchim, Montluçon, France). The mobile phase consisted of methanol and 0.005 M ammonium acetate buffer (pH 5, 50:50 v/v). The flow rate was 1 ml min⁻¹. The HPLC system was equipped with a UV detector (Shimadzu SPD-10A, Touzart et Matignon, Les Ulis, France), which was set at 230 nm. Data acquisition and analysis were performed with Millennium[®] software (Millennium[®] 2020 C/S, Waters, Saint-Quentin en Yvelines, France).

Major metabolite identification

In order to characterize the nature of 2nPQ metabolites produced by hlm, a system was developed coupling electrospray ionization-mass spectrometry detection with HPLC separation (ESI-LC/MS). The analytic system was equipped with an HP 1100 pump (High Pressure gradient, Hewlett Packard, Palo Alto, CA, USA), a Zorbax SBC 18 column (HP Product, 150×2.1 mm; particle size 3.5 μ m; Interchim, Montluçon, France) and a mass spectrometer. The mobile phase consisted of methanol with 0.005 M ammonium acetate buffer (pH 5, 45:55 v/v) starting isocratically for 15 min followed by a linear gradient to a 60:40 v/v mixture for 10 min before returning to the initial conditions. The flow rate was 0.25 ml min⁻¹. Data acquisition and analysis were performed using Bruker ESQUIRE-LC[®] software. The retention times of metabolites on total ionic current and mass spectra of metabolites were compared with those of mono-hydroxylated compounds of 2nPQ (standard compounds). The UV spectra of major metabolites were also compared with the UV spectra of the standard compounds with a coupled HPLC-photodiode array detector (150–400 nm, Waters 996, Saint-Quentin en Yvelines, France).

Preparation of human liver microsomes

Individual human liver samples ($n = 14$) termed M_x were obtained from transplant donors. Liver fragments were immediately stored in Eurocollins[®] solution (Fresenius, France) at -80°C until the microsomes were prepared. The protocol strictly followed French legal considerations and ethical committee approval was obtained before the study. All subjects were Caucasian, 13 men and one woman aged from 20 to 36 years old (median 24 years) weighing 67 ± 15 kg. Microsomal fractions were prepared as described by Dragacci *et al.* (1987) and stored at -80°C in a 0.1 M KH₂PO₄ buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT and glycerol (80:20 v/v) until used. The microsomal batches were identified as follows: M₄, M₅, M_{6a}, M_{6b}, M₇, M₈, M₉, M₁₁, M₁₂, M_{19b}, M_{26b}, M_{33b}, M₄₁ and M₄₂. The protein content of microsomal preparations was determined by Bradford's (1976) method.

Kinetic formation of 2nPQ metabolites in human liver microsomes

The incubation mixture consisted of 2nPQ, microsomal protein (1 mg ml⁻¹), NADPH (1 mM) in a 0.01 M phosphate buffer (pH 7.4). NADPH was dissolved in phosphate buffer immediately before incubation. Incubations were performed in duplicate at 37°C , in polypropylene locked tubes (200 μ l final volume), in a shaking water bath. Metabolite formation was linear up to 30 min with microsome concentrations up to 1 mg ml⁻¹. These two values were chosen for further experiments. After 30 min of incubation, the reaction was stopped by the addition of methanol (200 μ l at 4°C).

The kinetic formation of 2nPQ metabolites was assessed in microsomal preparations (M_{6a}, M₇, M₈, M_{6b}, M_{19b}, M_{26b}) at 2nPQ concentrations ranging from 5 to 1000 μ M. The apparent Michaelis-Menten constants K_m and V_{max} were estimated using GRAFIT[®] (Version 3.0, Erithacus Software, Staines, UK) a non-linear least-squares regression analysis software, by proportional weighting ($1/v^2$). The intrinsic clearance (Cl_{int}) was calculated from the ratio of V_{max} to K_m . Data were analysed by linear transformation (Lineweaver-Burk and Eadie-Hofstee plots) to determine whether 2nPQ biotransformation conformed to single- or two-enzyme Michaelis-Menten kinetics.

Inhibitory studies of 2nPQ metabolism using specific CYP form substrates or inhibitors

All incubations were performed in duplicate using four different batches of microsomes (M₄, M_{6a}, M₇, M₈). In order to identify the different enzymes involved in 2nPQ metabolism, two 2nPQ concentrations were chosen for the inhibition studies: 50 μ M for an enzyme with high affinity and 400 μ M for an enzyme with low affinity. For the chemical inhibition studies, different pathways may be involved at different concentrations. For example, ethoxycoumarin (10 and 200 μ M) is O-deethylated by CYP1A2 and CYP2E1, respectively (Carriere *et al.* 1996). 2nPQ concentrations were defined in accord with Iglarz *et al.* (1998).

2nPQ were incubated with hlm (1 mg ml⁻¹ final protein concentration) and 1 mM NADPH with or without selective CYP forms substrates or inhibitors. Their concentrations were chosen according to

the literature: coumarin (100 μ M, CYP2A6 substrate) (Bourrie *et al.* 1999), pilocarpine (5 and 10 μ M, CYP2A6 inhibitor) (Bourrie *et al.* 1996), sulfaphenazole (1 μ M, CYP2C9/10 inhibitor) (Leeman *et al.* 1993), quinidine (1 μ M, CYP2D6 inhibitor) (Ching *et al.* 1995), DETC (2.5 μ M, CYP2E1 inhibitor) (Tassaneeyakul *et al.* 1993), tolbutamide (100 μ M, CYP2C8/9/10 substrate) (Relling *et al.* 1990), *R,S*-mephenytoin (500 μ M, CYP2C19 substrate) (Hall *et al.* 1987), ketoconazole (0.2 μ M, CYP3A4 inhibitor) (Maurice *et al.* 1992) and α -naphthoflavone (1 μ M, CYP1A2 inhibitor) (Pastrakuljic *et al.* 1997). Inhibitors or substrates were added immediately before 2nPQ, except for DETC, which was pre-incubated for 15 min. The effect of 2.5 μ M DETC was also tested (Ono *et al.* 1996). Coumarin (0.5 μ M) was incubated with DETC and 7-hydroxycoumarin formation was compared with a control sample without inhibitor. 2nPQ metabolite formation was expressed as a percentage of the control sample. The results were expressed as mean (percentage of CYP residual activity) $\pm$ SD. Statistical comparisons between control samples and those with CYP-specific substrates or inhibitors were conducted with Dunnett's test post-hoc following ANOVA (Statview[®] software, Abacus Concept, Berkeley, CA, USA). Statistical significance was accepted as $p < 0.05$.

CYP2A6, CYP2E1, CYP2C9 and CYP3A4 activity assays

In the batches used to quantify CYP activities, coumarin 7-hydroxylation, chlorzoxazone 6-hydroxylation, tolbutamide hydroxylation and midazolam hydroxylation were used to probe CYP2A6, CYP2E1, CYP2C9 and CYP3A4, respectively. The hydroxylation of coumarin was determined as described by Yun *et al.* (1991) and Mäenpää *et al.* (1993), with a substrate concentration of 20 μ M. The hydroxylation of chlorzoxazone was studied according to Tassaneeyakul *et al.* (1993), with a substrate concentration of 250 μ M. The hydroxylation of tolbutamide was studied according to Miners *et al.* (1988), with a substrate concentration of 500 μ M. The hydroxylation of midazolam was studied as described by Gorski *et al.* (1994), with a substrate concentration of 400 μ M.

Correlation studies

Correlations between the rate of 2nPQ metabolite formation and the activity of CYP2A6, CYP2E1, CYP2C9 and CYP3A4 were investigated in microsomes from 12 human livers. Correlation coefficients were calculated according to Spearman's rank test with the statistically significant level fixed at 0.05 (Statview[®]). Incubations were performed in duplicate with 50 and 400 μ M 2nPQ.

2nPQ metabolism in human lymphoblasts expressing specific CYPs and in microsomes expressing human epoxide hydrolase

Human microsomal epoxide hydrolase microsomes (meh) and microsomes were prepared from a B-lymphoblastoid cell line expressing CYP: CYP1A2 (113 pmol P450 mg⁻¹ protein), CYP2A6 (202), CYP2C9 (62), CYP2D6 (128), CYP2E1 (229), CYP3A4 (56) and CYP2C19 (27). 2nPQ was incubated with human lymphoblasts expressing CYPs, under the same conditions as those previously described for liver microsomes except for human lymphoblasts expressing CYP2C9, which were incubated in a 100 mM Tris buffer (pH 7.5), according to the manufacturer's instructions. Identification of pathways involved in metabolite 1 and 4 formation:

2nPQ (50 μ M) was incubated for 30 min with human lymphoblasts expressing CYP2C9 (1 mg ml⁻¹). Hlm or human lymphoblasts expressing CYP2A6 or human lymphoblasts expressing CYP2E1 were then added for an additional 30-min incubation. 2nPQ (400 M) was incubated for 30 min with microsomal protein containing specific cytochromes P450C9 or P450E1 (1 mg ml⁻¹) and microsomal protein containing epoxide hydrolase (1 mg ml⁻¹).

Results

In the presence of hlm and its essential cofactor NADPH, four chromatographic peaks, labelled M1, M2, M4 and M5 according to their elution order under these chromatographic conditions, were observed (figure 2). The main metabolites were identified by ESI-LC/MS as a dihydroxy compound for M1 and mono-hydroxy derivatives of 2nPQ for M2 and M4. M1 was identified as dihydroxy-2n-propylquinoline ($\lambda_{\text{max}} = 294.9$ nm, average molecular weight of 205 with $[M + H]^+$ at m/z 206) but it was not possible to attribute the location to the 5–6 or 7–8 positions. M2 was identified as 3'-OH-PQ and M4 as 1'-OH-PQ ($\lambda_{\text{max}} = 228.7$ nm, average molecular weight of 187 with $[M + H]^+$ at m/z 188). The formation of M3 was observed only with lymphoblasts expressing specific

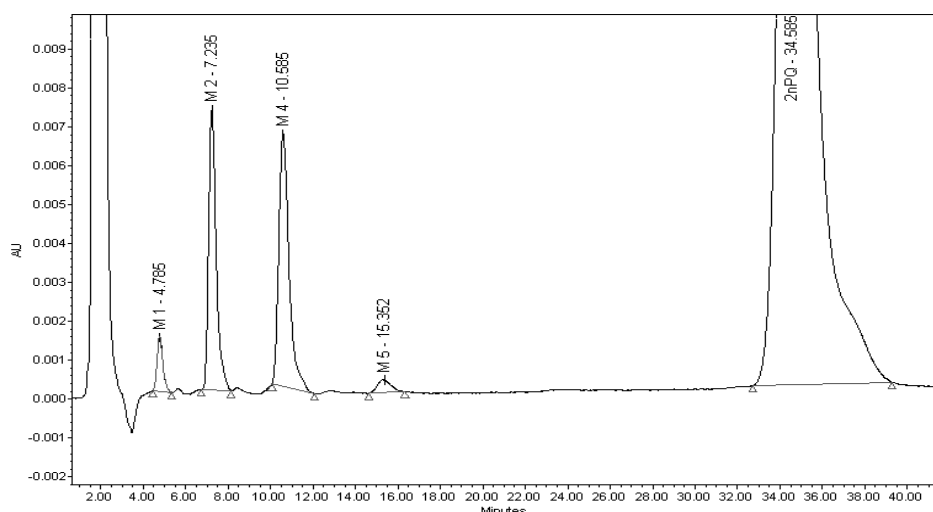


Figure 2. Typical chromatogram of 2*n*PQ oxidation by human liver microsomes (*M*₈ batch). Hepatic microsomal preparations (1 mg ml⁻¹) were incubated for 30 min with 2*n*PQ (400 μM) and NADPH (1 mM). After removal of precipitated proteins by centrifugation, the supernatant fluid was analysed by HPLC as described in the Materials and methods.

CYPs (CYP2E1, CYP2C9 and CYP3A4) and was identified as an epoxy-2*n*-propylquinoline derivative ($\lambda_{\max}$ = 294.9 nm, average molecular weight of 187 with $[M+H]^+$ at m/z 188). *M*₅, an hydroxy-2*n*-propylquinoline, was a minor metabolite produced only by CYP 3A4 ($\lambda_{\max}$ = 233.4 nm, average molecular weight of 187 with $[M+H]^+$ at m/z 188), and was not considered anymore in further studies.

Kinetics of 2nPQ metabolite formation in human liver microsomes

The formation of 2*n*PQ metabolites was CYP mediated (figure 1). They were not produced in the absence of CYP cofactor (NADPH), or in the presence of liver microsomes heated for 60 min at 56 °C (data not shown). Kinetic parameters were fitted to the Michaelis–Menten equation by non-linear regression analysis of the turnover rates. The K_m and $V_{\max}$ obtained for each major metabolite with four human liver preparations are summarized in table 1. Intrinsic clearance for *M*₁ was not calculated as this metabolite was not synthesized and available for quantification. Therefore, $V_{\max}$ for *M*₁ was expressed in arbitrary units. A high variability appeared in metabolite formation, probably due to variability in CYP expression in the different hlm preparations.

Lineweaver–Burk and Eadie–Hofstee curves for *M*₂ were monophasic (data not shown), implying that one or several enzymes with similar K_m values were involved ($K_{m2 \text{ CYP2A6}}$ = 113 μM and $K_{m2 \text{ CYP2E1}}$ = 311 μM). With microsomes from five subjects (*M*_{6b}, *M*₇, *M*₈, *M*_{19b}, *M*_{26b}), Lineweaver–Burk and Eadie–Hofstee curves for *M*₁ and *M*₄ were biphasic (data not shown), suggesting that several enzymes were involved in their formation (Eagling *et al.* 1998). Nevertheless, K_m values were not different enough to be determined by calculation or by Grafit[®] software. 2*n*PQ is biotransformed at a low rate in the liver: intrinsic

Table 1. Michaelis–Menten kinetic parameters for the formation of 2*n*-propylquinoline metabolites in human liver microsomes.

Microsome batches	Dihydroxy-2 <i>n</i> PQ (M1)		3'-Hydroxy-2 <i>n</i> PQ (M2)			1'-Hydroxy-2 <i>n</i> PQ (M4)		
	K_{m1}	V_{max1}^a	K_{m2}	V_{max2}	Cl_{int2}	K_{m4}	V_{max4}	Cl_{int4}
M _{6a}	41	2 161	86	67	0.78	226	114	0.50
M _{19b}	42	11 177	147	88	0.60	158	78	0.49
M _{26b}	538	143 372	222	325	1.46	1109	721	0.65
M _{6b}	49	10 441	213	82	0.39	972	183	0.19
M ₈	917	49 411	239	113	0.47	1320	379	0.29
M ₇	350	60 100	ND	ND	ND	ND	ND	ND
Mean	323	46 110	181	135	0.74	757	295	0.42
± SD	± 356	± 53 086	± 64	± 107	± 0.43	± 531	± 265	± 0.18

^a Arbitrary Units $\text{mg}^{-1} \text{min}^{-1}$ because this compound was not synthesized and, therefore, the concentration could not be determined.

ND, not determined; K_m , apparent Michaelis–Menten constant (μM); V_{max} , maximal velocity ($\text{pmol mg}^{-1} \text{min}^{-1}$); $Cl_{int} = V_{max}/K_m$ = intrinsic clearance ($\mu\text{l mg}^{-1} \text{min}^{-1}$).

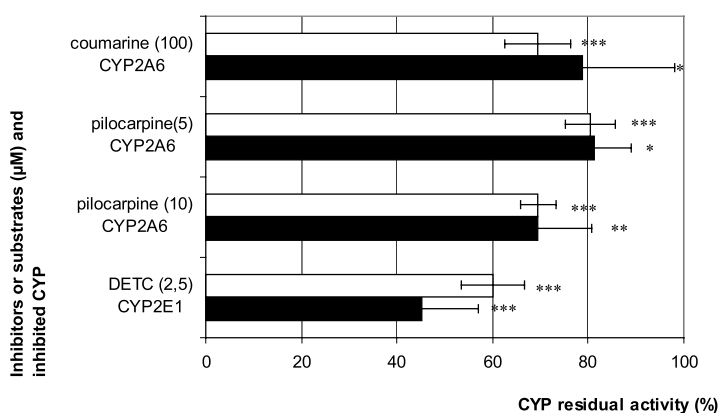
clearances of M2 and M4 ($0.74 \pm 0.43 \mu\text{l mg}^{-1} \text{min}^{-1}$ and $0.42 \pm 0.18 \mu\text{l mg}^{-1} \text{min}^{-1}$, respectively) were lower than intrinsic clearance of midazolam biotransformed by CYP3A4 ($Cl_{int} = 276.1 \mu\text{l mg}^{-1} \text{min}^{-1}$) (Gorski *et al.* 1994).

*Inhibition studies of 2*n*PQ metabolism using specific CYP substrates or inhibitors in human liver microsomes*

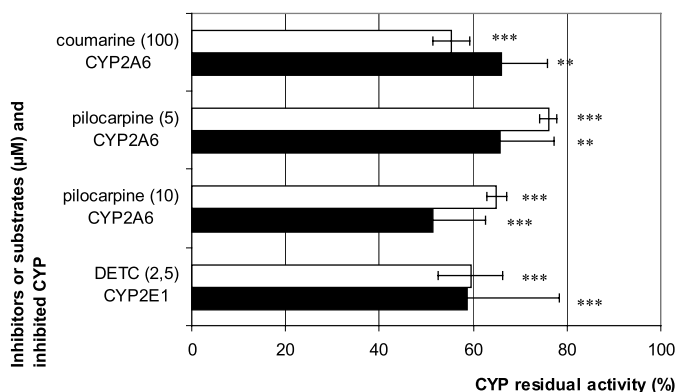
The effects of selective substrates or inhibitors on 2*n*PQ oxidation are shown in figure 3. Formation of M1, M2 and M4 by human CYP was inhibited by coumarin (100 μM), a CYP2A6 substrate (Nakajima *et al.* 1996), and by pilocarpine (10 μM), a CYP2A6 inhibitor (Kinonen *et al.* 1995). Formation of M1 and M2 was also inhibited by DETC (2.5 μM), a CYP2E1 inhibitor. In contrast, the usual inhibitory concentrations of other selective CYP substrates or inhibitors (alpha-naphthoflavone, tolbutamide, quinidine, ketoconazole, mephenytoin and sulphaphenazole) did not inhibit biotransformation of 2*n*PQ. Inhibition studies of 2*n*PQ metabolism by specific CYPs, using different substrates or inhibitors with hlm, suggested the involvement of CYP2A6 and CYP2E1 (figure 3). DETC (2.5 μM) did not inhibit the activity of CYP2A6. Indeed, the metabolism of coumarin (specific substrate of CYP2A6) in hlm was the same with or without DETC. Thus, the inhibition of M1 and M2 formation was not the consequence of a non specific inhibition of CYP2A6 by DETC.

*Correlation between CYP activities and 2*n*PQ biotransformation*

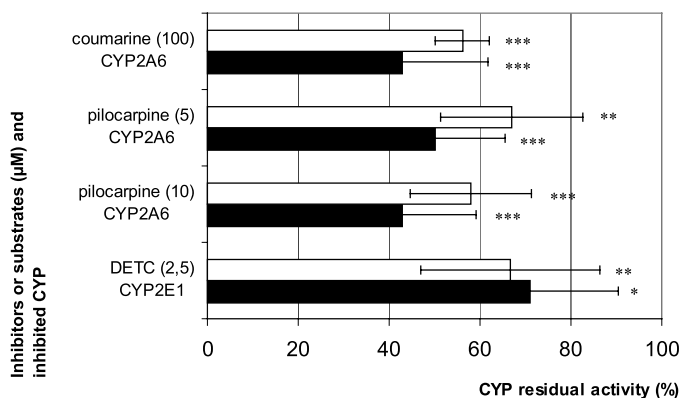
The correlation between the formation of 2*n*PQ metabolites with 2*n*PQ (50 and 400 μM) and the activities of CYP2A6, CYP2E1, CYP2C9 and CYP3A4 is shown in table 2. Strongest correlations were between: M1 and CYP2E1, CYP2C9; M2 and CYP2A6; and M4 and CYP2A6 for low concentrations of 2*n*PQ (50 μM), and between M1 and CYP2E1, CYP2C9; M2 and CYP2E1, CYP2C9; and M4 and CYP2C9, CYP3A4 for high concentrations of 2*n*PQ (400 μM). Furthermore, when correlation between CYPs were studied, we found that CYP2C9 and CYP2E1 activities correlated together ($r = 0.879$, $p < 0.005$), whereas CYP2A6, CYP3A4



(a)



(b)



(c)

Figure 3. Effect of specific substrates or specific inhibitors on 2*n*PQ oxidation (50 and 400 μM) in human liver microsomes. Black column: 2*n*PQ, 50 μM; white column: 2*n*PQ, 400 μM. Hepatic microsome preparations (1 mg ml⁻¹, M₄, M_{6a}, M₇, M₈) were incubated for 30 min with 50 or 400 μM 2*n*PQ and 1 mM NADPH with or without specific CYP probes (either substrates or inhibitors). (a) Formation of M1, (b) formation of M2, (c) formation of M4. Values are mean ± SD (*n* = 4). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, control samples = 100%.

Table 2. Correlation between specific CYP activities (CYP2A6, CYP2E1, CYP2C9 and CYP3A4) and 2*n*-propylquinoline biotransformation in human liver microsomes.

CYP	Reaction	Correlation coefficient					
		50 μM 2 <i>n</i> PQ			400 μM 2 <i>n</i> PQ		
		M1	M2	M4	M1	M2	M4
2A6	coumarin 7-hydroxylation	0.259	0.855***	0.916***	0.364	0.497	0.608*
2E1	chlorzoxazone 6-hydroxylation	0.942***	0.191	0.210	0.778**	0.827**	0.673*
2C9	tolbutamide 4-hydroxylation	0.818**	0.260	0.327	0.927***	0.855**	0.845**
3A4	midazolam 1-hydroxylation	0.622*	0.452	0.559	0.559	0.622*	0.804**

Hepatic microsomes were incubated (under optimal conditions with respect to incubation time and substrate concentration) with either 2*n*-propylquinoline or different specific CYP probes in the presence of NADPH cofactor, and the oxidation rate was determined on the basis of metabolite appearance, as described in the Materials and methods.

Values are Spearman correlation coefficients (*r*).

* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.005.

M1, M2, M4; metabolites 1, 2 and 4 of 2*n*PQ.

and CYP2E1 activities did not correlate (*r* = 0.580, *p* = 0.054; *r* = 0.196, *p* = 0.520; and *r* = 0.487, *p* = 0.106 respectively). CYP2C9 activity did not correlate with those of CYP2A6 and CYP3A4 (*r* = 0.427, *p* = 0.177; and *r* = 0.531, *p* = 0.08, respectively).

2nPQ metabolism in human lymphoblasts expressing specific CYPs and in microsomes expressing human epoxide hydrolase

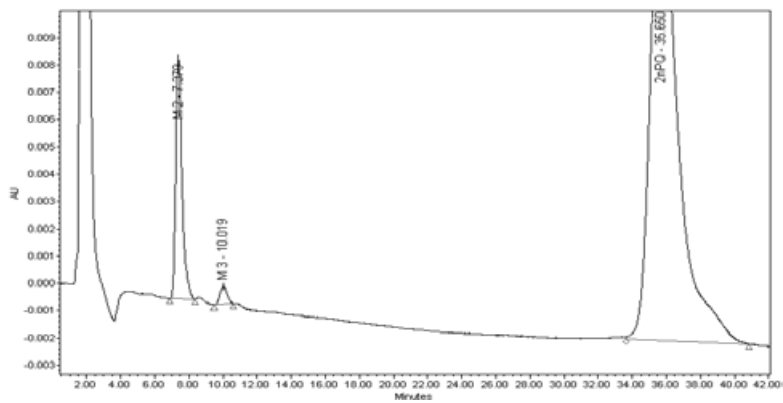
The kinetic parameters of 2*n*PQ oxidation in human lymphoblasts expressing specific CYPs are summarized in table 3. CYP1A2 and CYP2D6 did not support the metabolism of 2*n*PQ. CYP2E1 and, to a lesser extent, CYP2A6, CYP3A4, CYP2C9 and CYP2C19 supported the formation of M2, the major metabolite (figure 4a–d). CYP2A6 was the main CYP involved in the formation of M4, the

Table 3. Michaelis–Menten kinetic parameters for the formation of 2*n*-propylquinoline metabolites in lymphoblasts expressing specific CYPs.

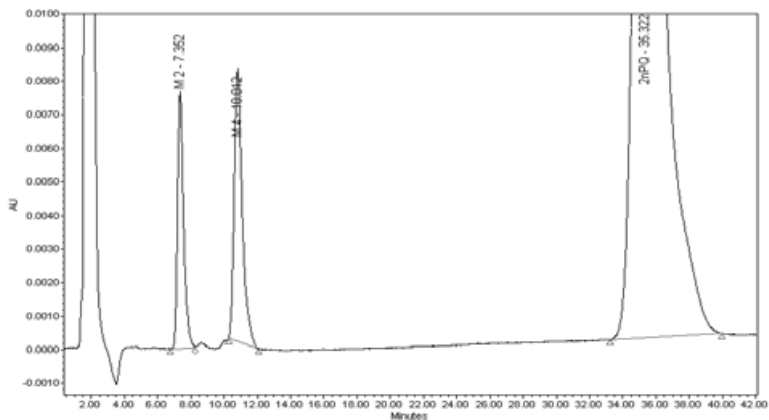
CYP	3'-Hydroxy-2 <i>n</i> PQ (M2)			Epoxy-2 <i>n</i> PQ (M3)		1'-Hydroxy-2 <i>n</i> PQ (M4)		
	<i>K</i> _{m2}	<i>V</i> _{max2}	Cl _{int2}	<i>K</i> _{m3}	<i>V</i> _{max3}	<i>K</i> _{m4}	<i>V</i> _{max4}	Cl _{int4}
2C9	1387	0.64	0.459	9309	4181.7 ^a	2778	2.68	0.965
2C19	154	0.05	0.338	*	*	342	1.42	4.150
2D6	*	*	*	*	*	*	*	*
2E1	311	0.78	2.5	342	23.7 ^a	*	*	*
1A2	*	*	*	*	*	*	*	*
2A6	113	0.05	0.478	*	*	127	0.13	1.039
3A4	1064	0.06	0.055	*	*	2688	0.97	0.359

* Undetectable. *K*_m (μM). *V*_{max} (pmol pmol⁻¹ P450 min⁻¹). Cl_{int} (μl nmol⁻¹ P450 min⁻¹). CYP, cytochrome P450.

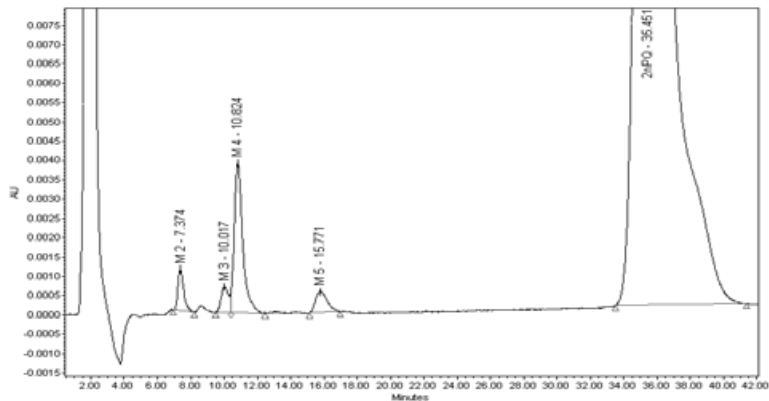
^a Arbitrary Units pmol⁻¹ P450 min⁻¹ because this compound could not be synthesized.



(a)



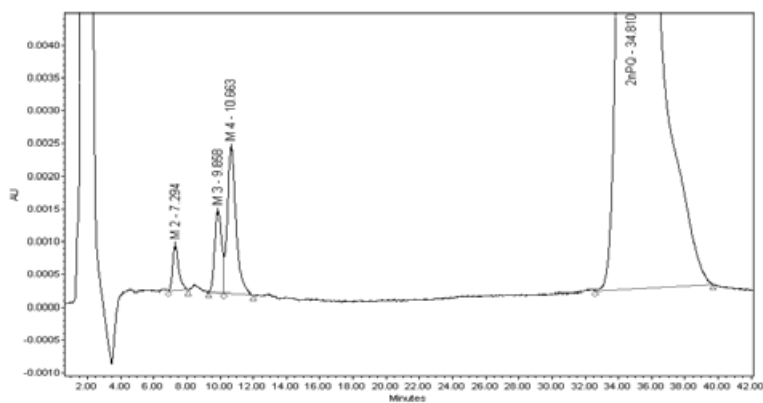
(b)



(c)

Figure 4. HPLC metabolite profiles following 2*n*PQ incubation with human lymphoblasts expressing CYPs: (a) 2E1, (b) 2A6, (c) 3A4, (d) 2C9. Specific CYPs (1 mg ml⁻¹) were incubated for 30 min with 2*n*PQ (400 μM) and NADPH (1 mM). After removal of precipitated proteins by centrifugation, the supernatant fluid was analysed by HPLC as described in the Materials and methods. (Continued.)

Figure



(d)

Figure 4. *Continued.*

second major metabolite. At high substrate concentration ($400\ \mu\text{M}$), $2n\text{PQ}$ was metabolized to M4 by CYP3A4, CYP2C9 and CYP2C19 (figure 4c, d).

The formation of M3 was observed only in human lymphoblasts expressing (CYP2E1, CYP2C9 mainly and CYP3A4) but never in hlm (figures 1 and 4a, c, d). $2n\text{PQ}$ was incubated with CYP2C9 or CYP2E1 for 30 min. Hlm ($1\ \text{mg ml}^{-1}$) were then added in each tube for another 30 min following incubation. HPLC analysis showed the disappearance of M3. The formation of M1 was not observed in human lymphoblasts expressing any CYP. M5 was not considered as a major metabolite as its formation by CYP3A4 was very low. Incubation of $2n\text{PQ}$ with CYP2E1 and epoxide hydrolase or with CYP2C9 and epoxide hydrolase, resulted in the formation of M1, M2 and M4 (figures 5 and 6). In these incubation mixtures, M3 was metabolized to M1 by epoxide hydrolase.

Discussion

The results observed with hlm and heterologous cDNA expression have demonstrated that CYP2A6, CYP2E1 and meh play a major role in the formation of three hydroxylated metabolites of $2n\text{PQ}$: M1 is dihydroxylated on the quinoline ring, whereas M2 (3'-OH-PQ) and M4 (1'-OH-PQ) are hydroxylated on the propylchain.

The formation of $2n\text{PQ}$ metabolites is partially CYP mediated. Metabolites were not produced in the absence of the CYP cofactor, NADPH, or in the presence of liver microsomes heated for 60 min at 56°C .

Discordant results according to M1 formation were obtained, namely M1 was produced when $2n\text{PQ}$ was incubated with microsomes and its formation was inhibited by CYP2A6 and CYP2E1 substrate and inhibitors (coumarin, pilocarpine and DETC, respectively). However, this metabolite was not produced by human lymphoblasts expressing these CYPs alone. Hence, other enzymes present in the microsomal preparation could be involved in M1 formation.

Moreover, M3 was identified only with human lymphoblasts expressing specific CYPs (CYP2E1, CYP2C9 and CYP3A4) but never with hlm, suggesting that M3 could be a substrate of enzymes other than the CYPs present in

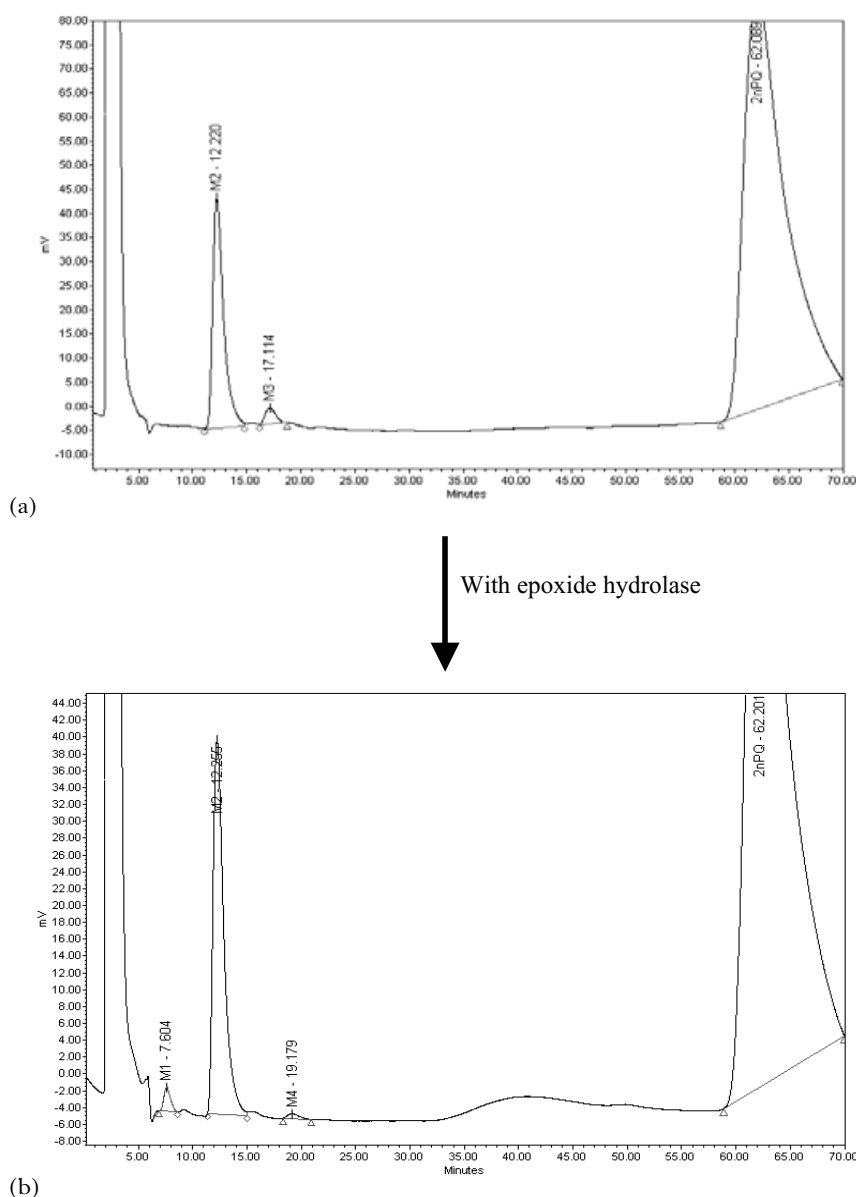


Figure 5. HPLC metabolite profiles following 2*n*PQ incubation with human lymphoblasts expressing CYP2E1 and CYP2E1 with epoxide hydrolase (a) CYP2E1 and (b) CYP2E1 with epoxide hydrolase. CYP2E1 (1 mg ml^{-1}) with microsomal proteins containing epoxide hydrolase (1 mg ml^{-1}) were incubated for 30 min with 2*n*PQ ($400 \mu\text{M}$) and NADPH (1 mM). After removal of precipitated proteins by centrifugation, the supernatant fluid was analysed by HPLC as described in the Materials and methods.

microsomes. 2*n*PQ incubation with heterologous expression systems in the presence of epoxide hydrolase supports the formation of M1 and it is concluded that M1 is derived from M3. M3 is mainly formed by CYP2C9 and CYP2E1, and is either the 5,6-epoxy-2*n*-propylquinoline or the 7,8-epoxy-2*n*-propylquinoline

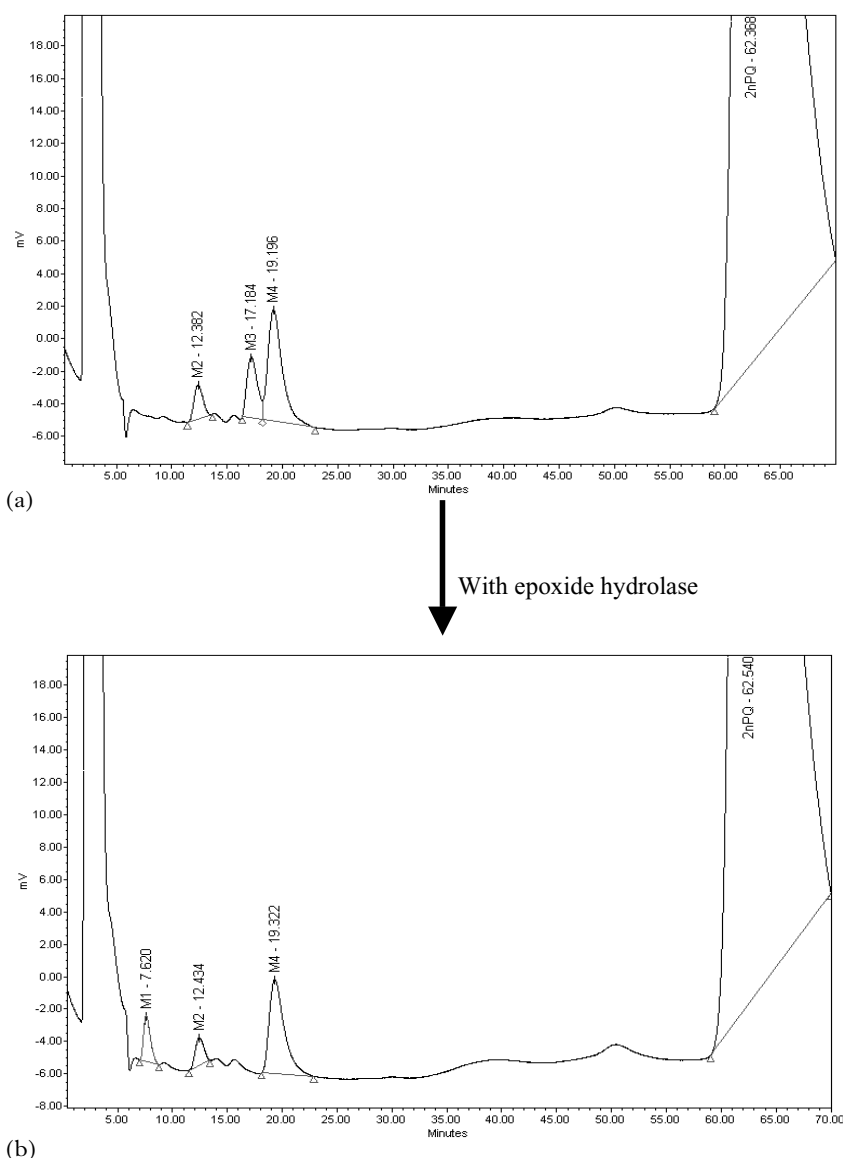


Figure 6. HPLC metabolite profiles following 2nPQ incubation with human lymphoblasts expressing CYP2C9 and CYP2C9 with epoxide hydrolase (a) CYP2C9 and (b) CYP2C9 with epoxide hydrolase. CYP2C9 (1 mg ml^{-1}) with microsomal proteins containing epoxide hydrolase (1 mg ml^{-1}) were incubated for 30 min with 2nPQ ($400 \mu\text{M}$) and NADPH (1 mM). After removal of precipitated proteins by centrifugation, the supernatant fluid was analysed by HPLC as described in the Materials and methods.

with a molecular weight of 187. Epoxide hydrolase metabolized M3 to M1, 5,6-dihydroxy-2n-propylquinoline or 7,8-dihydroxy-2n-propylquinoline (molecular weight of 205) (Armstrong 1999). As with quinoline metabolism, M1 is likely the *trans*-5,6-dihydroquinoline-5,6-diol (Boyd *et al.* 1991). Thus, CYP2C9 and CYP2E1 are indirectly involved in M1 formation. Correlation studies also showed

their involvement: the formation of M1 with 50 and 400 μM 2nPQ correlated with the activity of CYP2C9 ($r=0.818$, $p=0.01$; and $r=0.927$, $p=0.001$, respectively) and the activity of CYP2E1 ($r=0.942$, $p=0.001$; and $r=0.778$, $p=0.01$, respectively).

Table 1 shows the intermicrosome batch variability (interindividual variability) for 2nPQ metabolite formation. An important variability was also observed in the K_m ($K_{m1}=323 \pm 356 \mu\text{M}$). This variability could be due to the involvement of different enzymes (CYP2E1 and CYP2C9) in the formation of M3 and to the involvement of microsomal epoxide hydrolase in the biotransformation of M3 to M1 (Chalmers and Brown 1987). However, an analysis based on a two-enzyme model did not improve our findings, suggesting that the K_m values were not sufficiently different to be differentiated. The K_{m1} variation is likely due to the different amounts of CYP2E1, CYP2C9 and epoxide hydrolase expressed in hlm. This variability was also observed during the correlation study: coumarin, chlorzoxazone, tolbutamide and midazolam hydroxylation varied 16-, 5-, 8- and 18-fold, respectively.

Heterologous systems showed the role of CYP2A6 and for the high concentration of 2nPQ, the role of CYP2E1, CYP2C9 and CYP3A4 in 2nPQ metabolism. M4 was produced by CYP2C9 but never by human lymphoblast expressing specific CYP2E1. In correlation studies, the formation of M4 with 2nPQ (400 μM) correlated with the activity of CYP2C9. The correlation between the formation of M4 and the activity of CYP2E1 could be explained by the strong correlation between activities of CYP2C9 and CYP2E1 in our 12 hlm samples ($r=0.879$, $p=0.001$). Thus, the formation of M4 involves several CYPs. The involvement of different enzymes producing a single metabolite has been demonstrated for other compounds, including tolbutamide (Veronese *et al.* 1993) and caffeine (Tassaneeyakul *et al.* 1994).

The identification of specific enzymes involved in the biotransformation process is of great importance in several stages of drug development (Miners *et al.* 1988). Once the enzymes responsible for the metabolism of a particular drug are known, predictions may be made concerning drug-drug interactions likely to influence the pharmacokinetic, therapeutic responses and development of adverse reactions in specific patient groups (Lin and Lu 1998, Tanaka 1998). The major human CYPs responsible for the oxidation of 2nPQ were found to be CYP2A6 and CYP2E1, with minor contributions by other CYPs. Since CYP2A6 and CYP2E1 are not implicated to a significant degree in drug metabolism, the oxidative metabolism of 2nPQ should not play an important role in drug-drug interactions (Moltke *et al.* 1998).

In conclusion, in the human liver, 2nPQ oxidation is mediated by CYP2A6 and CYP2E1. CYP2A6 is involved at low 2nPQ concentrations (50 μM). Other CYPs, mainly CYP2E1, are involved at high 2nPQ concentrations (400 μM). Microsomal epoxide hydrolase also plays a role. In the human *in vitro* system, three main metabolites were formed: a dihydroxy-2n-propylquinoline (molecular weight of 205), 3'-hydroxy-2n-propylquinoline and 1'-hydroxy-2n-propylquinoline. This is an important finding because the co-administration of CYP substrates or inhibitors involved in drug interactions (i.e. CYP3A4, CYP2D6, CYP1A2 and CYP2C family) (Michalets *et al.* 1998) might not affect significantly the metabolism of 2nPQ.

Acknowledgements

The authors thank Dr A.-M. Taburet (Service de Pharmacie, Hôpital du Kremlin-Bicêtre, France) for her collaboration in the preparation of human hepatic microsomes. Grateful thanks also go to Dr C. Fernandez (Service de Pharmacie, Hôpital de la Pitié-Salpêtrière, France) for her help in the preparation of the manuscript.

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Curcumin is a potent inhibitor of phenol sulfotransferase (SULT1A1) in human liver and extrahepatic tissues

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Received 16 September 2002

1. Curcumin has anti-carcinogen effects and is under clinical evaluation as a potential colon cancer chemopreventive agent. The first aim was to see whether curcumin inhibited phenol sulfotransferase (SULT1A1) and, if so, to study the variability of the IC₅₀ of curcumin for SULT1A1 in 50 human liver samples. For comparative purposes, the inhibition of catechol sulfotransferase (SULT1A3) in five human liver specimens was studied. The second aim was to measure the IC₅₀ of curcumin against SULT1A1 in five samples of human duodenum, colon, kidney and lung.

2. Curcumin was a potent inhibitor of SULT1A1 in human liver; the mean $\pm$ SD and median of IC₅₀ were 14.1 ± 7.3 nM and 12.8 nM, respectively. The IC₅₀ ranged from 6.2 to 30.6 nM between the 5th and 95th percentiles and the fold of variation was 4.9. The distribution of IC₅₀ was positively skewed (skewness 1.2) and deviated from normality ($p = 0.0004$).

3. Curcumin inhibited human SULT1A3, and the inhibition was studied in five liver specimens with an IC₅₀ of 4324 ± 1026 nM. This inhibition was greater than the IC₅₀ of curcumin for SULT1A1 ($p < 0.0001$).

4. In the extrahepatic tissues, the IC₅₀ of curcumin for SULT1A1 was 25.9 ± 4.8 nM (duodenum), 25.4 ± 6.8 nM (colon), 23.4 ± 2.2 nM (kidney) and 25.6 ± 5.6 nM (lung). Inhibition in these tissues is greater than that of curcumin for SULT1A1 in human liver ($p < 0.0001$).

5. In conclusion, curcumin is a potent inhibitor of SULT1A1 in human liver, duodenum, colon, kidney and lung. The IC₅₀ of curcumin for SULT1A1 varied 4.9-fold in human liver. The comparison of the present data with those of the literature revealed that the IC₅₀ of curcumin in the liver and extrahepatic tissues is one order of magnitude lower than the peak serum concentration of curcumin after therapeutic doses of 4 g to humans.

Introduction

Curcumin (diferuloylmethane) (figure 1), the yellow pigment isolated from the root of the plant *Curcuma longa* L., has several biological effects, the most important being its anti-carcinogenic effect. Curcumin inhibits several carcinogenic stimuli in mice (Huang *et al.* 1997), inhibits diethylnitrosamine-induced murine hepatocarcinogenesis (Chuang *et al.* 2000), inhibits the cell cycle of

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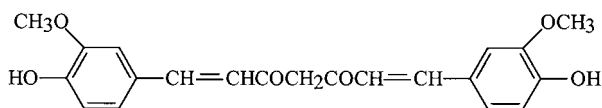


Figure 1. Chemical structure of curcumin.

immortalized human umbilical vein endothelial cells (Park *et al.* 2002) and inhibits the cell proliferation in human breast carcinoma (Shao *et al.* 2002). It is currently under clinical evaluation as a potential colon cancer chemopreventive agent (Ireson *et al.* 2002).

The anticarcinogenic effect of curcumin is complex and Eaton *et al.* (1996) reported that it is a potent inhibitor of SULT1A1 in human liver. Boberg *et al.* (1983) and Tsutsumi *et al.* (1995) have shown that sulfotransferase dramatically reduce the potency of sulfated activated carcinogens in both mice and hamsters.

There are several forms of phenol sulfotransferase (EC 2.8.2.1) (Raftogianis *et al.* 1996) and two forms have been characterized for substrate specificity, inhibitor sensitivity, thermal stability and regulation. One form is sensitive to the inhibition of 2,6-dichloro-4-phenol (Campbell *et al.* 1987), is relatively thermostable and is named TS-PST (Weinshilboum 1986) or P form (Bonham Carter *et al.* 1983), simply phenol sulfotransferase (Cappiello *et al.* 1990, Pacifici and De Santi 1995) or SULT1A1. The other form is thermolabile and is named TL-PST (Weinshilboum 1986) or M form (Bonham Carter *et al.* 1983), catechol sulfotransferase as its functional moiety is the catecholic group of dopamine (Cappiello *et al.* 1990, Pacifici and De Santi 1995) or SULT1A3. Here, the SULT1A1 and SULT1A3 will be used. The first aim of the current study was to determine the IC_{50} of curcumin for SULT1A1 in 50 human liver samples and to investigate its variation in human liver. For comparative purposes, the inhibition of SULT1A3 by curcumin in five human liver samples was also studied. Our second aim was to measure the IC_{50} of curcumin in duodenum, colon, kidney and lung.

Materials and methods

3'-Phosphoadenosine-5'-phosphosulphate- $[^{35}S]$ (PAPS, specific activity $1.71 \text{ Ci mmol}^{-1}$, radiochemical purity 99%) was obtained from NEN Life Science Products, Inc. (Brussels, Belgium). Curcumin, dithiothreitol (DTT) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (Milan, Italy). Monobasic and dibasic potassium phosphate, acetone, sodium hydroxide, barium hydroxide, barium acetate and zinc sulphate was obtained from Carlo Erba (Milan, Italy).

Biological material

The Ethical Committee of Pisa Medical School approved this project. Liver samples were obtained after clinically indicated partial hepatectomy from 50 patients (19 females and 33 males) whose age and liver function tests are summarized in table 1. A specimen of normal liver was sampled from a normal part of the organ as far as possible away from the tumour. The duodenum samples were derived from one female and four males with a mean $\pm$ SD age of 63 ± 8 years. The biopsies of duodenum were sampled after informed consent was obtained from the patients. Biopsies of duodenum of about 5 mg were sampled from the second portion of the duodenum in the five patients, who underwent clinically indicated gastrointestinal enteroscopy. Several biopsies of duodenum obtained from the same patient were combined and analysed as a single sample. The colon, kidney and lung samples were obtained from five patients undergoing clinically indicated surgery to remove tumours, and a specimen of normal tissue was sampled from a normal part of the tissue as far as possible away from the tumour. The colon samples were obtained from one female and four males, and the mean $\pm$ SD age was 63 ± 8 years. The kidney samples were derived from two females and three males, and their mean $\pm$ SD age was 57 ± 22

Table 1. Liver function tests of patients undergoing clinically indicated partial hepatectomy.

	Minimum	Maximum	Mean $\pm$ SD	Median	Normal
Age (years)	19	84	63 $\pm$ 11	65	—
Albumin (g dl ⁻¹)*	3.3	4.9	4.0 $\pm$ 0.3	4.0	3.5–5.5
Bilirubin (mg dl ⁻¹)**	0.2	12.1	1.1 $\pm$ 2.1	0.6	$\leq$ 1.1
GOT (U l ⁻¹)	11	83	22 $\pm$ 12	19	<45
GPT (U l ⁻¹)	4	61	20 $\pm$ 12	16	<45
PT (s)	11.0	37	12.3 $\pm$ 3.1	11.8	10.9–12.8

* Plasma albumin concentration; **total bilirubin plasma concentration; GOT, glutamic-oxalacetic transaminase activity; GPT, glutamic-pyruvic transaminase activity; PT, prothrombin time.

years. The lung samples were obtained from five males whose mean $\pm$ SD age was 64 $\pm$ 9 years. Tissue samples were frozen and stored at -80°C .

Isolation of the cytosolic fractions

About 1 g liver, colon mucosa, kidney and lung was homogenized in 5 vols 0.25 M sucrose dissolved in 5 mM potassium orthophosphate buffer (pH 7.4) using a glass-Teflon homogenizer. The homogenates were centrifuged at 12 000g for 15 min and the supernatants centrifuged again at 105 000g for 1 h. The high-speed cytosolic fractions were divided into aliquots, stored at -80°C and designated as the cytosolic fraction. The biopsies of the duodenum were homogenized in 0.3 ml 0.25 M sucrose (buffered at pH 7.4 with 5 mM potassium orthophosphate buffer) using a glass-Teflon homogenizer ('size 0', Thomas, Philadelphia, PA, USA). The homogenates were aspirated using a Pasteur pipette and transferred into centrifuge tubes. The grinding chamber and the pestle were rinsed with 0.2 ml 0.25 M sucrose (buffered at pH 7.4 with 5 mM potassium orthophosphate buffer) and the washings combined with the homogenates. The homogenates were centrifuged as described above.

Assays

Protein concentration was measured as described by Lowry *et al.* (1951) using BSA as a standard.

Assay of SULT1A1

SULT1A1 activity was measured with 4 μM 4-nitrophenol as the sulfate acceptor substrate and with 0.4 μM PAPS as the sulfate donor substrate. SULT1A1 activity assay was performed as described by Foldes and Meek (1973), as modified by Anderson and Weinshilboum (1980) and as adapted to the human liver by Cappiello *et al.* (1990). The incubation buffer was 8.3 mM orthophosphate potassium (pH 6.0) and the incubation time was 20 min. The liver cytosolic fractions were diluted 10 000-fold. Dilutions were carried out with 5 mM orthophosphate potassium buffer (pH 7.4) containing DTT (1.54 mg ml⁻¹) and BSA (0.625 mg ml⁻¹). The final protein concentration per millilitre of the incubation mixture ranged from 0.6 to 1.6 $\mu\text{g ml}^{-1}$ in the liver in a final incubation mixture of 150 μl . The duodenal cytosolic fraction was diluted 500-fold; the colon mucosa cytosolic fraction was diluted 250-fold. The kidney and lung cytosolic fractions were diluted 100-fold. The final protein concentration ($\mu\text{g ml}^{-1}$) ranged from 0.4 to 3.3 (duodenum), from 30 to 40 (colon), from 53 to 79 (kidney) and from 80 to 147 (lung). The given enzyme activities were measured under linear conditions of protein concentration and incubation time. Curcumin was dissolved in acetone and 2 μl were added to the incubation mixture (final volume 150 μl). This volume of acetone did not affect the phenol sulfotransferase activities.

Assay of SULT1A3 activity

SULT1A3 activity assay was performed as described by Foldes and Meek (1973), as modified by Anderson and Weinshilboum (1980) and as adapted to the human liver by Cappiello *et al.* (1990). The incubation mixture for SULT1A3 was as that for SULT1A1 with the following modifications. The sulfate acceptor substrate was 60 μM dopamine, the incubation mixture contained 1 mM pargyline and the incubation time was 5 min. The liver cytosolic fractions were diluted 100-fold. Dilutions were carried out with 5 mM orthophosphate potassium buffer, pH 7.4. The final protein concentration ranged from 60 to 160 $\mu\text{g ml}^{-1}$. The given activity was given under linear conditions of protein concentration and incubation time. Curcumin was dissolved in acetone and 2 μl were added to the incubation mixture (final volume 150 μl). This volume of acetone did not affect the activity of SULT1A3.

Inhibition of SULT1A1 and SULT1A3 activities by curcumin

The inhibition of SULT1A1 by curcumin was studied in 50 liver samples. The inhibition curves were constructed using 3.12, 6.25, 12.5, 25 and 50 nM curcumin. The concentrations of 4-nitrophenol and PAPS were kept constant at 4 and 0.4 μM, respectively. The inhibition of SULT1A3 rate by curcumin was studied in five liver samples. The inhibition curves were constructed using 1, 2, 4, 8 and 16 μM curcumin. The concentrations of dopamine and PAPS were kept constant at 60 and 0.4 μM, respectively.

Statistical analysis

Descriptive statistical analysis was performed using the software package Statistica (StatSoft, Tulsa, OK, USA). IC₅₀ were calculated with the software package Dose–Effect Analysis (Biosoft, Cambridge, UK). The difference in IC₅₀ in the liver, duodenum, colon, kidney and lung were compared by ANOVA test followed by the Tukey–Kramer multiple comparisons test. The correlations were tested by the Spearman-correlation test, while the difference between groups was tested by the Mann–Whitney *U*-test using the software package Statistica (StatSoft).

Results and discussion

The activity of SULT1A1 mean (± SD, *n* = 50) was 537 ± 76 pmol min^{−1} mg^{−1}. The IC₅₀ of curcumin for SULT1A1 was measured in 50 liver samples with mean (± SD) and medians of 14.1 ± 7.3 and 12.8 nM, respectively, and statistical analysis of the data are summarized in table 2. The IC₅₀ of curcumin was positively skewed and the skewness was 1.2. The IC₅₀ of curcumin for SULT1A1 did not correlate with the age of liver donors (*r* = −0.0987, *p* = 0.495) and was not different between males and females (*p* = 0.337). The probit plot of IC₅₀ of curcumin for SULT1A1 (figure 2) shows that the IC₅₀ deviated from normality. The activity of SULT1A1 did not correlate with the IC₅₀ (*r* = −0.210, *p* = 0.125). The IC₅₀ of curcumin for SULT1A1 was also determined in five samples of duodenum (25.9 ± 4.8 nM), colon (25.4 ± 6.8 nM), kidney (23.4 ± 2.2 nM) and lung (25 ± 5.6 nM), and the inhibition curves are shown in figure 3. The IC₅₀ of curcumin in these tissues were higher than that in the liver (*p* < 0.0001). The IC₅₀ of curcumin for SULT1A3 were measured in five samples of liver and had a mean (± SD) of 4324 ± 1026 nM (figure 3). It was different from that of SULT1A1 (*p* < 0.0001) (figure 3).

The present results demonstrate that curcumin is a potent inhibitor of SULT1A1 both in human liver and extrahepatic tissues. SULT1A3 is less susceptible than SULT1A1 to the inhibition by curcumin and the IC₅₀ of

Table 2. Descriptive statistics of the IC₅₀ of curcumin for SULT1A1 in human liver.

Number of cases	50
Mean*	14.1
± SD	7.3
Median*	12.8
5th percentile*	6.2
95th percentile*	30.6
Variation**	4.9
Skewness	1.2
W-statistic§	0.904 84
<i>p</i> †	0.0004

* nM; ** variation was calculated dividing the 95th percentile by the 5th percentile; § critical value of the Wilk–Shapiro test; † distribution deviates from normality when *p* < 0.05.

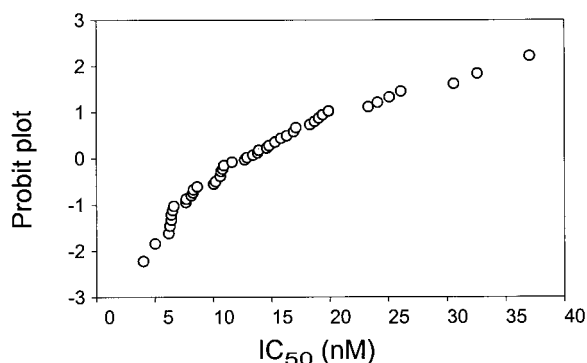


Figure 2. Probit plot of the IC_{50} of curcumin for phenol sulfotransferase (SULT1A1). The IC_{50} was measured in 50 human liver samples.

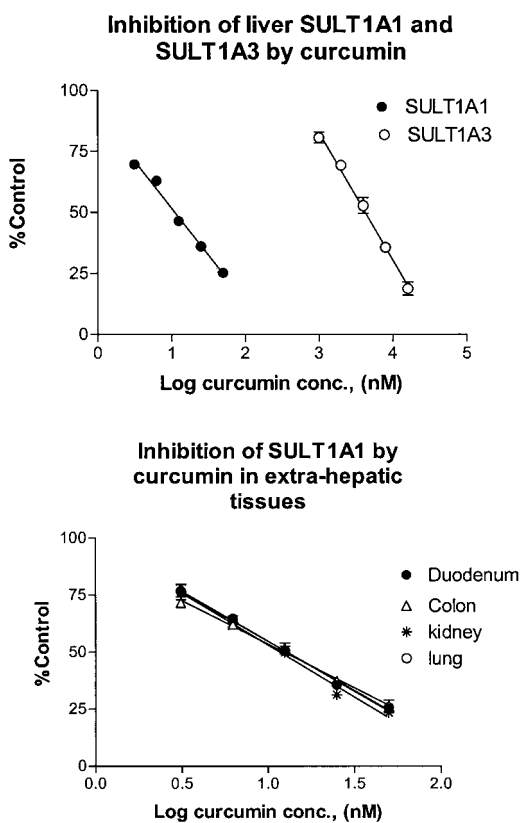


Figure 3. (top) Inhibition of phenol sulfotransferase (SULT1A1) and catechol sulfotransferase (SULT1A3) by curcumin in the human liver. (bottom) Inhibition of phenol sulfotransferase (SULT1A1) by curcumin in extrahepatic tissues. In the non-inhibited samples, the activity of SULT1A1 ($\text{pmol min}^{-1} \text{mg}^{-1}$) was 537 ± 76 (liver), 289 ± 13 (duodenum), 41 ± 31 (colon), 34 ± 16 (kidney) and 17 ± 11 (lung). The liver activity of SULT1A3 was $37 \pm 27 \text{ pmol min}^{-1} \text{mg}^{-1}$.

SULT1A3 was 307-fold greater than that of SULT1A1 in human liver. The only information on the inhibition of SULT1A1 by curcumin in human liver was reported by Eaton *et al.* (1996) who measured the IC₅₀ of curcumin in two liver samples. They reported an IC₅₀ of 380 nM in human liver, which is an order of magnitude greater than that reported here.

There is increasing interest of curcumin as an anti-carcinogenic drug (Cheng *et al.* 2001, Park *et al.* 2002, Shao *et al.* 2002, Shim *et al.* 2002). Harris *et al.* (1998) suggested that the prophylactic use of salicylic acid, an inhibitor of SULT1A1, as an anti-carcinogen is due to the inhibition of sulfotransferase which prevents the formation of the sulfated compounds into the final carcinogenic forms. Boberg *et al.* (1983) and Tsutsumi *et al.* (1995) reported that sulfotransferase inhibitors dramatically reduce the potency of sulfated activated carcinogens in both mice and hamster.

The IC₅₀ of curcumin for SULT1A1 varied 4.9-fold within 5th and 95th percentiles, indicating that it varies considerably in human liver. The liver with a relatively high IC₅₀ of curcumin will be less susceptible to the inhibition of SULT1A1 by curcumin than those with relatively low IC₅₀. Curcumin is administered in doses of several grams and the highest dose used in a clinical trial was 12 g (Cheng *et al.* 2001). After chronic treatment with 4 g curcumin, the serum concentration of this drug was (mean $\pm$ SD) 0.51 ± 0.11 μ M (Cheng *et al.* 2001). This serum concentration is one order of magnitude greater than the IC₅₀ of curcumin in liver and extrahepatic organs and SULT1A1 should be therefore inhibited *in vivo* after treatment with curcumin.

Curcumin inhibited SULT1A1 in the duodenum, colon, kidney and lung, indicating that it inhibits the sulfation of SULT1A1 in the main metabolic organs. In contrast, hepatic SULT1A3 is less susceptible to the inhibition by curcumin than SULT1A1. This result is in accord with studies conducted with human liver. By comparison, 2,6-dichloro-4-nitrophenol (Campbell *et al.* 1897), several fenamates (Vietri *et al.* 2000a) and salicylic acid (Vietri *et al.* 2000b) have lower IC₅₀ for SULT1A1 than for SULT1A3.

Acknowledgements

This work was supported by Contract No. BMH1-CT94-1622 of the European Commission (Brussels, Belgium), by the Italian Ministry of the University (Rome, Italy) and the Italian Association for *Cancer Research* (Milan, Italy).

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Differential metabolism of midazolam in mouse liver and intestine microsomes: a comparison of cytochrome P450 activity and expression

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Received 15 September 2002

1. Although multiple cytochrome P450s (CYP) contribute to hepatic phase I metabolism, CYP3A is the principal subfamily present in human and mouse small intestine.

2. Differences in phase I metabolism were investigated using midazolam (MDZ) hydroxylation in mouse liver and intestinal microsomes. The net MDZ metabolite formation rate in intestinal microsomes was approximately 30% that of liver microsomes (at 250 μ M MDZ).

3. Quantitative Western blotting with anti-CYP3A1 antibody detected two bands of immunoreactive protein in both liver and intestinal samples, 2.24 ± 0.27 (mean $\pm$ SD, $n = 3$) and 0.64 ± 0.08 pmol mg^{-1} protein, respectively. Qualitative Western blotting with anti-CYP2C11 antibody detected a single band of immunoreactive protein in liver microsomes and no signal in intestinal samples (1 μ g sample).

4. Ketoconazole potently inhibited formation of both α - and 4-OH-MDZ metabolites in intestinal microsomes (IC_{50} 's of 0.126 ± 0.010 and 0.0955 ± 0.014 μ M, respectively) and of 4-OH-MDZ formation in mouse liver microsomes (IC_{50} of 0.041 ± 0.003 μ M). However, ketoconazole (5 μ M) did not produce 50% inhibition of α -OH-MDZ formation in mouse liver microsomes. Inhibition by ritonavir (5 μ M) produced similar results.

5. MDZ hydroxylation is predominately CYP3A dependent in mouse intestine (compared with mouse liver) since CYP2C is not expressed in the intestine. The importance of CYP3A in the mouse intestine appears to mirror that in humans.

Introduction

Although hepatic metabolism is usually considered the major route of drug clearance in humans and animals, recent clinical and *in vitro* studies have demonstrated the importance of intestinal metabolism when drugs are administered orally (Doherty and Charman 2002). Cytochrome P450 (CYP) 3A enzymes are the major phase I-metabolizing enzymes found in human liver, kidney and small intestine (Watkins 1997), and metabolize more than 50% of drugs used in clinical practice (Thummel and Wilkinson 1998). Whereas the CYP3As have differential substrate specificity across species, it remains a dominant and ubiquitous enzyme in many species, responsible for the metabolism of numerous of endogenous and exogenous molecules (Thummel and Wilkinson 1998, Quattrochi and Guzelian 2001). In humans, the CYP3A forms account for more than 30% of total CYP protein in the liver (Shimada *et al.* 1994) and 70% in the intestine

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(McKinnon *et al.* 1995). Similarly, CYP3A is a major enzyme subfamily in the mouse liver (Quattrochi and Guzelian 2001) and appears to be the major CYP in mouse intestine, based on immunoblot and metabolic activity analysis (Emoto *et al.* 2000a).

The importance of intestinal metabolism as a means of drug clearance in humans has become increasingly apparent. Intestinal metabolism in mice is not well understood, even though mouse models (and knockout mice) are commonly used to study drug metabolism (Nebert and Duffy 1997, Perloff *et al.* 1999, Schellens *et al.* 2000, Warrington *et al.* 2000). The importance of mice as a model in biomedical research is exemplified with The National Cancer Institute's continued work with the Mouse Cancer Genome Anatomy Project and the Mouse Models of Human Cancer Consortium. Emoto *et al.* (2000b) reported that CYP3A forms are the major CYP family in the mouse intestine, but the relative *in vivo* pharmacological implications of CYP3A in the mouse intestine, compared with liver, have not been established.

The benzodiazepine derivative midazolam (MDZ) has been used as a CYP3A probe substrate in multiple clinical studies (Thummel *et al.* 1996, Wandel *et al.* 1998, Tsunoda *et al.* 1999, Venkatakrishnan *et al.* 2000). MDZ metabolism, yielding α -OH and 4-OH metabolites, is considered a pure index reaction for human CYP3A-mediated metabolism both clinically and *in vitro* (Kronbach *et al.* 1989, Thummel *et al.* 1994, Schmider *et al.* 1996, von Moltke *et al.* 1996, Wandel *et al.* 1998). However, hepatic metabolism of MDZ in mice has a significant CYP2C and CYP3A component (Perloff *et al.* 2000) and mouse intestinal microsomes appear to have very low CYP2C expression and activity (Emoto *et al.* 2000b). The following study was designed to compare CYP-mediated metabolism of MDZ and CYP expression in mouse liver and intestinal microsomes, thereby exploring the relevance of the intestinal component.

Materials and methods

Materials

Midazolam (MDZ), α -OH-midazolam (α -OH-MDZ) and 4-OH-midazolam (4-OH-MDZ) were kindly provided by Hoffman La Roche Inc. (Nutley, NJ, USA) or purchased from Ultrafine Chemicals (Oxford, UK). Ketoconazole (KTZ) was a gift from Janssen Co. (Beerse, Belgium). Ritonavir (RIT) was kindly provided by Abbott Laboratories (Chicago, IL, USA). Other chemical inhibitors and reaction cofactors (NADP⁺, DL-isocitric acid, magnesium chloride, isocitric dehydrogenase and potassium phosphate buffer solutions) were obtained from Sigma Chemical Co (St. Louis, MO, USA). A polyclonal antibody to rat CYP3A1 and control serum developed in rabbit were obtained from Xenotech (Phoenix, AZ, USA). A polyclonal antibody to rat CYP2C11 and control serum developed in goat were obtained from Gentest (Woburn, MA, USA). Per the manufacturer, this polyclonal antibody immunoblots rat CYP2C11 with a minor cross-reactivity to rat CYP2C13 in male liver microsomes. Cross-reactivity with mouse CYP2C enzymes was confirmed by the manufacture and demonstrated in Perloff *et al.* (2000) and Warrington *et al.* (2000).

Liver microsomal preparation

Mouse livers were taken from three male Crl:CD-1(ICR)BR (CD1) mice (8–10 weeks old) (Charles River Laboratories, Wilmington, MA, USA). Mice had been maintained on a 12-h light/dark cycle and given food *ad libitum*. All microsomes were prepared using standard techniques (von Moltke *et al.* 1993). In brief, microsomes were prepared by ultracentrifugation; microsomal pellets were resuspended in 0.1 M potassium phosphate buffer containing (pH 7.56) 20% glycerol and stored at -80°C until use. Total protein concentration was determined by a bicinchoninic acid protein assay (BCA-Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard.

Intestinal microsomal preparation

Mouse intestinal microsomes, from the same CD-1 mice used for liver microsomes, were prepared as described previously (Cotreau *et al.* 2001, Kotegawa *et al.* 2002). In brief, all tissue and reagents were kept at 4 °C and phenylmethylsulfonyl fluoride (40 µg ml⁻¹, PMSF) was used throughout the procedure. Immediately after decapitation, beginning at the pyloric sphincter, 20 cm of intestine was isolated. The segments were flushed and then incubated in solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄, pH 7.4) for 15 min. Segments were then filled with solution B (8.7 mM KH₂PO₄, 1.56 mM Na₂HPO₄, 96 mM NaCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.4). After shaking horizontally in an ice bath, solution B lumen fractions (5, 10, 15, 25, 35 min) were collected and pooled separately for each mouse. The cell fraction was then pelleted and resuspended in solution C (5 mM histidine, 0.25 M sucrose, 0.5 M EDTA, pH 7.4). Cell pellets were rinsed, then homogenized in a hand homogenizer, and centrifuged at 15 000g for 10 min. The supernatant was collected, and CaCl₂ was added such that the final concentration was 7.9 mM. Tubes were gently mixed, then allowed to stand for 15 min. Fractions were then centrifuged at 25 000g for 10 min. The resulting microsomal pellets were then suspended in 0.1 M potassium phosphate buffer (pH 7.56) containing 20% glycerol. Total protein concentration was determined by a BCA protein assay.

Incubation

Incubation mixtures contained 50 mM phosphate buffer, 5 mM Mg²⁺, 0.5 mM NADP⁺ and an isocitrate/isocitric dehydrogenase-regenerating system (3.75 mM DL-isocitric acid and 1 U ml⁻¹ isocitrate dehydrogenase). For inhibition studies, MDZ (in methanol) solution was aliquoted into incubation tubes to yield final concentrations of 50 µM. The solvent was evaporated to dryness at 40 °C under mild vacuum. Incubations were performed at 0–600 µM MDZ to establish kinetic parameters (K_m , V_{max}) for metabolite formation in mouse liver and intestinal microsomes (Perloff *et al.* 1999, Cotreau *et al.* 2001). Reaction mixtures with both liver and intestinal microsomes were incubated at a protein concentration of 0.1 mg ml⁻¹. Formation of MDZ metabolites with both liver and intestinal microsomes was linear with respect to incubation time (to 7 min) and microsomal protein (to 0.5 mg ml⁻¹) concentration. Incubations were initiated by the addition of microsomal protein. After 7 min at 37 °C, reactions were stopped by cooling on ice and the addition of acetonitrile (100 µl). Phenytoin (25 ng) was added as the internal standard, the incubation mixture was centrifuged and the supernatant transferred to an autosampling vial for HPLC analysis. All kinetic samples were done in triplicate. The formation rate of individual metabolites in reaction mixtures was determined based on calibration curves constructed from a series of standards containing varying known amounts of metabolite standards together with internal standard. Reaction velocities were calculated in units of nmoles product formed per min per mg microsomal protein. Control incubations with no cofactor, no protein and/or no MDZ substrate were performed concurrently to validate CYP-dependent metabolism. The identity of metabolites was verified by comparing HPLC retention time with authentic standards.

Chemical inhibition

Inhibition studies with KTZ (0.05–5 µM) and RIT (0.5–5 µM) were performed by co-addition to MDZ incubations. Chemical inhibition incubations were performed as described above. The time-dependant mechanism-based inhibitor, troleandomycin (TAO, 50 and 500 µM), was pre-incubated (20 min) with microsomes and cofactor before the addition of MDZ. The incubation was then stopped 7 min after the addition of MDZ by cooling and addition acetonitrile as described above. Controls with no inhibitor were performed at the beginning and end of each inhibition experiment. All inhibition samples were done in duplicate. Metabolite formation was expressed as a per cent of control, based on metabolite to internal standard peak–height ratio (PHR), in the inhibition studies and IC₅₀ analysis.

Immunoinhibition

Serum from goats or rabbits containing polyclonal anti-CYP antibodies has previously been shown to inhibit the activity of human and mouse CYPs in a concentration-dependent manner (Kronbach *et al.* 1989, Schmider *et al.* 1996, Perloff *et al.* 1999). Additionally, MDZ hydroxylation in mouse liver microsomes has been shown to be both CYP3A and CYP2C dependent (Perloff *et al.* 2000, Warrington *et al.* 2000). Antiserum to CYP3A1 and CYP2C11 was tested for each antibody's activity against MDZ hydroxylation in mouse liver and intestinal microsomes at a single polyclonal serum to microsomal protein ratio (10:1). In preliminary experiments, 20:1 ratios were also tested, demonstrating minimal increase in inhibition over 10:1 ratios. In each experiment, 50 µM MDZ and 10 µg microsomal protein were pre-incubated for 30 min at 37 °C with 0 or 100 µg serum protein from immunized rabbits or goats, or the same quantities of appropriate control pre-immune serum. Incubations were initiated by the addition of cofactor solution and stopped after 7 min with cooling and acetonitrile as described above.

High-performance liquid chromatography (HPLC)

For MDZ, α -OH-MDZ and 4-OH-MDZ, the HPLC mobile phase consisted of 200 ml acetonitrile, 350 ml methanol and 450 ml 10 mM phosphate buffer; with a flow rate of 1.5 ml min⁻¹. The analytical column (25 cm $\times$ 2.5 mm) was a Beckman C18 'ODS' (5 μ m) (Beckman-Coulter, Fullerton, CA, USA). Column effluent was monitored by ultraviolet absorbance at 220 nm.

Quantitative CYP3A Western blot

Western blot procedures were similar to those previously described (Perloff *et al.* 1999, Warrington *et al.* 2000). Microsomal protein was separated by SDS-PAGE in 7.5% polyacrylamide gels. Sample wells were loaded with 1.0 μ g liver or intestinal microsomes. Human cDNA expressed CYP3A4 (Gentest, Woburn, MA, USA) was used to generate calibration standards at concentrations of 0.375, 0.75, 3.0 and 6.0 pmoles/well. Samples were run at 80 V for 2 h in 25 mM Tris buffer/0.2 M glycine/0.1% SDS (sodium dodecyl sulfate) (pH 8.3). Samples were then transferred to Immobilon-P (PVDF membrane) (Millipore, Bedford, MA, USA) for 1 h at 100 V in 25 mM Tris buffer (pH 7.8)/20% methanol. Blots were blocked with 3% dry milk for 1 h, incubated with polyclonal rabbit anti-CYP3A1 (1:3000) for 2 h and then reblocked for 30 min. Blots were then probed with the secondary antibody, donkey anti-rabbit Ig-horse radish peroxidase (1:10 000) for 1 h. Super Signal Cl-HRP Substrate System (Pierce) was used to activate the HRP signal. Blots were then exposed to a cold camera imaging system (Kodak Image Station 440, Kodak Imaging, New Haven, CT, USA). Quantification of protein was completed via computer image analysis with Kodak image software. A standard curve of sum pixel density versus pmoles CYP3A4 was created and fit to the equation: $y = mx + b$.

Qualitative CYP2C Western blot

Sample wells were loaded with 1.0 or 10.0 μ g liver or intestinal microsomes. Blots were blocked with 3% dry milk for 1 h, incubated with polyclonal goat anti-CYP2C11 (1:500) for 2 h and then reblocked for 30 min. Blots were then probed with the secondary antibody, sheep anti-goat Ig-horse radish peroxidase (1:10 000) for 1 h. All other CYP2C Western blot procedures were identical to CYP3A Western blot procedures described above.

Data analysis

Kinetic parameters for MDZ biotransformation, without addition of inhibitors, were determined by non-linear least-squares regression analysis of untransformed data. In liver microsomes, 4-OH-MDZ formation data were fit to a substrate activation (Hill) model (1) and α -OH-MDZ formation data were fit to a model incorporating Michaelis–Menten kinetics with uncompetitive substrate inhibition (2) (Segel 1975, Perloff *et al.* 1999, Venkatakrishnan *et al.* 2001). In intestinal microsomes, both 4-OH-MDZ and α -OH-MDZ formation data were fit to a two enzyme model with Michaelis–Menten kinetics for high-affinity component and linear approximation of low-affinity component (3) (Segel 1975, Venkatakrishnan *et al.* 2001):

$$V = \frac{(V_{\max} \cdot S^a)}{(K_m^a + S^a)} \quad (1)$$

$$V = \frac{(V_{\max} \cdot S)}{(K_m + S + (S^2/K_s))} \quad (2)$$

$$V = \frac{(V_{\max} \cdot S)}{(K_m + S)} + zS, \quad (3)$$

where V is the reaction velocity (nmoles mg⁻¹ protein min⁻¹), $V_{\max}$ is the maximum reaction velocity, S is substrate concentration (μ M), K_m is the Michaelis–Menten constant (μ M) (the substrate concentration at which $V = 0.5V_{\max}$), 'a' is the Hill coefficient, K_s is the uncompetitive substrate inhibition constant (μ M), and z is the linear approximation of low affinity enzyme intrinsic clearance.

IC₅₀'s (concentration corresponding to a 50% reduction in metabolite formation velocity) for KTZ were determined by non-linear regression of relative reaction velocities at a single substrate concentration in the presence of varying inhibitor concentrations (von Moltke *et al.* 1998). These functions (equations 4 and 5) are based on the Hill equation (Segel 1975), and can be used for an empirical determination of IC₅₀ (Venkatakrishnan *et al.* 1998):

$$f = 100 \left[1 - \frac{I_{\max} \cdot I^b}{I^b + IC^{b_50}} \right] \quad (4)$$

$$IC_{50} = \frac{IC}{(2I_{\max} - 1)^{1/b}}, \quad (5)$$

where f is per cent reaction velocity of uninhibited control, I is the inhibitor concentration (μM), I_{max} is the maximum inhibition (%), IC is the inhibitor concentration that corresponds to half of I_{max} , IC_{50} is the inhibitor concentration that corresponds to 50% of the uninhibited control ($IC_{50} = IC$, when $I_{\text{max}} = 100\%$) and b is the exponent in IC_{50} determination.

Results

Midazolam biotransformation in mouse liver and intestinal microsomes

Kinetic parameters for MDZ hydroxylation in the pooled mouse liver microsomes were $V_{\text{max}} = 0.76$ and $0.58 \text{ nmoles min}^{-1} \text{ mg}^{-1}$ protein with $K_m = 0.72$ and $17.7 \mu\text{M}$ for α -OH- and 4-OH-MDZ formation, respectively (figure 1A). Apparent substrate inhibition ($K_s = 911 \mu\text{M}$) was evident in the kinetics of MDZ α -hydroxylation. Kinetic parameters for MDZ hydroxylation in the pooled mouse intestinal microsomes were fit to a two-enzyme model. The low-affinity enzyme was approximated by a linear relation to substrate concentration, and parameters for the high-affinity enzyme were $V_{\text{max}} = 0.074$ and $0.075 \text{ nmoles min}^{-1} \text{ mg}^{-1}$ protein and $K_m = 0.66$ and $13.0 \mu\text{M}$ for α -OH- and 4-OH-MDZ formation, respectively (figure 1C). Eadie-Hofstee graphic analysis (Segel 1975) of α -OH-MDZ formation in mouse liver microsomes demonstrated a characteristic sickle shape associated with substrate inhibition, whereas 4-OH-MDZ formation was

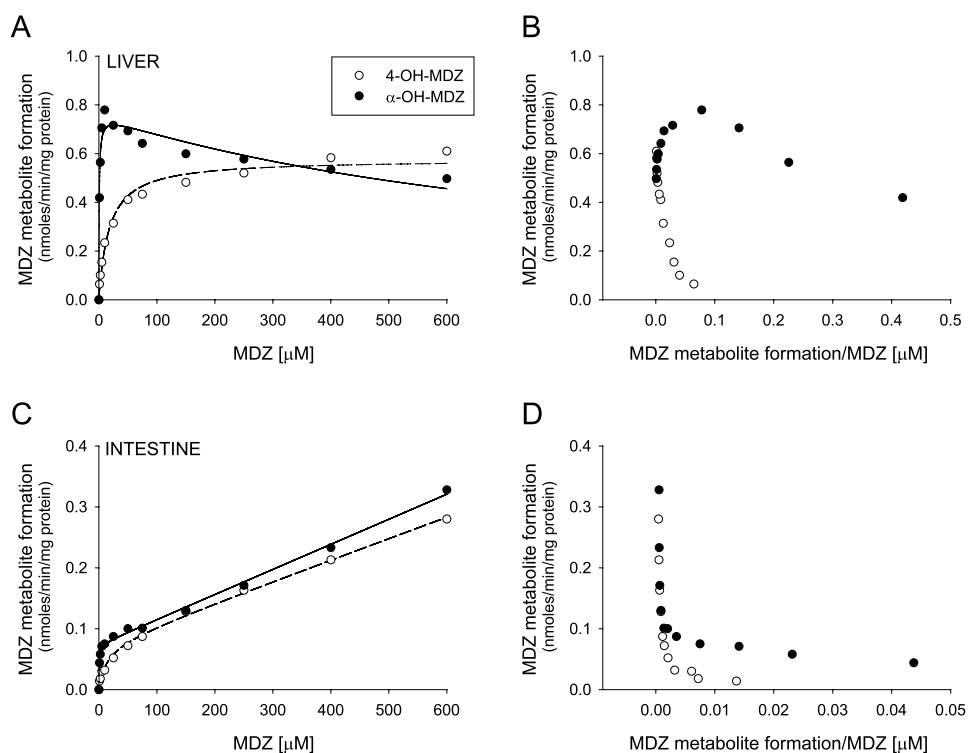


Figure 1. Rates of formation of α -OH-MDZ and 4-OH-MDZ in relation to MDZ concentration by mouse liver (A) and intestinal (C) pooled microsomal preparations. Units for V_{max} and K_m are $\text{nmoles min}^{-1} \text{ mg}^{-1}$ protein and μM , respectively. (B, D) are Eadie-Hofstee transformations of the enzyme kinetics in (A, C). Lines represent functions determined by non-linear least-squares regression analysis. Data points are derived from three determinations.

linear and considered to follow a Michaelis–Menten model (figure 1B). Eadie–Hofstee graphic analysis of both MDZ metabolite’s formation in mouse intestinal microsomes demonstrated a characteristic ‘L’ shape associated with a two enzyme model (figure 1D).

Inhibition of midazolam biotransformation with ketoconazole, ritonavir and troleandomycin

KTZ IC₅₀ determinations were performed at 50 μM MDZ, which exceeds the *K_m*’s. In mouse liver microsomes, KTZ potently inhibited 4-OH-MDZ forma-

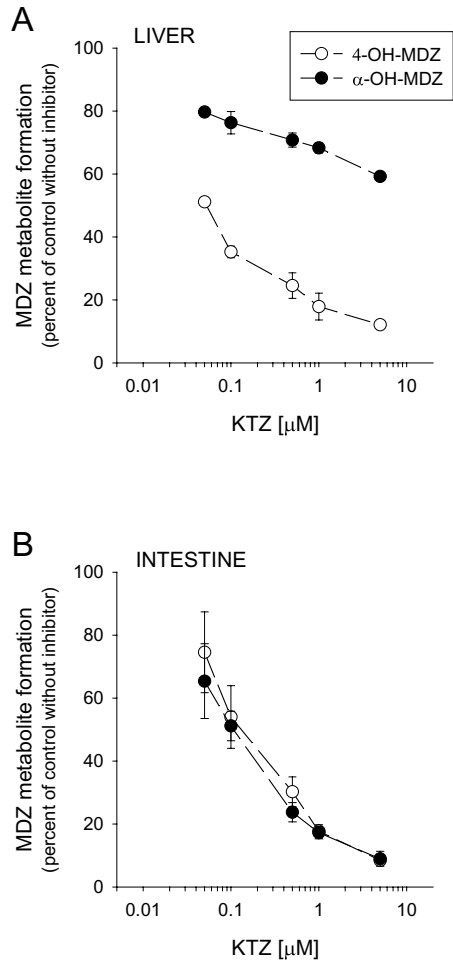


Figure 2. Effect of ketoconazole on midazolam metabolite formation in mouse liver and intestinal microsomes. Rates of formation of α-OH-MDZ and 4-OH-MDZ (50 μM MDZ) in the presence of increasing concentrations of KTZ are expressed as a per cent relative to the rate of formation with no KTZ present in mouse liver (A) and intestinal microsomes (B). Each point represents the mean (± SD, *n* = 3). In liver microsomes, KTZ inhibited 4-OH-MDZ formation, IC₅₀ = 0.041 ± 0.003 μM, but did not produce 50% inhibition of α-OH-MDZ formation (A). KTZ inhibited both 4-OH- and α-OH-MDZ formation in mouse intestinal microsomes, with IC₅₀’s of 0.0955 ± 0.014 and 0.126 ± 0.010 μM, respectively (B).

tion, $IC_{50} = 0.041 \pm 0.003 \mu\text{M}$ (mean SD, $n = 3$), but did not produce 50% inhibition of α -OH-MDZ formation, with reaction velocity reduced to only 60% of control values at $5 \mu\text{M}$ KTZ (figure 2A). However, KTZ was a potent inhibitor of both 4-OH- and α -OH-MDZ formation in mouse intestinal microsomes, with $IC_{50} = 0.0955 \pm 0.014$ and $0.126 \pm 0.010 \mu\text{M}$, respectively (figure 2B). RIT ($5 \mu\text{M}$) potently inhibited ($50 \mu\text{M}$ MDZ), 4-OH-MDZ formation in mouse liver and intestinal microsomes, and α -OH-MDZ formation in intestinal microsomes to less than 10% of control values (table 1). In mouse liver microsomes, $5 \mu\text{M}$ RIT reduced α -OH-MDZ formation only to 54.0% of control values. RIT (0.5 and $5 \mu\text{M}$) demonstrated a very similar inhibition profile to that of KTZ in mouse liver and intestinal microsomes (table 1). TAO demonstrated a concentration-dependent reduction ($50 \mu\text{M}$ MDZ) in 4-OH- and α -OH-MDZ formation in mouse liver microsomes to 46.4 and 70.0% of control values, respectively, at $500 \mu\text{M}$ TAO. TAO (50 and $500 \mu\text{M}$) did not significantly reduce ($>10\%$) 4-OH- or α -OH-MDZ formation in mouse intestinal microsomes (table 1).

Inhibition of midazolam biotransformation by polyclonal antibody

MDZ metabolism was also investigated by co-incubating polyclonal antibody serum to CYP3A and CYP2C with mouse microsomes (10:1 protein ratios). These specific antibodies have previously been shown to inhibit CYP reactions (Schmider *et al.* 1996), and have been used to verify CYP3A- and CYP2C-mediated metabolism of MDZ in mouse liver microsomes (Perloff *et al.* 2000). In pooled mouse liver microsomes (duplicate assay), serum containing anti-CYP3A1 antibodies reduced 4-OH- and α -OH-MDZ metabolite formation to 20.1 and 50.2% of control values, respectively (table 1). Serum containing anti-CYP2C11 antibodies inhibited liver α -OH-MDZ metabolite formation to 68.2% of

Table 1. Effect of various inhibitors on midazolam metabolite formation in mouse liver and intestinal microsomes. Experiments were undertaken with a fixed concentration of midazolam (MDZ, $50 \mu\text{M}$) using pooled mouse liver microsomes and intestinal microsomes.

Inhibitor	Per cent of control metabolite formation			
	Liver microsomes		Intestinal microsomes	
	4-OH-MDZ	α -OH-MDZ	4-OH-MDZ	α -OH-MDZ
Ketoconazole				
0.5 μM	24.6	70.8	30.3	23.8
5 μM	12.1	59.2	8.5	9.0
Ritonavir				
0.5 μM	19.6	62.4	21.2	23.1
5 μM	8.0	54.0	6.9	7.2
Troleandomycin				
50 μM	83.1	90.5	115.9	123.7
500 μM	46.4	70.0	131.1	98.4
Anti-CYP3A 1:10	20.1	50.2	89.7	114.3
Anti-CYP2C 1:10	93.8	68.2	119.2	116.8

Troleandomycin (TAO), anti-CYP3A and anti-CYP2C antibodies were pre-incubated with microsomes (and cofactor for TAO) before the addition of MDZ; all other inhibitors were co-incubated with MDZ.

Each value represents the average of duplicate incubations. In no case did the duplicates differ by greater than 10%.

control values, but did not inhibit 4-OH-MDZ formation in mouse liver microsomes (table 1). In pooled mouse intestinal microsomes (duplicate assay), serum containing anti-CYP3A1 or anti-CYP2C11 antibodies did not significantly reduce (>10%) 4-OH- or α -OH-MDZ metabolite formation.

CYP3A and CYP2C immunoreactive expression in mouse liver and intestinal microsomes

Western blotting detected two CYP3A immunoreactive bands in both mouse liver and intestine microsomes with a molecular weight of approximately 57–58 kD. Bands were quantified by computer image analysis and the mean quantity of immunoreactive CYP3A was 2.24 ± 0.27 (mean SD, $n = 3$) pmol mg⁻¹ protein in liver microsomes and 0.64 ± 0.08 pmol mg⁻¹ protein in mouse intestinal microsomes (figure 3). Western blotting detected a single CYP2C immunoreactive band in mouse liver microsomes (figure 4). Immunoreactive CYP2C was undetectable in mouse intestinal microsomes when 1 μ g protein was loaded, while liver microsomes demonstrated an intense immunoreactive band with an approximate molecular weight of 53 kD. When loading 10 μ g of sample, a weak immunoreactive CYP2C band was detectable in mouse intestinal microsomes. Taken together, results indicate that CYP2C expression appears to be two orders of magnitude lower in the mouse intestine compared with the liver.

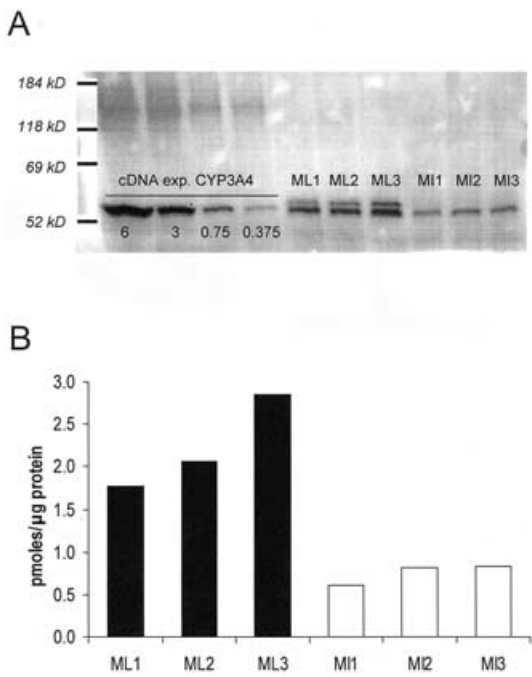


Figure 3. Immunoquantification of CYP3A in mouse liver and intestinal microsomes. Each lane (A) represents a sample from an individual mouse (pair matched, 1 μ g protein loaded). cDNA expressed CYP3A4 (6, 3, 0.75 and 0.375 pmoles) was used as a standard curve. The bars (B) represent quantities of immunoreactive hepatic (ML) and intestinal (MI) CYP3A in individual samples as detected and quantified by Western blotting.

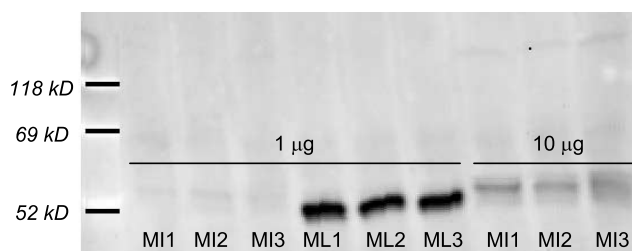


Figure 4. Immunoblotting CYP2C in mouse liver and intestinal microsomes. Each lane represents a sample from an individual mouse (pair matched, 1 or 10 µg protein loaded). Bands represent immunoreactive hepatic (ML) and intestinal (MI) CYP2C in individual samples as detected by Western blotting.

Discussion

In humans, MDZ hydroxylation is generally considered to be a pure probe reaction for CYP3A-mediated metabolism (Kronbach *et al.* 1989, Thummel *et al.* 1994, von Moltke *et al.* 1996, Wandel *et al.* 1998, Tsunoda *et al.* 1999, Venkatakrishnan *et al.* 2001). In the present study, MDZ metabolite formation in mouse intestinal microsomes was significant compared with liver microsomes. Because MDZ metabolite formation in intestinal microsomes followed a two-enzyme kinetics model (figure 1D), comparison of liver and intestinal $V_{\max}$, or intrinsic clearance, was not possible. However, since MDZ intestinal microsomal metabolism could be virtually eliminated with KTZ (figure 2B), it appears that CYP3A forms mediate MDZ intestinal metabolism, perhaps CYP3A11 and CYP3A13 (Yanagimoto *et al.* 1994). Accordingly, comparison of CYP3A-mediated 4-OH-MDZ formation in liver (Perloff *et al.* 2000) and intestinal microsomes between 100 and 200 µM MDZ (plateau region in 4-OH-MDZ liver microsomal kinetics) demonstrated significant intestinal activity, approximately 20–30% that of liver microsomes (figure 1). This CYP3A activity estimate corresponded very well with quantified CYP3A immunoreactive protein in liver and intestinal microsomes, where intestinal microsomal CYP3A protein was approximately 25% of that seen in liver microsomes (figure 3). These results suggest that CYP3A is highly expressed in mouse intestine and mediates the majority of MDZ intestinal metabolism. Although the specific CYP enzymes expressed (potentially various CYP3A forms) were not determined, activity correlated strongly with CYP3A based on Western blots and inhibition studies. Both α -OH and 4-OH-MDZ formation kinetics in intestinal microsomes followed a two-enzyme model, and while the high-affinity component appears to be mediated by CYP3A, the low-affinity component could be mediated by non-specific activity of various microsomal enzymes. Previous studies reported that MDZ hydroxylation in intestinal microsomes was approximately 10% of that in liver microsomes at 100 µM MDZ and qualitative Western blotting demonstrated CYP3A immunoreactive protein signal in intestinal microsomes (Emoto *et al.* 2000a). With quantification of immunoreactive protein, enzyme kinetics and inhibition profiles, the current study has demonstrated that the mouse intestine is a significant contributor of CYP3A-mediated metabolism, in addition to that of the liver.

In humans, the $V_{\max}$ of α -OH-MDZ formation kinetics decreases distally from the duodenum to the ileum, but median intestinal intrinsic clearance in intestinal microsomes is similar to hepatic microsomes (157 and 200 µmoles min⁻¹ mg⁻¹

protein, respectively) (Paine *et al.* 1997). Clinical studies have demonstrated a significant intestinal component to MDZ phase I metabolism (Tsunoda *et al.* 1999). Even with complete oral absorption of MDZ, bioavailability is only approximately 50% of the predicted values based on the hepatic contribution to presystemic extraction. Pharmacokinetic estimates indicate that the MDZ extraction ratio of liver and intestinal components are roughly equal (Thummel *et al.* 1996, Tsunoda *et al.* 1999).

CYP3A represents 70% of CYP expression in the human small intestine (McKinnon *et al.* 1995) and is the only CYP with appreciable levels (compared with CYP1A, 2C, 2D or 2E) in mouse intestine (Emoto *et al.* 2000a). Whereas CYP2C40 has been identified as the major CYP2C form in mouse intestine (Tsao *et al.* 2000), immunoblotting with anti-rat CYP2C in our (figure 4) and previous studies (Emoto *et al.* 2000a) produced little to no immunoreactive protein signal. Accordingly, with minimal CYP2C protein expression in the intestine, MDZ metabolism to α -OH- and 4-OH-MDZ appears to be totally mediated by CYP3A enzymes in mouse intestinal microsomes. Both 4-OH-MDZ and α -OH-MDZ formation was reduced to less than 20% in the presence of 1 μ M KTZ in mouse intestinal microsomes, very similar to 4-OH-MDZ inhibition with KTZ in mouse liver microsome studies (figure 2). As in previous studies (Perloff *et al.* 2000, Warrington *et al.* 2000), α -OH-MDZ formation in mouse liver microsomes was only partially attributable to CYP3A, being inhibited less than 40% at 5 μ M KTZ (figure 2). Inhibition of MDZ metabolite formation with RIT was very similar to that seen with KTZ (table 1).

With respect to clinically relevant CYP3A inhibition, the antifungal KTZ and the protease inhibitor RIT represent two of the most potent CYP3A inhibitors (Thummel and Wilkinson 1998, Venkatakrishnan *et al.* 2001). RIT is often co-administered to increase the bioavailability of other coadministered HIV medications (Hsu *et al.* 1998). Troleandomycin (TAO) is considered a relatively specific mechanism-based CYP3A inhibitor in humans at concentrations up to 1000 μ M (Newton *et al.* 1995, Ono *et al.* 1996) and has been used as a CYP3A-specific inhibitor in mice at similar concentrations (Perloff *et al.* 2000, Honda *et al.* 2001). Whereas KTZ and RIT fully inhibit CYP3A-mediated formation of 4-OH-MDZ in liver microsomes and both metabolites in mouse intestinal microsomes (table 1), moderate inhibitors (with high K_i , albeit specific for CYP3A) TAO and anti-CYP3A antibody failed to significantly reduce MDZ metabolism in mouse intestinal microsomes (table 1). Similar results were seen with moderate CYP3A inhibitors, α -naphthoflavone (10 μ M) and terfenadine (100 μ M), inhibiting CYP3A-mediated metabolism 50% in mouse liver microsomes, but failing to cause inhibition in mouse intestinal microsomes. However KTZ eliminated CYP3A-mediated testosterone metabolism in both (Emoto *et al.* 2000a, 2001). KTZ has also been shown to eliminate CYP3A-mediated metabolism in both human liver and intestinal microsomes (Gibbs *et al.* 1999). Although KTZ substantially inhibits MDZ metabolism in mouse intestinal microsomes, KTZ IC_{50} 's for α - and 4-OH-MDZ formation in intestinal microsomes was two-fold higher than the IC_{50} for 4-OH-MDZ (CYP3A dependent) formation in liver microsomes, demonstrating some reduction in potency. Taken collectively, MDZ metabolite formation kinetics, immunoblotting, and inhibition studies in mouse intestinal and liver microsomes suggest that MDZ metabolite formation is CYP3A-dependent in the mouse intestine. Reduced KTZ inhibition potency on

MDZ metabolism and lack of inhibition with TAO and anti-CYP3A antibody appear to be the result of a confounding component within the intestinal microsomes, perhaps relating to non-specific binding.

The current in vitro study has demonstrated that MDZ metabolism in mice has a significant intestinal component that appears to be solely CYP3A mediated. In mouse liver microsomes, MDZ has a CYP3A and CYP2C component, but with absence of significant CYP2C expression in the mouse intestine, CYP3A alone appears to mediate MDZ biotransformation. Based on in vitro assays, CYP3A expression and CYP3A-mediated MDZ metabolism in mouse intestinal microsomes was approximately 30% of that in hepatic microsomes. Additionally, although MDZ is not a substrate for P-glycoprotein-mediated drug transport (Kim *et al.* 1999), drug transport 'recycling' (absorbed drugs being pumped back into the intestinal lumen) may enhance CYP3A-mediated intestinal metabolism in vivo for other CYP3A/P-glycoprotein substrates (Watkins 1997, Benet and Cummins 2001). Differential metabolism of MDZ in mouse liver and intestinal microsomes suggests that in vitro-in vivo scaling models of first-pass metabolism, solely based on hepatic metabolism, may be limited in their accuracy.

Acknowledgements

The work was supported in part by Grants MH-58435, DA-13209, DA-13834, DK/AI-58496, DA-05258 and RR-00054 from the Department of Health and Human Service

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Uptake of rosuvastatin by isolated rat hepatocytes: comparison with pravastatin

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Received 21 August 2002

1. The liver is the target organ for the lipid-regulating effect of rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, and liver-selective uptake of this drug is therefore a desirable property. The uptake kinetics of rosuvastatin were investigated and compared with those of pravastatin using isolated rat hepatocytes.

2. Uptake for both drugs involved both active transport and passive diffusion processes. The Michaelis constant (K_m) of uptake rate for rosuvastatin (9.17 μ M) was approximately half that for pravastatin (16.5 μ M). However, the maximum uptake rate (V_{max}) and carrier-mediated uptake clearance (V_{max}/K_m) of rosuvastatin were significantly ($p < 0.01$) greater than those of pravastatin, and a larger contribution of carrier-mediated uptake clearance to total uptake clearance was shown for rosuvastatin (contribution ratio 0.903 versus pravastatin 0.654).

3. Sodium and chloride ions did not play a significant role in the uptake of rosuvastatin and pravastatin, but the uptake of both drugs was inhibited both by depletion of cellular ATP and by organic anions such as bromosulfophthalein.

4. Rosuvastatin competitively inhibited the uptake of pravastatin, with an inhibition constant (K_i) (2.75 μ M) relatively similar to its K_m .

5. The results suggest that an organic anion transport protein is the main mediator of the hepatic uptake of rosuvastatin and pravastatin, which occurs in an ATP-dependent manner. Our results indicated that rosuvastatin was taken up by the hepatocytes via the same transport systems as pravastatin, but with a greater affinity and efficiency than pravastatin.

Introduction

Rosuvastatin (Crestor[®]) is a new and highly efficacious 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor that has completed Phase III clinical development at AstraZeneca for the treatment of patients with dyslipidaemia. Rosuvastatin, calcium bis[(+)-(3*R*,5*S*,6*E*)-7-[4-(*p*-fluorophenyl)-6-isopropyl-2-(*N*-methylmethane-sulfonamido)-5-pyrimidinyl]-3,5-dihydroxy-6-heptenoate] was originally synthesized at Shionogi and Co., Ltd. (Watanabe *et al.* 1997). In clinical trials, rosuvastatin (1–80 mg) produced significant dose-dependent reductions in low-density lipoprotein cholesterol (up to 65%), total cholesterol and apolipoprotein B, and the drug was well tolerated (Olsson *et al.* 2001). Reductions in triglycerides and increases in high-density lipoprotein cholesterol were consistently observed with rosuvastatin.

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Liver-selective uptake of this drug is a desirable property because the liver is the target organ for the lipid-regulating effect of rosuvastatin. In studies involving rats, rosuvastatin was distributed selectively into the liver (Nezasa *et al.* 2002a). Furthermore, the liver-selective uptake of rosuvastatin was more efficient than that of pravastatin (Nezasa *et al.* 2002b). The aim of the present study was to investigate and compare with pravastatin the uptake kinetics of rosuvastatin using isolated rat hepatocytes.

Materials and methods

Materials

Rosuvastatin (content on the anhydrous basis 96.8%), ^{14}C -rosuvastatin ($31.0\mu\text{Ci mg}^{-1}$; radiochemical purity 99.4%), pravastatin (content on the anhydrous basis 99.4%) and ^{14}C -pravastatin ($31.5\mu\text{Ci mg}^{-1}$; radiochemical purity 99.0%) were synthesized at Shionogi and Co., Ltd. (Osaka, Japan) (figure 1).

^{14}C -dextran (0.71mCi g^{-1}) and ^3H -water (1.0mCi ml^{-1}) were purchased from New England Nuclear (Boston, MA, USA). Heparin sodium was purchased from Shimizu Pharmaceutical Co., Ltd. (Shizuoka, Japan). Trypan blue was purchased from ICN Biomedica, Inc. (Aurora, OH, USA). *O,O'*-bis (2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA), collagenase, and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bovine serum albumin fraction V (BSA), mineral oil, rotenone, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and bromosulphophthalein (BSP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Silicone oil and carbonyl cyanide-*p*-(trifluoromethoxy)-phenylhydrazone (FCCP) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Taurocholate sodium salt (TCA) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents were commercially available guaranteed reagent grade.

Animals

Male rats (aged 10–12 weeks, Jcl:Sprague–Dawley strain) served as liver donors and were purchased from CLEA Japan, Inc. (Osaka, Japan).

Isolation of hepatocytes

A modified version of the method reported by Moldeus *et al.* (1978) was used to isolate rat hepatocytes. Rats were administered heparin (200 units/200 μl) intravenously under diethyl ether anaesthesia. The liver was perfused via a portal vein cannula with Hanks solution (NaCl 140 mM, KCl 5.4 mM, KH_2PO_4 0.44 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.81 mM, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.34 mM, NaHCO_3 25 mM and HEPES 13 mM, pH 7.4) containing EGTA 0.5 mM and 1% BSA (Hanks-I buffer) at 37°C (30 ml min^{-1} for 5 min). The liver was further perfused with Hanks solution containing 0.05% collagenase and $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 4 mM (Hanks-II buffer) at 37°C (30 ml min^{-1} for 3 min), and the liver was removed from the animal.

The liver surface was washed twice with Krebs–Henseleit buffer (KHB) containing HEPES 12.5 mM (pH 7.4) and 1% BSA (KHB-I) at 37°C . The liver was placed in a beaker containing KHB-I (about 100 ml at 37°C) and shaken to disperse the hepatocytes. The suspension was then filtered

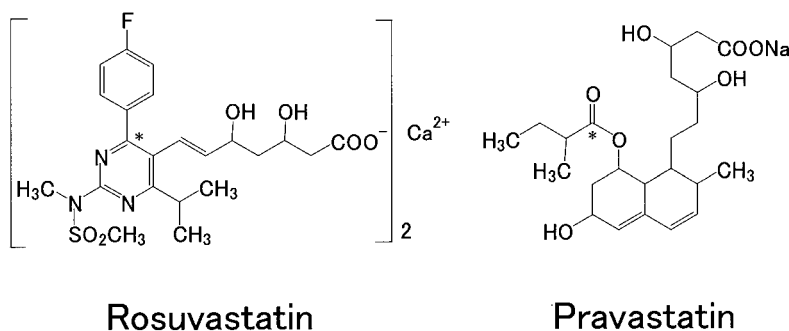


Figure 1. Chemical structures of ^{14}C -rosuvastatin and ^{14}C -pravastatin. *Labelled position.

through gauze (Type I, four sheets) and the filtrate was centrifuged (70g) for 3 min at 4 °C. The supernatant fraction was discarded and the precipitated hepatocytes were washed twice with KHB-I at 4 °C by centrifugation.

To prepare the hepatocyte suspension, the cell pellet was added to a 4-fold volume of KHB containing HEPES 12.5 mM (pH 7.4) and glucose 10 mM (KHB-II) at 4 °C. A 50- μ l aliquot of the hepatocyte suspension was added to 950 μ l 0.4% trypan blue solution. The number of hepatocytes was then counted using a Bürker–Türk cell-counting plate in order to calculate cell viability (subsequently confirmed to be >90%).

Uptake experiments

The hepatocyte suspension was diluted with KHB-II to achieve an approximate concentration of 2 mg protein ml⁻¹. An 800- μ l aliquot of the diluted hepatocyte suspension was placed in a glass test tube and pre-incubated at 37 °C for 5 min. Following pre-incubation, ¹⁴C-rosuvastatin or ¹⁴C-pravastatin were added to the test tube, which was then incubated for a scheduled period.

Following incubation, 200 μ l of the mixture were collected and layered on the top layer of a 400- μ l Eppendorf tube containing a mixture of silicone and mineral oil (4:1, density = 1.008) 100 μ l and 3N KOH 50 μ l. The Eppendorf tube was then centrifuged in a tabletop centrifuge for 10 s. As a result of this procedure, the hepatocytes were centrifuged down through the oil layer to the alkaline layer and the uptake process was stopped. The tubes were allowed to stand overnight to solubilize the hepatocytes in the alkaline layer.

The top layer of the Eppendorf tube was transferred into a counting vial as the medium fraction. The tube at the position of the oil layer was cut off from the alkaline layer by a razor, and the alkaline layer was transferred into another counting vial as the cell fraction. Pico-Fluor 40 (Packard Instrument Co., Meriden, CT, USA) 10 ml was added to each vial, and the radioactivity measured by a liquid scintillation counter (Tri-Carb 2200-CA, Packard).

Correction for adhering fluid and intracellular volume measurement

On centrifugation, the hepatocytes, together with a small amount of adhering medium, passed through the oil layer into the alkaline layer. To calculate the amount of adhering fluid, the hepatocytes were incubated with impermeable ¹⁴C-dextran for 1 min and the radioactivity present in the cell fraction was measured. Values of approximately 1.6–3.5 μ l mg⁻¹ protein were obtained. Using the calculated adhering fluid values, the amount of substrate taken up by the cell fraction was corrected. To evaluate the intracellular volume, hepatocytes were incubated with ³H-water for 1 min and the radioactivity present in the cell fraction was measured. The intracellular volume was calculated by subtracting the amount of adhering fluid from that of ³H-water in the cell fraction, because ³H-water was distributed into the cell and adhering fluid of the cell surface. The cell volume was about 2.7 μ l mg⁻¹ protein and used in the calculation of the time-course of the cell-to-medium (C/M) ratios.

Protein determination

The protein concentration was determined according to the method of Bradford (1976); a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used.

Data analysis

Calculation of kinetic parameters. The kinetic parameters for *in-vitro* hepatic uptake of rosuvastatin were calculated according to the following equation:

$$V_0 = V_{\max} \cdot S / (K_m + S) + P_{\text{dif}} \cdot S, \quad (1)$$

where V_0 is the initial uptake rate (nmol min⁻¹ mg⁻¹ protein), $V_{\max}$ is the maximum uptake rate (nmol min⁻¹ mg⁻¹ protein), S is the substrate concentration (μ M), K_m is the Michaelis constant of uptake rate (μ M) and P_{dif} is the uptake clearance via passive diffusion (non-specific diffusion) (μ l min⁻¹ mg⁻¹ protein). The observed data were fitted (1/ Y^2 weighting) to equation (1) by means of a non-linear least-squares method (MULTI) (Yamaoka *et al.* 1981) and the kinetic parameters were calculated.

Statistical analysis

Statistical significance was examined using a Student's *t*-test or Welch's test for paired values, and Dunnett's test for multiple values.

Calculation of the inhibition constant (K_i). The K_i of rosuvastatin for inhibiting the initial uptake of pravastatin was calculated using WinNonlin Professional Version 3.3 (Pharsight Corporation, Mountain View, CA, USA) according to the following equation:

$$V_0 = V_{\max} \cdot S / [K_m \cdot (1 + i/K_i) + S] + P_{\text{dif}} \cdot S, \quad (2)$$

where i is the rosuvastatin concentration (μM) and K_i is the inhibition constant (μM). The data were fitted using the Gauss–Newton (Levenberg and Hartley) method.

Calculation of in-vivo hepatic uptake. The *in-vivo* hepatic uptake clearance of rosuvastatin was calculated using the kinetic parameters obtained in the present study and the following models (Yamazaki *et al.* 1993):

$$\text{Model 1 (well-stirred model): } CL_{\text{uptake}} = Qh_B \cdot f_P \cdot PS_{\text{influx}} / (Qh_B + f_P \cdot PS_{\text{influx}} / R_B)$$

$$\text{Model 2 (parallel-tube model): } CL_{\text{uptake}} = Qh_B \cdot R_B \cdot [1 - \exp(-f_P \cdot PS_{\text{influx}} / Qh_B / R_B)],$$

where CL_{uptake} is the hepatic uptake clearance ($\text{ml min}^{-1} \text{g}^{-1}$ liver), Qh_B is the hepatic blood flow ($\text{ml min}^{-1} \text{g}^{-1}$ liver), f_P is the unbound-protein fraction in the plasma, PS_{influx} is the membrane permeability clearance (PS denotes the permeability surface area product) ($\text{ml min}^{-1} \text{g}^{-1}$ liver) and R_B is the ratio of the blood concentration to the plasma concentration.

Results

Time-course of uptake by isolated rat hepatocytes

The C/M ratios (i.e. the value obtained by dividing the concentration in the cell fraction with the concentration in the medium fraction) of rosuvastatin and pravastatin ($50 \mu\text{M}$) uptake by isolated rat hepatocytes are shown in figure 2. The C/M ratios of rosuvastatin and pravastatin increased substantially with incubation time for up to 60 s after starting the incubation; steady-state was reached approximately 300 s after starting the incubation. The C/M ratios at steady-state were 14.9 for rosuvastatin and 10.9 for pravastatin, which demonstrated that both substrates were taken up into hepatocytes via active transport mechanisms.

Both rosuvastatin and pravastatin showed a linear increase of the cellular uptake in proportion to time for up to 20 s after starting the incubation. By 60 s after starting the incubation, over 90% was unchanged for both substrates. Thus, efflux of metabolites from the cells was negligible and evaluation of the initial uptake rate at the 20-s data point was valid. Based on this finding, the incubation time was set at 20 s in subsequent experiments for calculation of the initial uptake rate.

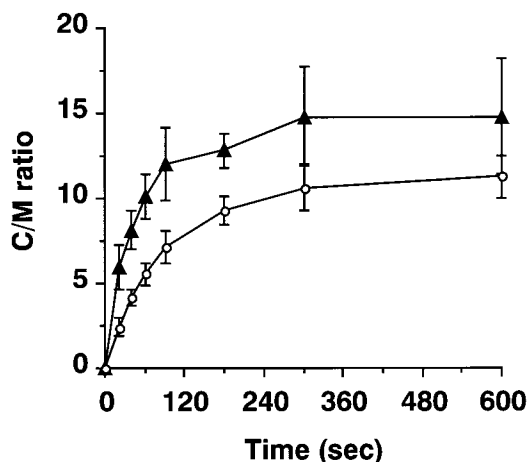


Figure 2. Time-course of the cell-to-medium (C/M) ratios of rosuvastatin $50 \mu\text{M}$ (closed triangles) and pravastatin $50 \mu\text{M}$ (open circles) uptake by isolated rat hepatocytes. Each symbol represents the mean $\pm$ SD of three to four experiments.

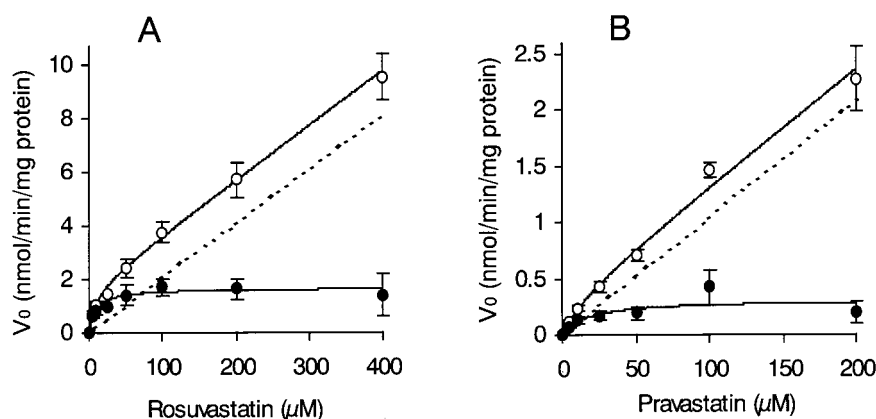


Figure 3. Effect of substrate concentration on the initial uptake rate (V_0) of rosuvastatin (A) and pravastatin (B) by isolated rat hepatocytes. Open circles (total uptake) indicate observed data. Dashed lines (non-specific diffusion, P_{dif}) were calculated by fitting the observed data to equation (1) using MULTI. Closed circles (carrier-mediated uptake) were calculated by subtracting the non-specific diffusion from the total uptake. Solid lines were drawn by fitting the kinetic parameters to equation (1) (see the Materials and methods). Each symbol represents the mean $\pm$ SD of three to five experiments.

Concentration dependency of initial uptake rate by isolated rat hepatocytes

Figure 3 shows the effect of substrate concentration on the initial uptake rate of rosuvastatin and pravastatin by isolated rat hepatocytes. The initial uptake rate of both drugs increased in a concentration-dependent manner.

The kinetic parameters for the uptake of rosuvastatin and pravastatin are shown in table 1; these were calculated by fitting the observed data (total uptake) to equation (1) (see above) using MULTI. The K_m of rosuvastatin was approximately half that of pravastatin, but the V_{max} , V_{max}/K_m (carrier-mediated uptake clearance in the linear range) and P_{dif} of rosuvastatin were significantly ($p < 0.01$) greater than those of pravastatin. The $V_{\text{max}}/K_m + P_{\text{dif}}$ (total uptake clearance in the linear range) was about sevenfold greater for rosuvastatin than for pravastatin. The ratio of the carrier-mediated uptake clearance to the total uptake clearance (0.903 for rosuvastatin and 0.654 for pravastatin) demonstrated a greater contribution of carrier-mediated uptake for rosuvastatin than for pravastatin.

Table 1. Comparison of kinetic parameters for rosuvastatin and pravastatin uptake.

Kinetic parameter	Rosuvastatin	Pravastatin
K_m (μM)	9.17 ± 3.27	16.5 ± 9.6
V_{max} ($\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$)	$1.67 \pm 0.48^{**}$	0.31 ± 0.13
V_{max}/K_m ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$)	$190 \pm 42^{***}$	19.7 ± 3.6
P_{dif} ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$)	$20.4 \pm 2.1^{***}$	10.4 ± 1.8
$V_{\text{max}}/K_m + P_{\text{dif}}$ ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$)	210	30.1
$(V_{\text{max}}/K_m)/(V_{\text{max}}/K_m + P_{\text{dif}})$	0.903	0.654

K_m is the Michaelis constant of uptake rate; V_{max} is the maximum uptake rate; V_{max}/K_m is the carrier-mediated uptake clearance in the linear range; P_{dif} is the uptake clearance via passive diffusion (non-specific diffusion); $V_{\text{max}}/K_m + P_{\text{dif}}$ is the total uptake clearance in the linear range; and $(V_{\text{max}}/K_m)/(V_{\text{max}}/K_m + P_{\text{dif}})$ is the ratio of the carrier-mediated uptake clearance to the total uptake clearance.

Each value is the mean $\pm$ SD of 3–5 experiments.

** $p < 0.01$ versus pravastatin; *** $p < 0.001$ versus pravastatin.

Mechanisms of hepatic uptake by isolated rat hepatocytes

Figure 4 shows the effects of ion substitution and various other substances on the initial uptake rate of rosuvastatin ($10\text{ }\mu\text{M}$) by isolated rat hepatocytes. The initial uptake rate of rosuvastatin was not affected by the substitution of the Na^+ ion with the choline $^+$ ion or the Cl^- ion with the gluconate $^-$ ion. The metabolic inhibitors rotenone and FCCP (30 and $10\text{ }\mu\text{M}$, respectively) significantly ($p < 0.05$) decreased the initial uptake rate of rosuvastatin. The anion exchange inhibitor DIDS (300 and $1000\text{ }\mu\text{M}$), and the bile acid TCA ($100\text{ }\mu\text{M}$), also caused significant ($p < 0.05$) decreases in the initial uptake rate of rosuvastatin; these decreases occurred in a concentration-dependent manner. The organic anion BSP ($3\text{--}30\text{ }\mu\text{M}$) caused non-significant decreases in the initial uptake rate of rosuvastatin.

Figure 5 shows the effects of ion substitution and the various other substances on the initial uptake rate of pravastatin $10\text{ }\mu\text{M}$ by isolated rat hepatocytes. There was no effect of substitution of the Na^+ ion with the choline $^+$ ion or the Cl^- ion with the gluconate $^-$ ion on the initial uptake rate of pravastatin. Rotenone ($30\text{ }\mu\text{M}$) and FCCP ($10\text{ }\mu\text{M}$) significantly ($p < 0.05$) decreased the initial uptake rate of pravastatin. DIDS, TCA and BSP at higher concentrations caused significant ($p < 0.05$) decreases in the initial uptake rate of pravastatin.

The effects of rosuvastatin on the initial uptake rate of pravastatin are shown in figure 6. The uptake rates of pravastatin at concentrations of 5 , 10 and $50\text{ }\mu\text{M}$ were inhibited, in a concentration-dependent manner, by the addition of rosuvastatin ($1\text{--}10\text{ }\mu\text{M}$). The observed data were best fitted using a competitive inhibition model with the lowest AIC compared with other inhibition models (non-competitive, uncompetitive and mixed inhibition). The K_i of rosuvastatin for inhibiting the initial uptake of pravastatin was as $2.75 \pm 1.14\text{ }\mu\text{M}$ (estimate $\pm$ SE).

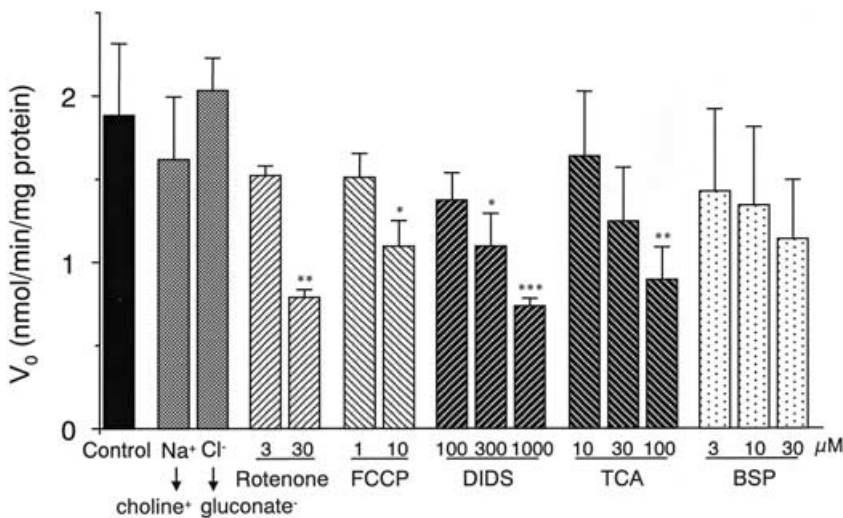


Figure 4. Effect of ion substitution (Na^+ with choline $^+$ and Cl^- with gluconate $^-$), metabolic inhibitors (rotenone and FCCP), an anion exchange inhibitor (DIDS), a bile acid (TCA) and an organic anion (BSP) on the initial uptake rate (V_0) of rosuvastatin $10\text{ }\mu\text{M}$ by isolated rat hepatocytes. Each column represents the mean $\pm$ SD of three experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control.

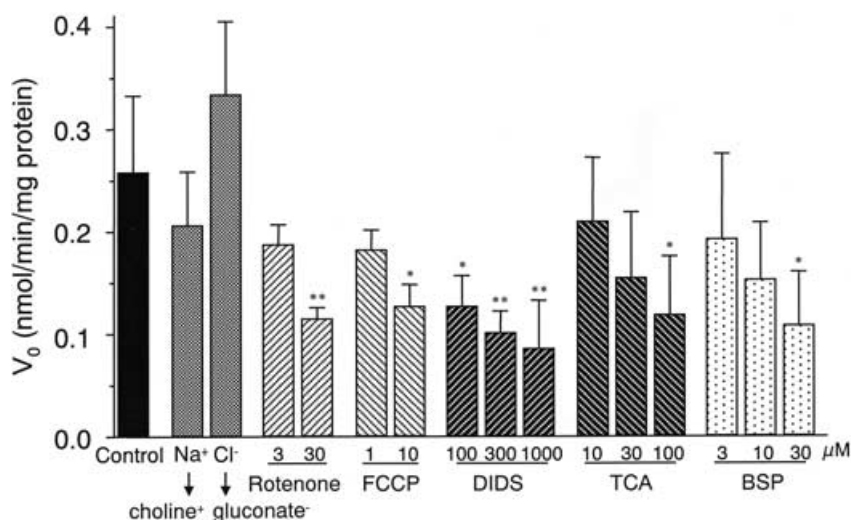


Figure 5. Effect of ion substitution (Na^+ with choline^+ and Cl^- with gluconate^-), metabolic inhibitors (rotenone and FCCP), an anion exchange inhibitor (DIDS), a bile acid (TCA) and an organic anion (BSP) on the initial uptake rate (V_0) of pravastatin $10\text{ }\mu\text{M}$ by isolated rat hepatocytes. Each column represents the mean $\pm$ SD of three experiments. * $p < 0.05$ and ** $p < 0.01$ versus control.

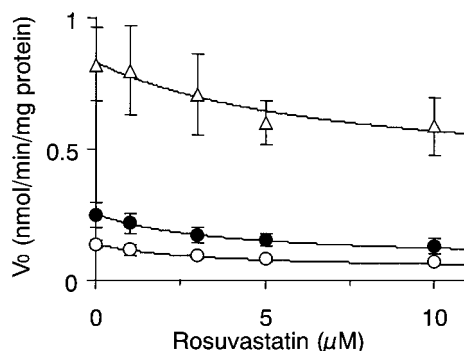


Figure 6. Inhibiting effect of rosuvastatin on pravastatin uptake by isolated rat hepatocytes. The concentrations of pravastatin were $5\text{ }\mu\text{M}$ (open circles), $10\text{ }\mu\text{M}$ (closed circles) and $50\text{ }\mu\text{M}$ (open triangles). Solid lines are the fitting curves estimated using equation (2) (see the Materials and methods). Each symbol represents the mean $\pm$ SD of three experiments.

In-vivo hepatic uptake

The $\text{CL}_{\text{uptake}}$ of rosuvastatin *in vivo* was calculated according to models (1) and (2). The total uptake clearance in the linear range ($V_{\text{max}}/K_m + P_{\text{dif}}$) of rosuvastatin *in vitro* was $210\text{ }\mu\text{l min}^{-1}\text{ mg}^{-1}\text{ protein}$ (table 1); this corresponded to $17.1\text{ ml min}^{-1}\text{ g}^{-1}\text{ liver}$ [$1\text{ mg protein} = 1.6 \times 10^6\text{ cells}$ (Seglen 1976); $1\text{ g liver} = 1.3 \times 10^8\text{ cells}$ (Seglen 1973)]. Since blood flow rate and protein binding were not accounted for in the *in-vitro* uptake experiments, the total uptake clearance observed was regarded as $\text{PS}_{\text{influx}}$. Regarding Q_{hB} as $1.5\text{ ml min}^{-1}\text{ g}^{-1}\text{ liver}$ (Dedrick *et al.* 1973), f_p as 0.039 (data not shown) and R_B as 0.59 (data not shown), the $\text{CL}_{\text{uptake}}$ in models (1) and (2) were 0.38 and $0.47\text{ ml min}^{-1}\text{ g}^{-1}\text{ liver}$,

respectively. The CL_{uptake} of rosuvastatin determined *in vivo* (by integration plot) was $0.89 \text{ ml min}^{-1} \text{ g}^{-1}$ liver (Nezasa *et al.* 2002b).

Discussion

The liver is the target organ for the lipid-regulating effect of HMG-CoA reductase inhibitors, therefore liver-selective uptake of these drugs is a desirable property. A previous study has reported that the liver-selective uptake of rosuvastatin was more efficient than that of pravastatin (Nezasa *et al.* 2002b). In the present study, we investigated and compared with pravastatin the uptake kinetics of rosuvastatin using isolated rat hepatocytes.

According to the time courses of the C/M ratios (figure 2), the uptake of rosuvastatin and pravastatin by the isolated hepatocytes reached steady-state after about 5 min, and a concentrating uptake of both drugs was demonstrated. The C/M ratio of pravastatin at steady-state was lower (10.9) than that reported previously (18.0) (Yamazaki *et al.* 1993); the differences would be attributed to differences in the concentration of pravastatin in the cell suspensions.

The uptake clearance by carrier-mediated active transport in the linear range (V_{max}/K_m) for rosuvastatin was $190 \mu\text{l min}^{-1} \text{ mg}^{-1}$ protein, which was significantly ($p < 0.001$) greater than the value for pravastatin ($19.7 \mu\text{l min}^{-1} \text{ mg}^{-1}$ protein). Furthermore, because of the larger contribution of carrier-mediated uptake clearance to total uptake clearance (contribution ratio for rosuvastatin 0.903 versus 0.654 for pravastatin), rosuvastatin was considered to be taken up by the liver more efficiently than pravastatin.

According to a model presented by Sugiyama and Ito (1998), the predicted plasma concentration of rosuvastatin in the human portal vein will be less than $0.1 \mu\text{M}$. Hepatic uptake of rosuvastatin in such a low concentration range is likely to be mediated mainly by a carrier process, which supports active transport of rosuvastatin to the liver in humans.

The mechanism for the uptake of pravastatin by rat hepatocytes has been investigated in detail, and a Na^+ -independent multispecific anion transporter using ATP as the driving force has been reported to play a role (Komai *et al.* 1992, Yamazaki *et al.* 1993). Recently, the organic anion transporting polypeptide (OATP2) (Kakyo *et al.* 1999, Tokui *et al.* 1999) and the liver-specific organic anion transporter (LST-1) (Abe *et al.* 1999) have been cloned from the livers of rats and humans, respectively, and have been shown to be responsible for the hepatic uptake of pravastatin.

Based on the pravastatin hepatic uptake findings, the present study investigated the mechanism for the uptake of rosuvastatin by rat hepatocytes (figure 4). The initial uptake rates of rosuvastatin in the medium free from Na^+ or Cl^- showed no significant difference (although the absence of Na^+ caused a slight decrease in the initial uptake rate), which indicated that neither the Na^+ - nor the Cl^- -gradient served as the driving force for rosuvastatin uptake. Rotenone and FCCP, agents that deplete cellular ATP, significantly decreased the initial uptake rate of rosuvastatin. The anion exchange inhibitor DIDS and TCA, a substance taken up by an ATP-dependent mechanism, also inhibited the uptake of rosuvastatin. Based on these results, an organic anion transport protein using ATP as the driving force – or an organic anion transport protein using an ion gradient that is

lost by the decrease of cellular ATP more rapidly than is the Na^+ ion gradient – was considered to be involved in the uptake of rosuvastatin.

BSP, an organic anion taken up by hepatocytes in an Na^+ - and ATP-independent manner (Yamazaki *et al.* 1992) tended to inhibit the uptake of rosuvastatin in a concentration-dependent manner, although it was not significant. This indicated that the uptake of rosuvastatin was catalysed partly by an ATP-independent transport system in addition to an ATP-dependent transport system.

Although BSP significantly inhibited the uptake of pravastatin, the results of the same experiments with pravastatin were very similar to those obtained with rosuvastatin (figure 5). This indicated that rosuvastatin was transported (at least partially) by the same transport systems as pravastatin (including OATP2 – although the driving force for OATP2 still remains to be clarified; Tokui *et al.* 1999). A small difference between the effects of BSP on rosuvastatin and pravastatin might be attributed to the difference in the contribution of the ATP-independent transport system to the two compounds. A recent study involving oocytes expressing OATP-C or OATP-A suggested that rosuvastatin is a substrate for OATP-C but not for OATP-A (Brown *et al.* 2001).

The mechanism of uptake experiments used rosuvastatin and pravastatin at a concentration of $10\text{ }\mu\text{M}$. The contribution ratios of the active transport system to the total uptake of rosuvastatin and pravastatin (both at $10\text{ }\mu\text{M}$) were 81.0 and 52.9%, respectively (calculated according to equation (1) using the kinetic parameters presented in table 1). Therefore, passive diffusion was considered to account for part of the total uptake. This may have led to insufficient inhibition by the various inhibitors used in the experiments. It should be noted that at a lower substrate concentration range (i.e. at a range showing a large contribution ratio of active transport), the inhibitory effects of the various inhibitors will be much greater than those observed in the present study.

Rosuvastatin inhibited the uptake of pravastatin in a concentration-dependent manner that was thought to be competitive. The K_i of rosuvastatin for inhibiting the uptake of pravastatin was $2.75\text{ }\mu\text{M}$; this was relatively similar to the K_m for the uptake of rosuvastatin ($9.17\text{ }\mu\text{M}$).

We have already reported that rosuvastatin is distributed to the liver more selectively than pravastatin and simvastatin in an *in-vivo* tissue distribution study (Nezasa *et al.* 2002b), which supports a greater pharmacological effect (McTaggart *et al.* 2001, Paoletti *et al.* 2001) and a less toxicological effect (Olsson *et al.* 2001) of rosuvastatin from the aspect of pharmacokinetics. When *in-vitro* and *in-vivo* data were compared, the $\text{CL}_{\text{uptake}}$ values calculated on the basis of the *in-vitro* results (0.38 and $0.47\text{ ml min}^{-1}\text{ g}^{-1}$ liver) were very similar to the $\text{CL}_{\text{uptake}}$ determined *in vivo* ($0.89\text{ ml min}^{-1}\text{ g}^{-1}$ liver) (Nezasa *et al.* 2002b). Therefore, the *in-vitro* uptake clearance of rosuvastatin found in isolated rat hepatocytes is consistent with the *in-vivo* uptake clearance of rosuvastatin and the high liver-selective distribution of rosuvastatin observed *in vivo* would be ascribed to the high affinity and high capacity of rosuvastatin for hepatic transport systems.

In conclusion, the results indicate that rosuvastatin is taken up by isolated rat hepatocytes via the same transport systems as pravastatin, but with a greater affinity and efficiency than pravastatin.

Acknowledgements

The authors thank T. Nagasaki, Y. Katsuyama and M. Segawa for synthesizing and purifying ^{14}C -rosuvastatin and ^{14}C -pravastatin.

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Glucose inhibition of the induction of CYP2E1 mRNA expression by ethanol in FGC-4 cells

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Received 30 September 2002

1. Rats fed intragastrically with ethanol-containing diets made with low levels of carbohydrates have greater CYP2E1 induction than rats fed similar diets made with high carbohydrate levels.

2. FGC-4 rat hepatoma cells were used to test the hypothesis that carbohydrates could down-regulate ethanol-induced CYP2E1 induction.

3. FGC-4 cells grown in a glucose-free media and treated with 1–100 mM ethanol for 24 h exhibited a dose-dependent increase ($p < 0.05$) in CYP2E1, with maximum mRNA steady-state (3.8-fold) or protein (3.1-fold) levels measured at 30 or 100 mM ethanol, respectively.

4. In cells treated with 30 mM ethanol, a glucose concentration-dependent inhibition ($p < 0.05$) of CYP2E1 mRNA was observed between 2.5 and 10 mM glucose.

5. Induction by 30 mM ethanol of CYP2E1 protein was reduced in cells co-treated with 1 mM or greater glucose concentration and complete inhibition was measured with 5 mM glucose co-treatment.

6. These data demonstrate that under culture conditions of extremely low carbohydrate concentrations: (1) ethanol treatment of FGC-4 cells results in elevated steady-state levels of CYP2E1 mRNA and protein; and (2) glucose inhibits this increase.

7. It is concluded that glucose can negatively regulate CYP2E1 expression and could at least partially explain the greater induction of hepatic CYP2E1 in rats fed low carbohydrate ethanol-containing diets compared with high carbohydrate diets at the same ethanol level.

Introduction

Alcoholism is a major health concern, with millions of people affected by it world-wide. Alcoholic liver disease (ALD) encompasses a continuum of pathophysiological conditions ranging from fatty liver (steatosis) to alcoholic hepatitis and cirrhosis (Rao *et al.* 1992). There is considerable evidence that ethanol/nutritional interactions are important mediators of ALD. While it is clear that some of the pathological consequences of drinking can be attributed to ethanol directly, others appear to be indirect effects mediated via ethanol metabolites, in particular free radicals produced as a consequence of ethanol and free fatty acid metabolism catalysed by the cytochrome P450 enzyme CYP2E1 (Knecht *et al.* 1990, Lieber 1990, Nordmann *et al.* 1992, Ingelman-Sundberg *et al.* 1993, Albano *et al.* 1996, Ronis *et al.* 1996). Ethanol, unsaturated fat and increases in fat/carbohydrate ratio all result in induction of CYP2E1 (Yoo *et al.* 1991). In male rats

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infused ethanol intragastrically as part of a low carbohydrate diet (total enteral nutrition), hepatic CYP2E1 apoprotein levels increased approximately fourfold compared with rats infused an ethanol-containing, high carbohydrate diet (Korourian *et al.* 1999). In addition, focal necrosis was observed only in ethanol-fed rats infused with the low carbohydrate diet compared with rats fed the high carbohydrate diet. It is our hypothesis that: (1) CYP2E1-mediated radical formation is the principal factor involved in the aetiology of ethanol-induced liver injury; and (2) dietary factors play an important role in the inducibility of CYP2E1 expression and the development of ethanol-induced hepatotoxicity.

Ethanol regulation of *CYP2E1* is complex and occurs at numerous levels including transcription, mRNA stabilization and protein stabilization (Badger *et al.* 1993). Studies attempting to identify the gene regulatory regions involved in ethanol-regulated *CYP2E1* gene transcription are hampered by the lack of a good cell culture model that retains *CYP2E1* gene expression. We have identified cell culture conditions that maintain *CYP2E1* mRNA expression in FGC-4 rat hepatoma cells and using this *in vitro* model we have measured ethanol induction of *CYP2E1* mRNA. In the present study, we tested the hypothesis that carbohydrates negatively regulate ethanol-induced CYP2E1 expression. Our results provide *in vitro* support for this hypothesis.

Materials and methods

Chemicals and reagents

All chemicals unless otherwise specified were purchased from Sigma Chemical Co. (St Louis, MO, USA). Radionuclides were purchased from DuPont NEN (Boston, MA, USA). A rabbit polyclonal antibody previously reported to have high specificity against rat CYP2E1 (Johansson *et al.* 1988) was a gift from Dr. Magnus Ingelman-Sundberg, Karolinska Institute, Sweden. Horseradish peroxidase-conjugated donkey-anti-rabbit IgG was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, USA).

Cell culture and ethanol/glucose treatments

FGC-4 rat hepatoma cell line (a gift from Mary C. Weiss, Pasteur Institute, Paris, France) was maintained in Dulbecco's modified eagle medium (pH 7.3) supplemented with 0.37% NaHCO₃ (DMEM), 5% foetal bovine serum (FBS), 100 units ml⁻¹ penicillin:100 µg ml⁻¹ streptomycin sulfate (Ab/Am) at 37 °C in a humidified atmosphere of 5% CO₂/95% air. For experiments, cells were seeded in T25 flasks at 1.75×10^5 cells cm⁻² in DMEM + Ab/Am + 5% FBS and allowed to attach overnight. The next day, cells were washed once with PBS and the media was changed to glucose free DMEM + Ab/Am + 5% FBS supplemented with 1 mM sodium pyruvate. Ethanol and/or glucose were also added at this time as indicated in the individual experiments. The cells were allowed to equilibrate for 30 min in the incubator and then the caps were closed for the remainder of the experiment to prevent the ethanol from evaporating. After 24 h, cells were harvested directly into 1.0 ml TRI Reagent (MRC, Inc., Cincinnati, OH, USA) as described below.

Multiplex relative RT-PCR analysis

Total RNA was extracted using the TRI Reagent according to the manufacturer's directions. First-strand cDNAs were synthesized from total RNA using the Reverse Transcription System (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. Multiplex relative RT-PCR was performed in a total volume of 25 µl PCR buffer containing 1.1 mM MgCl₂, 0.2 µM each primer (CYP2E1 and β -actin), 1 unit RediTAQ DNA polymerase (Sigma) and 2 µl first-strand cDNA containing deoxynucleoside triphosphates. The CYP2E1 sequences were selected from Gene Bank (NM031543) and primers specific for CYP2E1 (forward: 61-CTCCTCGTCATATCCATCTG-80) and (backward: 534-GCAGCCAATCAGAAATGTGG-515) were end-labelled with polynucleotide kinase and [γ -³²P]ATP and PCR conditions were optimized to produce the desired products in the range of linearity. Primers specific for β -actin (forward: 243-TATGGAGAAGATTTGGCACC-262) and (backwards: 1033-TAGAGCCACCAATCCACACAG-1012) were used at 1:2 end-labelled to unlabelled. The PCR cycle started at 95 °C for 5 min followed by a three-step cycling: denaturation at

95 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 1.5 min for 20 cycles. This was followed by a final extension step at 72 °C for 10 min. The PCR products were fractionated through a denaturing 6% polyacrylamide, 8M urea gel at 250 V and visualized by autoradiography. The bands were scanned and analysed by densitometry; the areas of the peaks were calculated in arbitrary units. The relative value was generated by calculating the ratio of the arbitrary units of the *CYP2E1* gene to that of β -actin in the same PCR, since we had previously demonstrated that ethanol does not affect β -actin expression of in FGC-4 cells (McGehee *et al.* 1994).

Western immunoblot analysis

Cell proteins from whole cell homogenates (30 μ g/well) were resolved on 10% polyacrylamide gels and transferred to a Hybond-P membrane (Amersham Pharmacia Biotech). Membranes were blocked ≥ 8 h at room temperature with shaking in TBST [10 mM Tris buffered saline, 0.13 M NaCl, pH 7.4, 0.05% (v/v) Tween-20] plus 10% (w/v) milk powder. Membranes were then incubated with primary antibody diluted 1:1000 in TBST plus 5% (w/v) milk powder overnight at 4 °C with shaking. After washing three times in TBST, the membranes were incubated for 1 h at room temperature in TBST plus 5% (w/v) milk powder containing horseradish peroxidase-conjugated anti-rabbit Ig (1:10000). Membranes were washed three times in TBST and the proteins visualized using the enhanced chemiluminescence plus system (ECL Plus; Amersham Pharmacia Biotech) and detected by autoradiography. Immunoquantitation was obtained by densitometric scanning of the resulting autoradiographs using a Bio-Rad GS525 molecular imager (Richmond, CA, USA).

Statistical analysis

Statistical analysis was performed by Student's *t*-test, one- and two-way ANOVA with Student-Newman-Keuls Multiple Comparison using the SigmaStat Software (Jandel Scientific Software, San Rafael, CA, USA) with $p < 0.05$ being considered as statistically significant. Data are mean $\pm$ SEM.

Results

Ethanol induction of CYP2E1

The ethanol dose-dependent induction of CYP2E1 is presented in figures 1 and 2. Figure 1 demonstrates CYP2E1 mRNA results from multiplex RT-PCR analysis. Treatment for 24 h with 3, 10, 30 or 100 mM ethanol increased ($p < 0.05$) the expression of *CYP2E1* mRNA steady-state levels with a maximal induction level of

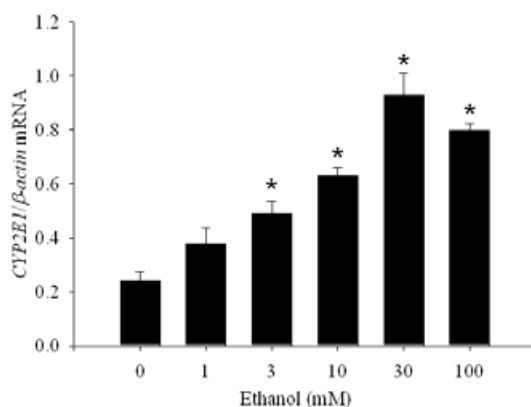


Figure 1. Ethanol concentration-dependent induction of CYP2E1 mRNA steady-state levels in FGC-4 cells. Relative multiplex RT-PCR was conducted on $n = 6$ samples/group. The bars represent mean ($\pm$ SEM) densities of bands visualized on autoradiographs of polyacrylamide gels following electrophoresis of PCR products from samples of each group. Values are arbitrary units following densitometry and asterisks indicate differences ($p < 0.05$) from the respective samples exposed to no ethanol as determined by one-way ANOVA; Student-Newman-Keuls Multiple Comparison.

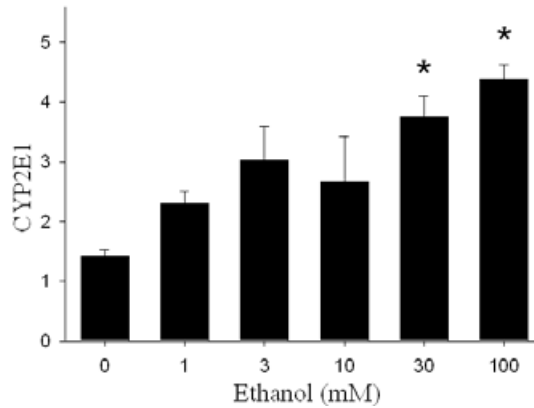


Figure 2. Ethanol concentration-dependent induction of CYP2E1 protein levels in FGC-4 cells. Western analysis was conducted on $n = 6$ samples/group. The bars represent mean ($\pm$ SEM) densities of bands visualized on autoradiographs of polyacrylamide gel following electrophoresis and visualization using enhanced chemiluminescence. Values are arbitrary units following densitometry and asterisks indicate differences ($p < 0.05$) from the respective samples exposed to no ethanol as determined by one-way ANOVA; Student–Newman–Keuls Multiple Comparison.

3.8-fold observed in cells treated with 30 mM ethanol. There was also an ethanol-dependent increase ($p < 0.05$) in CYP2E1 measured by Western analysis of cells treated with 30 or 100 mM ethanol (figure 2). The ethanol concentrations used in this experiment are equivalent to those reported *in vivo* (300–500 mg dl⁻¹) where hepatic CYP2E1 was induced 10-fold (Korourian *et al.* 1999).

Glucose repression of ethanol-induced CYP2E1

The concentration-dependent effects of glucose on 30 mM ethanol 24 h induction of CYP2E1 mRNA steady-state levels were measured by multiplex RT-PCR. Figure 3 shows the mean ($\pm$ SEM) densitometric values of six flasks per treatment

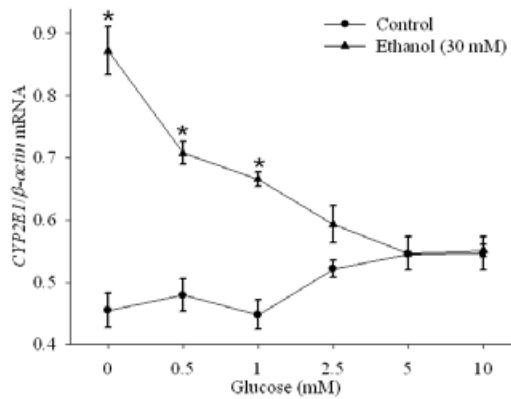


Figure 3. Physiological concentrations of glucose inhibit ethanol-induced CYP2E1 mRNA steady-state levels. Data represent the means ($\pm$ SEM) of $n = 6$ samples per treatment group. The asterisks signify increases ($p < 0.05$) in CYP2E1 mRNA of ethanol-treated samples relative to the appropriate non-ethanol-treated samples as determined by two-way ANOVA and Student–Newman–Keuls Multiple Comparison.

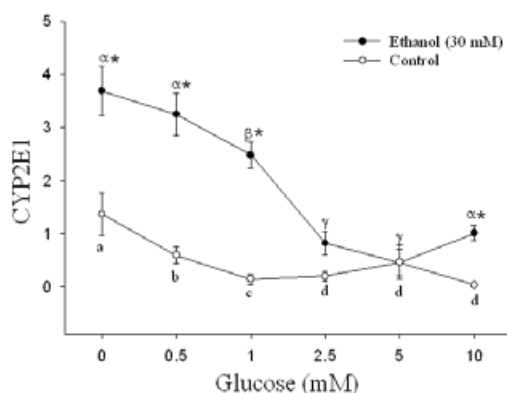


Figure 4. Glucose concentration-dependent inhibition of ethanol-induced CYP2E1 protein levels in FGC-4 cells. Circles represent means ($\pm$ SEM) of $n = 6$ samples. The asterisks signify increases ($p < 0.05$) in CYP2E1 protein of ethanol-treated samples relative to the respective non-ethanol-treated samples as determined by two-way ANOVA and Student-Newman-Keuls Multiple Comparison. Circles bearing different Greek symbols differ statistically from each other ($p < 0.05$). Circles bearing different Roman letters differ statistically from each other ($p < 0.05$).

group. Ethanol induction of *CYP2E1* mRNA steady-state levels was observed in cells cultured without glucose (1.9-fold) and in cells co-treated with 0.5 mM (1.5-fold) or 1 mM (1.5-fold) glucose ($p < 0.05$). However, in cells co-treated with 30 mM ethanol and 2.5, 5 or 10 mM glucose, there was no significant ethanol-induction of *CYP2E1* mRNA expression measured compared with glucose only treated cells. In addition, complete inhibition was measured in cells co-treated with either 5 or 10 mM glucose plus 30 mM ethanol. In the absence of ethanol, glucose concentrations of 0.5 mM or greater caused a decrease ($p < 0.05$) in CYP2E1 protein levels with maximum decrease ($p < 0.05$) measured at 10 mM glucose (figure 4). There was a glucose-dependent inhibition of ethanol-induced CYP2E1 protein measured in cells co-treated with 30 mM ethanol and increasing glucose. Co-treatment of cells with 1 mM glucose reduced ($p < 0.05$) ethanol induction of CYP2E1 compared with cells cultured without glucose. Ethanol-induced CYP2E1 was completely inhibited ($p < 0.05$) in cells co-treated with 2.5 or 5 mM glucose.

Discussion

As reviewed by Ronis and Ingelman-Sundberg (1996), CYP2E1 appears to be regulated by ethanol at many levels including transcription, translation and protein stability/degradation; however, the precise mechanisms for these regulatory schemes have not been determined. Results from our laboratory suggest that ethanol induces CYP2E1 in a two-step mechanism that involves post-translational events at blood ethanol concentrations $< 300 \text{ mg dl}^{-1}$ that are independent of alterations in mRNA level, and additional transcriptional, mRNA stabilization and translational components at blood alcohol concentrations $> 300 \text{ mg dl}^{-1}$ (Badger *et al.* 1993, Ronis *et al.* 1993). A significant portion of ethanol-induced liver toxicity may result from the generation of oxygen free radicals formed as a

by-product of the oxidation of ethanol and unsaturated fatty acids via CYP2E1 (Lieber 1997).

In this regard, two reports have demonstrated an inverse relationship between dietary carbohydrate levels and CYP2E1 expression. Tuskada *et al.* (1998) and Korourian *et al.* (1999) reported that rats fed low carbohydrate/high ethanol diets had greater CYP2E1 protein and activity levels than rats fed high carbohydrate/high ethanol diets, even though the ethanol intake was the same. Importantly, in studies that compared the effects of different carbohydrate levels, there were increases in ethanol-induced liver pathology with low carbohydrate/high ethanol treatment relative to high carbohydrate/high ethanol treatment (Lindros and Jarvelainen 1998, Tuskada *et al.* 1998, Korourian *et al.* 1999). Thus, the putative role for carbohydrate in regulation of ethanol induction of CYP2E1 and in development of ALD has been suggested in these latter reports, but this has been not been well explored and it is difficult to study *in vivo* because when reducing the dietary carbohydrate levels there must be an increase in fat to keep the diets isocaloric. Therefore, two variables were changed at the same time, making it uncertain if the effects were to lower carbohydrate levels or higher fatty acids levels.

In the present study, we used rat FGC-4 cells to investigate further the mechanism of ethanol regulation of CYP2E1, since these cells were derived from a rat H4IIEC3 hepatoma and express a wide spectrum of liver specific functions (Angrand *et al.* 1990). Using this model, we previously reported that ethanol induced CYP2E1 protein expression in a dose-dependent manner, but we could not demonstrate increased *CYP2E1* mRNA expression (McGehee *et al.* 1994). We have since modified the cell culture conditions used in the previous study and here we report that ethanol can be a direct inducer of CYP2E1 protein and mRNA steady-state levels when the glucose concentration in the culture media is <2.5 mM.

CYP2E1 mRNA and protein levels are induced in response to ethanol *in vivo* (Johansson *et al.* 1988, Kim *et al.* 1990, Badger *et al.* 1993, Ronis *et al.* 1993). Ethanol-induces CYP2E1 protein levels through both increased translational efficiency and stabilization of the protein from degradation, which appears to occur primarily through ubiquitination and proteasomal degradation (Yang and Cederbaum 1996). Similarly, ethanol-induced *CYP2E1* mRNA may occur through mRNA stabilization (McGehee *et al.*, unpublished data) as well as through increased gene transcription (Badger *et al.* 1993). The ethanol concentration that induced *CYP2E1* mRNA *in vitro* in the current study (3–100 mM) is within the blood ethanol concentrations that have been reported to induce *CYP2E1* *in vivo* (Korourian *et al.* 1999). We do not yet know whether the ethanol-increased *CYP2E1* mRNA in FGC-4 cells occurred through increased gene transcription, mRNA stabilization or both.

In vitro experiments have revealed that CYP2E1 expression is affected by the glucose homeostatic hormones insulin and glucagon (Novak and Woodcroft 2000). In the absence of any other hormonal or metabolic alterations, insulin substantially decreased *CYP2E1* mRNA and protein levels in primary cultured rat hepatocytes (Woodcroft and Novak 1997, 1999a, b). In contrast, treatment of primary rat hepatocytes with glucagon markedly increased *CYP2E1* mRNA levels (Woodcroft and Novak 1999b), suggesting antagonistic regulation of CYP2E1 by insulin and glucagon. In addition, CYP2E1 is increased in both chemically-induced and spontaneous diabetic rats, indicating insulin regulation *in vivo* (Song *et al.* 1987,

Bellward *et al.* 1988, Dong *et al.* 1988). Glucose effects on CYP2E1 expression were also recently demonstrated *in vivo* in hypophysectomized rats that had a 20% reduction in serum glucose with a corresponding increase in hepatic CYP2E1 mRNA and glucose feeding reversed this (Son *et al.* 2000). Thus, *in vitro* and *in vivo* evidence suggests a link between glucose metabolism and CYP2E1 regulation. Our *in vitro* results demonstrate that glucose inhibits ethanol induction of CYP2E1 mRNA at concentrations (2.5–10 mM glucose) similar to that found in serum.

In addition, since the cell culture media contained only a low level of insulin contained in the foetal bovine serum and the medium was not supplemented with additional insulin, this suggests that the inhibition of ethanol-induced CYP2E1 mRNA measured is a result of glucose or a glucose metabolite.

Based on these results, CYP2E1 appears to be under negative control by carbohydrates or metabolites of carbohydrates. This finding is supported by studies demonstrating a physiological role of CYP2E1 in the gluconeogenic pathway, since the CYP2E1 inhibitor, imidazole, abolishes the conversion of ^{14}C acetone to ^{14}C glucose (Johansson *et al.* 1986). Therefore, the regulation of CYP2E1 may resemble that of phosphoenolpyruvate carboxykinase, the rate-limiting enzyme of gluconeogenesis, which is also negatively regulated by carbohydrates (Hanson and Reshef 1997). In addition, ethanol has been reported to decrease hepatocellular glucose concentrations (Pirola and Lieber 1972, Mezey 1985). These results are consistent with the hypothesis that glucose can negatively regulate CYP2E1 expression and could explain the increased induction reported in high ethanol, low carbohydrate diets leading to necrosis.

Like most models designed to study the effects of alcohol on the liver, the FGC-4 cell line was used here merely as a model and there are pitfalls and drawbacks. The FGC-4 cells are transformed cells, and as demonstrated by comparison of the results of current study with those of the earlier paper from our laboratory by McGehee *et al.* (1994), the response to ethanol varies with the culture conditions. Thus, whether the results using the FGC-4 cells are relevant to what occurs *in vivo* are not known. However, the data from the current *in vitro* study demonstrate that under the conditions used, carbohydrates alone can negatively regulate CYP2E1 expression and these data correlate with *in vivo* results of Tsukada *et al.* (1998) and Korourian *et al.* (1999) in which greater CYP2E1 induction by ethanol occurs with low carbohydrate/high fat diets than with high carbohydrate/lower fat, adding further to the evidence suggesting that the level of dietary carbohydrates alone may be an important factor in alcohol-induced liver damage.

Acknowledgements

The authors thank their laboratory colleague, Britt Young, for valuable assistance. Funding was provided by the National Institutes of Health Grant NIAAA 08645 (T. M. B.). The work was presented in part at the Society of Toxicology meeting, San Francisco, CA, 2001.

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***In vivo* and *in vitro* disposition profile of tezosentan, an intravenous dual endothelin receptor antagonist, in humans**

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Received 16 September 2002

1. The plasma concentration–time profile of the ^{14}C -labelled endothelin receptor antagonist tezosentan in healthy male volunteers after a 1-h infusion at 100 mg h^{-1} followed a biphasic decline with half-lives of 3–5 min for the initial disposition phase and approximately 4 h for the terminal phase.

2. Tezosentan was predominantly excreted unchanged into faeces, whereas less than 5% of the dose was excreted as unchanged drug in urine. Two isomeric, hydroxylated metabolites (M1, M2) were detected in faeces representing 2–5% of the total radioactivity.

3. *In vitro*, with human liver microsomes and primary hepatocytes, tezosentan was metabolized at very low rates. Upon prolonged incubation with human hepatocytes for 24 h, formation of the hydroxylated metabolite M1 and a glucuronic acid conjugate, M3, was observed.

4. No relevant inhibition of the human cytochrome P450 (CYP) forms, CYP1A2, 2C9, 2C19, 2D6 and 3A4, was observed *in vitro* at tezosentan concentrations largely exceeding those observed in clinical trials.

5. In human blood, tezosentan was highly bound to plasma proteins, mainly albumin, and hardly penetrated into red blood cells.

Introduction

Tezosentan (VeletriTM) and bosentan (TracleerTM (figure 1) are two structurally closely related dual endothelin receptor antagonists with a high affinity to endothelin A and B receptors. Endothelin-1 appears to play an important role in several pathological processes such as renal and heart failure (Kaddoura and Poole-Wilson 1996, Rabelink *et al.* 1996, Lüscher and Barton 2000). Bosentan, an orally active drug, was recently approved as the first endothelin receptor antagonist for the treatment of pulmonary arterial hypertension (Rubin *et al.* 2002). In humans, bosentan is almost entirely excreted into faeces (Weber *et al.* 1999) after extensive metabolism by the cytochrome P450 (CYP) enzymes 2C9 and 3A4. Tezosentan, in contrast, is a highly water-soluble analogue specifically designed for parenteral use (Clozel *et al.* 1999). In early clinical trials, tezosentan followed dose-proportional, two-compartmental pharmacokinetics characterized by a pronounced first disposition phase with a half-life of 6 min and a terminal elimination half-life of approximately 3 h (Dingemans *et al.* 2002a, b). In the present paper, we describe the disposition of ^{14}C -labelled tezosentan in healthy volunteers together with

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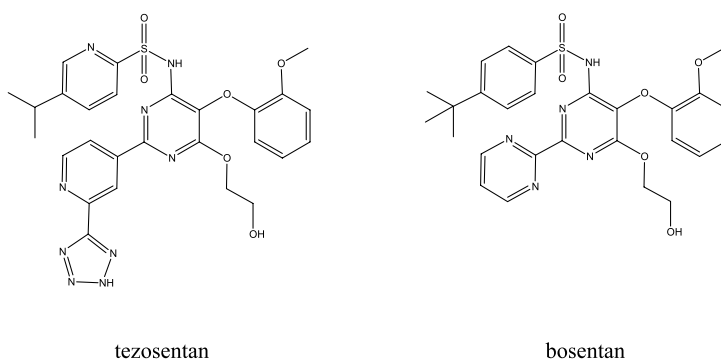


Figure 1. Chemical structure of the endothelin receptor antagonists tezosentan and bosentan.

complementary data on drug metabolism, plasma protein binding, plasma-to-blood distribution and CYP inhibition.

Materials and methods

In vivo study

The clinical part of this open label study was conducted at Swiss Pharma Contract, Allschwil, Switzerland, after approval of the study protocol by an Ethics Committee (Ethikkommission beider Basel, Basle, Switzerland). The study was conducted in full conformity with the principles of the Declaration of Helsinki and its amendments. Subjects were assessed to be healthy based on physical examination including a 12-lead ECG, medical history and clinical laboratory tests performed within 3 weeks before drug administration. Testing for illicit drugs in blood and urine was also performed. Subjects smoking more than 10 cigarettes a day were not included. No concomitant medications were allowed during the study, with the exception of medications to treat adverse events. Restrictions were applied regarding the intake of methylxanthine-containing beverages and food. The subjects were hospitalized from approximately 12 h before to 168 h after drug infusion. At the end of the study, a medical examination was performed at discharge. Four healthy male Caucasians (47–63 years of age and weighing 66–75 kg) were administered a 1-h i.v. infusion of 100 mg ^{14}C -labelled tezosentan (radioactive dose 50 μCi). Adverse events were assessed by spontaneous reports, observations and questioning at regular intervals. Supine blood pressure, pulse rate and body temperature were measured frequently. Clinical laboratory tests were performed before and at 72 h after the tezosentan infusion.

For the analysis of total ^{14}C -radioactivity and tezosentan in plasma, blood samples of 6 ml (in the period 0–8 h) and 11 ml (10–72 h) volume were drawn into EDTA-containing tubes from an indwelling catheter, inserted in the arm contralateral to the drug infusion site and by venipuncture. Additional samples for determination of radioactivity in whole blood (2 ml) and for the determination of tezosentan metabolites (10 ml) were taken at preselected time points during and after drug infusion. Plasma and whole blood samples were stored at -20°C pending analysis. Urine and faeces were quantitatively collected at specified intervals for 168 h following the start of drug infusion. After determination of volume and weight, samples were stored at -70°C . Faeces were homogenized with 2–3 equivalents (w/v) of water. Breath sampling was performed at several time points until 24 h after start of the infusion by letting the subjects expire into 4 ml of a trapping solution consisting of a 1:1 mixture of 1 N hyamine hydroxide and ethanol with thymolphthalein as pH indicator. The subjects expired normally into the solution until the colour change indicated the absorption of an equimolar amount of CO_2 . The vials were stored at 4°C pending analysis for total radioactivity.

Pharmacokinetic evaluation

The pharmacokinetic evaluation for tezosentan was performed with model-dependent and -independent methods (Gibaldi and Perrier 1982). The actual doses, as determined by analysis of the infusion solutions, were used for the pharmacokinetic evaluations. The area under the concentration-time curve (AUC) was estimated using the linear trapezoidal rule and extrapolation to infinity with the help of the terminal elimination rate constant λ_z . The latter was determined by log-linear regression analysis of the terminal phase. The terminal half-life ($T_{1/2}$) was calculated by dividing $\ln 2$ by λ_z . The systemic clearance (CL) and the volume of distribution at steady state (V_{ss}) were calculated using

standard algorithms (Gibaldi and Perrier 1982). Concentrations of tezosentan were fitted into an open two-compartment pharmacokinetic model using the WinNonlin software package (Professional version 3.1, Pharsight Corporation, Mountain View, CA, USA). Pharmacokinetic parameters were descriptively analysed, calculating geometric means and 95% confidence limits.

Analytical and spectroscopic equipment

Bioanalysis of plasma and urine samples. Concentrations of tezosentan and M1 in plasma and urine were determined using an API365 (PE Applied Biosystems, Foster City, CA, USA) mass spectrometer operating in positive-ion mode. (For reasons of consistency, metabolites were sequentially numbered M1–3. M1 has also been named Ro 64-1538 in Dingemans *et al.* (2002a, b) and van Giersbergen *et al.* (2002) accounting for its origin from research performed by F. Hoffmann-La Roche, Basle, Switzerland.) After acidification with phosphoric acid, plasma and urine samples were extracted with two equivalents *t*-butyl methyl ether (v/v) containing known quantities of the tetra-deuterated analogues of both analytes as internal standards. The compounds were separated on a 2.1×50 mm reversed-phase column (Waters, Symmetry C18, 100 Å, 3.5 µM) with a linear elution method using a 50:50 mixture of acetonitrile and 0.02% aqueous trifluoroacetic acid as mobile phase at a flow rate of 0.4 ml min^{-1} . The limit of quantification was 1.0 ng ml^{-1} for tezosentan and 2.5 ng ml^{-1} for M1. The performance of the chromatographic method was monitored by simultaneous analysis of independently prepared quality control samples. The assay was linear in a concentration range of 1.00–512 and 2.50–512 ng ml^{-1} for tezosentan and M1, respectively. The interassay coefficient of variation was below 12.7%, whereas the inaccuracy ranged from –6.5 to 6.3%.

Radioactivity determinations. ^{14}C -radioactivity in samples of the infusion solutions, whole blood, plasma, urine, faeces and expired air was determined in triplicate using a TRI-CARB TR 2500 liquid scintillation counter (Packard, Zurich, Switzerland). Faecal extracts were combusted using a biological oxidizer (Packard Oxidizer 306). The performance of the determinations was monitored by running alongside quality control samples containing known activities of ^{14}C -stearic acid.

Metabolic profiles. Metabolic profiles were recorded on a Shimadzu LC10 Liquid Chromatograph with a SPD 10A UV/VIS detector, SCL 10A System Controller, and a SIL 10A Auto Injector (Shimadzu Scientific Instruments, Kyoto, Japan). Product detection and quantification were performed either with UV at a wavelength of $\lambda = 274 \text{ nm}$ or a LB 506 C-1 radioactivity flow monitor (Berthold Technologies, Regensdorf, Switzerland) equipped with a 150-ml-yttrium glass cell (counting efficiency 85%). Data acquisition and peak integration was performed by using the WinFlow software package (version 1.21.60, Berthold Technologies). Chromatographic separations were achieved with two in-line connected Superspher columns (RP-18 endcapped, 100 Å, 5 µm, $125 \times 4 \text{ mm i.d.}$, Merck, Darmstadt, Germany) preceded by a Superspher guard column (same type, $25 \times 4 \text{ mm i.d.}$). Mobile phases consisted of 25 mM aqueous citric acid (pH 2.3) and acetonitrile in mixtures of 80:20 (phase A) and 30:70 (B). A linear gradient from 0 to 100% of phase B within 50 min was applied with a flow rate of 1.0 ml min^{-1} . For the isolation of metabolites, a slightly modified chromatographic system was used. Owing to the limited solubility of tezosentan at low pH, the pH of mobile phase A was adjusted to 3.3 using 25% ammonia and the gradient was started with 5% of phase B and increased to 95% within 20 min.

Structure elucidation. ^1H -NMR spectra of tezosentan and M1 were recorded in D_3 -acetonitrile on an AMX-500 spectrometer (Bruker, Rheinstetten, Germany). About 4000 scans were accumulated to obtain spectra of sufficient quality. Structural assignments were made with the help of 2D ^1H , ^1H -COSY spectra and by comparison with the spectrum of tezosentan recorded in the same solvent. Mass spectrometry for structure elucidation was carried out on an API300 triple quadrupole mass spectrometer (PE Applied Biosystems) combined with two Shimadzu LC10 pumps. Single mass and product ion spectra were acquired in positive-ion mode. For the generation of product ion spectra, collision energy settings were tuned to yield maximum signal and fragmentation. Chromatographic separation was achieved on an Inertsil ODS-3, 5 µm, microbore ($150 \times 1.0 \text{ mm}$) column applying a gradient from 0 to 100% of phase B within 20 min at a flow rate of 0.2 ml min^{-1} . Mobile phases were 1% aqueous formic acid and acetonitrile in mixtures of 90:10 (phase A) and 10:90 (B). The eluent was directed into the ion-spray interface of the mass spectrometer without splitting.

In vitro studies

Chemicals and reagents. ^{14}C -radiolabelled tezosentan (disodium salt), unlabelled tezosentan (disodium salt, chemical purity 98.9%) and M1 (free acid, chemical purity 97.4%) were obtained from Actelion-internal stocks. The radiolabelled material had a specific activity of $84.8 \text{ } \mu\text{Ci mg}^{-1}$, a radiochemical purity of 97% and the radiolabel introduced into the tetrazole ring. Mixed glucuronidase/sulphatase was purchased from IBF Biotechnics (Villeneuve, La Garenne, France), with the following enzyme

activities: 10^5 Fishman units ml^{-1} for β -glucuronidase and 10^6 Roy units ml^{-1} for sulphatase. c-DNA-expressed human CYP enzymes and fluorimetric probes for the interaction assays were obtained from Gentest Corporation (Woburn, MA, USA). Purified water was prepared by ultrafiltration (Millipore). All other chemicals and solvents used in this study were of highest commercially available purity.

Sample preparation for in vivo metabolic profiles

Plasma. Plasma proteins were precipitated by the addition of three equivalents of acetonitrile (v/v). Samples were then vortex mixed, sonicated and centrifuged for 10 min at 1050g. The supernatant was removed and evaporated to near dryness under a stream of nitrogen gas. The residue was reconstituted in an 80:20 mixture of water and acetonitrile and the solution was again vortex mixed, sonicated and centrifuged at 1050g for 10 min before HPLC analysis.

Urine. Urine samples were directly subjected to HPLC analysis after precipitation of proteins with three equivalents of acetonitrile (v/v), vortex mixing and centrifugation at 1050g for 10 min.

Faeces. Weighed portions (about 1–2 g) were repeatedly extracted with 5–10 ml acetonitrile by a combination of vortex mixing and sonication and then centrifuged for 10 min at 1050g. The supernatant solutions were combined and evaporated to a small volume under a stream of nitrogen gas. The extract was reconstituted in an 80:20 mixture (v/v) of water and acetonitrile and subjected to HPLC analysis.

Metabolic profiles with human liver tissue preparations. Human liver tissue for the preparation of microsomes and cultures of primary hepatocytes was obtained from surgical resections at the Hôpital Hautepierre (Strasbourg, France) in accordance with the guidelines of the Local Ethics Committee. Primary hepatocyte cultures (Seglen 1973) and microsomes (Lowry *et al.* 1951, von Bahr *et al.* 1980) were prepared following literature procedures.

Incubations with liver microsomes were performed in a total volume of 1.0 ml at a ^{14}C -tezosentan concentration of $12.5 \mu\text{M}$ with 1.0 mg ml^{-1} protein in the presence of an NADPH-regenerating system. Incubations were continued for up to 1 h and samples taken at intermediate points. Control experiments in the absence of microsomal protein or the NADPH-regenerating system were run to verify the dependency of metabolite formation on CYP activity. ^{14}C -tezosentan was incubated in the presence of human hepatocytes for up to 24 h at a substrate concentration of $10 \mu\text{M}$ and a cell density of 0.75×10^6 cells ml^{-1} . Intra- and extracellular media were collected separately at selected time points. Proteins in both types of samples were precipitated by addition of one equivalent (v/v) of acetonitrile and storage on ice for 1 h. After centrifugation at 16 100g for 10 min, supernatants were supplemented with 25 mM aqueous citric acid (pH 2.3) to achieve a final solvent composition of about 80:20 and subjected to HPLC analysis.

Enzymatic cleavage of tezosentan glucuronide. A 0.2-ml aliquot of the incubate of tezosentan with primary hepatocytes was added to a solution of 0.1 ml glucuronidase/sulphatase enzyme mixture in 0.7 ml 0.2 M acetate buffer (pH 5.0). Incubation was continued for 20 h at 37°C , stopped by the addition of 1 equivalent (v/v) of acetonitrile, and proteins were allowed to precipitate for 1 h on ice. After centrifugation for 10 min at 16 100g, the supernatant was evaporated to dryness, reconstituted in 0.25 ml of an 80:20 mixture of 25 mM aqueous citric acid/acetonitrile and subjected to HPLC analysis. The peak corresponding to the tezosentan glucuronide had completely disappeared in the radiochromatogram of this sample.

Inhibition of human CYP enzymes. The potential of tezosentan for inhibition of the main human CYP enzymes, i.e. CYP1A2, 2C9, 2C19, 2D6 and 3A4, was evaluated by the use of recombinant CYP forms expressed in baculovirus-infected Sf9 insect cells with co-expressed reductase. Tezosentan was used in a concentration range up to $100 \mu\text{M}$. The marker reactions for the five CYP enzymes investigated, determined IC_{50} values, and reference values for known strong inhibitors of each isoform are summarized in table 1. Experiments were performed around the respective K_m values of the marker substrates and metabolite formation was monitored fluorimetrically (Crespi and Stresser 2000, Miller *et al.* 2000).

Blood-to-plasma partitioning. The blood-to-plasma distribution ratio in human blood was studied at five different concentrations in the range of $0.1\text{--}50 \mu\text{g ml}^{-1}$ using ^{14}C -labelled tezosentan and heparinized blood from two individuals. For the determination of radioactivity in plasma, 0.4 ml blood was transferred to a plasma separator tube (Becton Dickinson, Franklin Lakes, USA) without anticoagulant and immediately centrifuged for 5 min at 3220g. Exactly 0.1 ml plasma was transferred to a preweighed liquid scintillation vial, 2.0 ml Ultima Gold was added and radioactivity measured on a TRI-CARB TR 1900 scintillation counter (all Packard). Radioactivity in whole blood was determined

by transferring 0.2-ml aliquots of blood into combustion cones, air-drying and combustion in an OX200 oxidizer (Harvey, Hillsdale, NY, USA). The generated $^{14}\text{CO}_2$ was trapped in Picosolve C-300 (Packard) and quantified as described above.

Plasma protein binding. The *in vitro* plasma protein binding of tezosentan was determined by equilibrium dialysis in a concentration range of $0.2\text{--}60\text{ }\mu\text{g ml}^{-1}$ using ^{14}C -labelled tezosentan and human plasma using EDTA as anticoagulant. Dialysis was continued for 4 h. Aliquots of 0.05–0.10 ml plasma and 0.25–1.0 ml buffer were removed, supplemented with Ultima Gold and the radioactivity was quantified with a TRI-CARB TR 1900 scintillation counter (Packard). The concentrations of total protein and albumin in plasma were determined with a Cobas Fara[®] autoanalyser (Roche Diagnostica Systems) using the biuret and bromocresol methods, respectively (Johnson 2001, Dumas *et al.* 1981). The concentration of α_1 -acid glycoprotein was measured by single radial immunodiffusion (NOR-Partigen[®] plates, Behringwerke AG, Marburg, Germany).

Results

All four subjects completed the study according to the protocol and none reported any adverse event. No pattern of abnormal laboratory values or vital signs was detected during the study to suggest a treatment effect.

Profiles of tezosentan and total radioactivity in plasma and blood

Figure 2 presents the mean concentration–time profiles of tezosentan in plasma and of total radioactivity in plasma and whole blood. Within 30 min after termination of the infusion, mean concentrations of tezosentan as well as total radioactivity dropped to less than 10% of the values attained at the end of the infusion period. The pharmacokinetic parameters for tezosentan in plasma, total radioactivity in plasma and total radioactivity in blood are summarized in table 1.

M1 was found only in some of the plasma samples and its concentrations never exceeded 1% of that of tezosentan. Since the activity of M1 towards endothelin receptors is 10–20-fold lower than that of tezosentan (Dingemanse *et al.* 2002a), its contribution to the overall pharmacological effect is considered negligible. A separate pharmacokinetic analysis of M1 was therefore not performed.

Excretion profiles in faeces and urine

The cumulative excretion profile of radioactivity in urine and faeces is shown in figure 3. The major part of the radioactive dose was excreted into faeces and

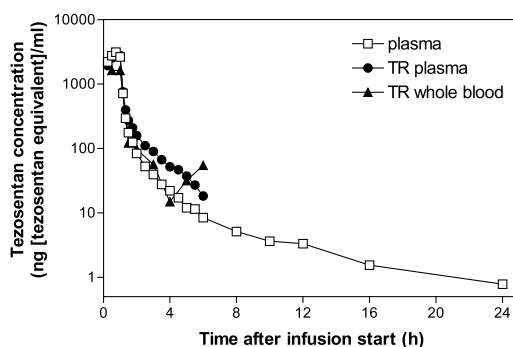


Figure 2. Mean concentration–time profiles of tezosentan in plasma and of total radioactivity (TR) in plasma and whole blood upon i.v. infusion of 100 mg h^{-1} ^{14}C -labelled tezosentan for 1 h ($n = 4$).

Table 1. Pharmacokinetic parameters of tezosentan in plasma and of total radioactivity in plasma and whole blood upon i.v. infusion of 100 mg h⁻¹ ¹⁴C-labelled tezosentan for 1 h (n = 4).¹

	AUC _{0-∞} (ng h ml ⁻¹)	C _{max} (ng ml ⁻¹)	T _{1/2} (α) ² (h)	T _{1/2} (β) (h)	CL (l h ⁻¹)	V _{ss} (l)
Tezosentan in plasma	2978 (2270, 3907)	3170 (2035, 4939)	0.08 (0.07, 0.09)	4.3 (1.6, 12)	34 (24, 48)	20 (6.4, 64)
Total radioactivity in plasma	2972 (2102, 4204)	2910 (2112, 4011)	0.08 (0.07, 0.10)	– ³	34 (26, 45)	20 (16, 24)
Total radioactivity in blood	2055 (1200, 3519)	1682 (1067, 2651)	0.05 (0.02, 0.15)	– ³	49 (30, 81)	64 (45, 92)

¹ Values are expressed as geometric mean (and 95% confidence limits); ² values for T_{1/2}(α) were estimated by compartmental modeling; ³ not given, estimations using total radioactivity in plasma and blood result in underestimated values for T_{1/2}(β) most likely due to the lower limit of quantification.

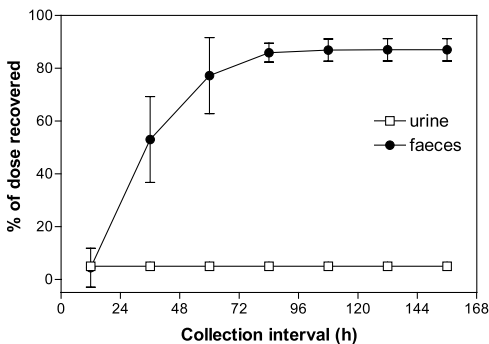


Figure 3. Mean ± 50 cumulative excretion profile of total radioactivity in faeces and urine upon i.v. infusion of 100 mg h⁻¹ ¹⁴C-labelled tezosentan for 1 h (n = 4).

excretion was virtually complete within 4 days. The total radioactivity excreted in faeces accounted for 87.0 ± 4.3% of the administered dose. Total radioactivity in urine was 5.0 ± 0.9%, which was close to the amount of unchanged tezosentan in urine (4.0 ± 0.7%) determined by mass spectrometry. The amount of M1 in urine was small and represented 0.007 ± 0.005% of the administered dose. The radioactivity determined in the breath samples was below the limit of quantification.

Metabolic profiles in vivo

Representative HPLC radiochromatograms and radiohistochromatograms of a plasma sample withdrawn immediately before termination of the infusion, of a urine sample collected over the 1 h after the start of the infusion, and from faeces samples of two subjects are presented in figures 4–6. In the 1-h plasma sample, unchanged tezosentan accounted for 94% of the total radioactivity. None of the six minor metabolites detected in the radiohistochromatogram accounted for more than 3% of the total radioactivity. Owing to the low concentrations of tezosentan metabolites in plasma, no attempts were undertaken to elucidate their structures. Unchanged tezosentan was also the main component detected in urine. Analysis of the radiohistochromatogram revealed the presence of five minor metabolites. Since excretion of total radioactivity in urine accounted for only 5% of the administered dose, most of which was unchanged tezosentan, efforts to identify these metabo-

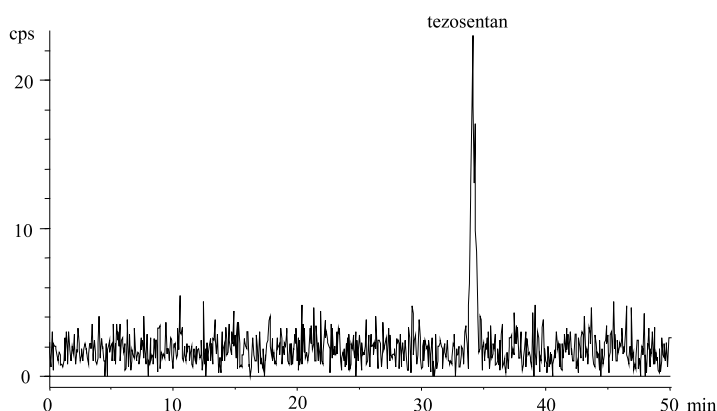
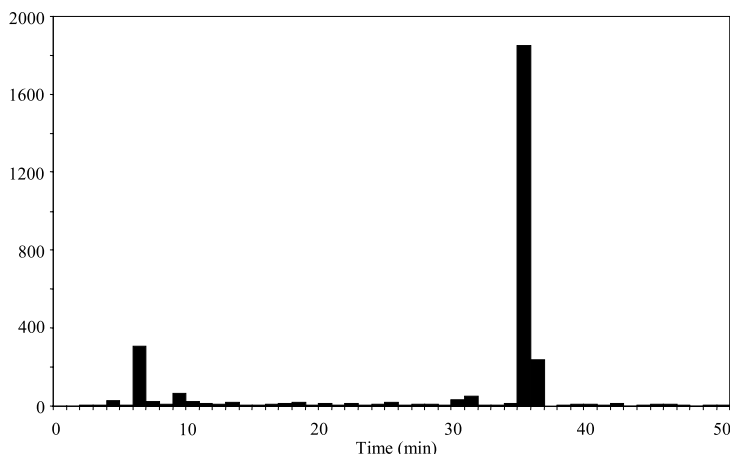
Radiochromatogram**Radiohistochromatogram**

Figure 4. HPLC radiochromatogram and radiohistochromatogram of a plasma sample taken immediately before termination of the infusion of ^{14}C -labelled tezosentan.

lites structurally were not undertaken. Most of the radioactive material excreted into faeces was again unchanged tezosentan. In addition, small amounts of two metabolites (M1, M2) were present in the radiochromatograms accounting for about 2–5% of the administered dose. Interestingly, the relative abundance of both metabolites varied among the four subjects. Whereas M1 was present in all four subjects, the amount of M2 varied from undetectable (subject 003) to equally abundant (subject 002).

Metabolic profiles in vitro

The conversion of radiolabelled tezosentan in the presence of liver microsomes was very low with more than 96% of radioactivity accounting for unchanged tezosentan after 1 h of incubation (data not shown). The same held true for

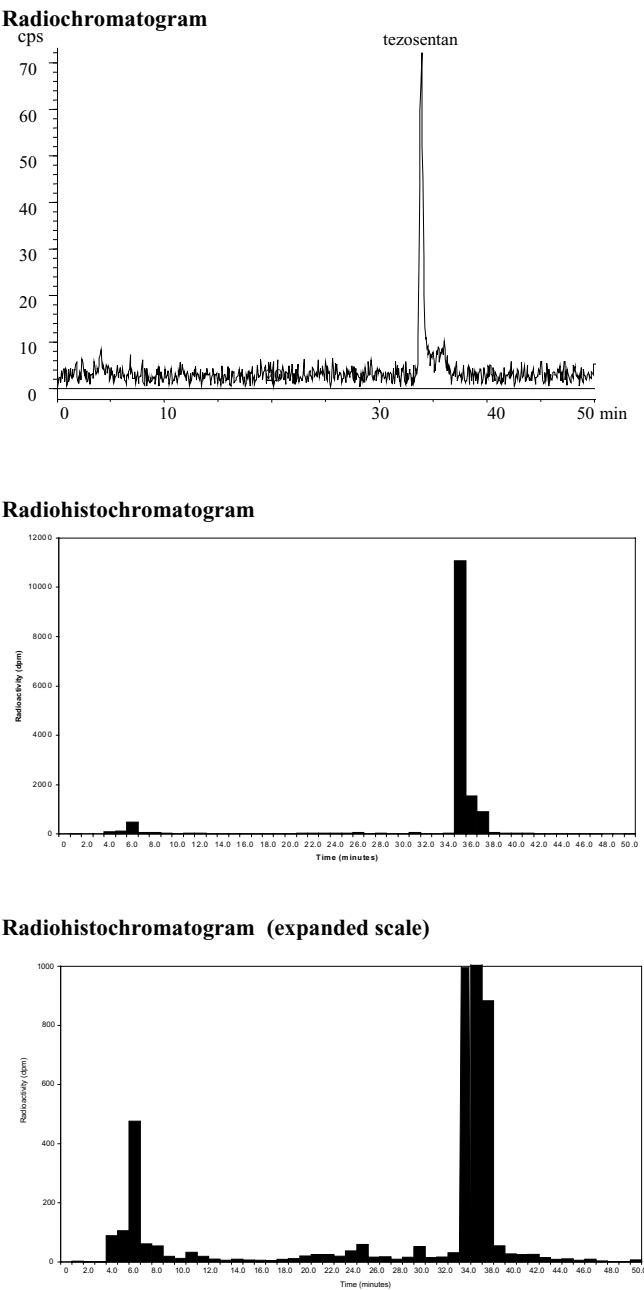


Figure 5. HPLC radiochromatogram and radiohistochromatograms of a urine sample collected over 1 h after the start of the infusion of ^{14}C -labelled tezosentan.

incubations with human hepatocytes. Separate analysis of intracellular and extra-cellular matrix yielded similar metabolic profiles and revealed that significant amounts of metabolites were only formed after prolonged incubation for 24 h. Two major metabolites, M1 and M3, were detected together with three minor products none of the latter exceeding 2% of the total radioactivity.

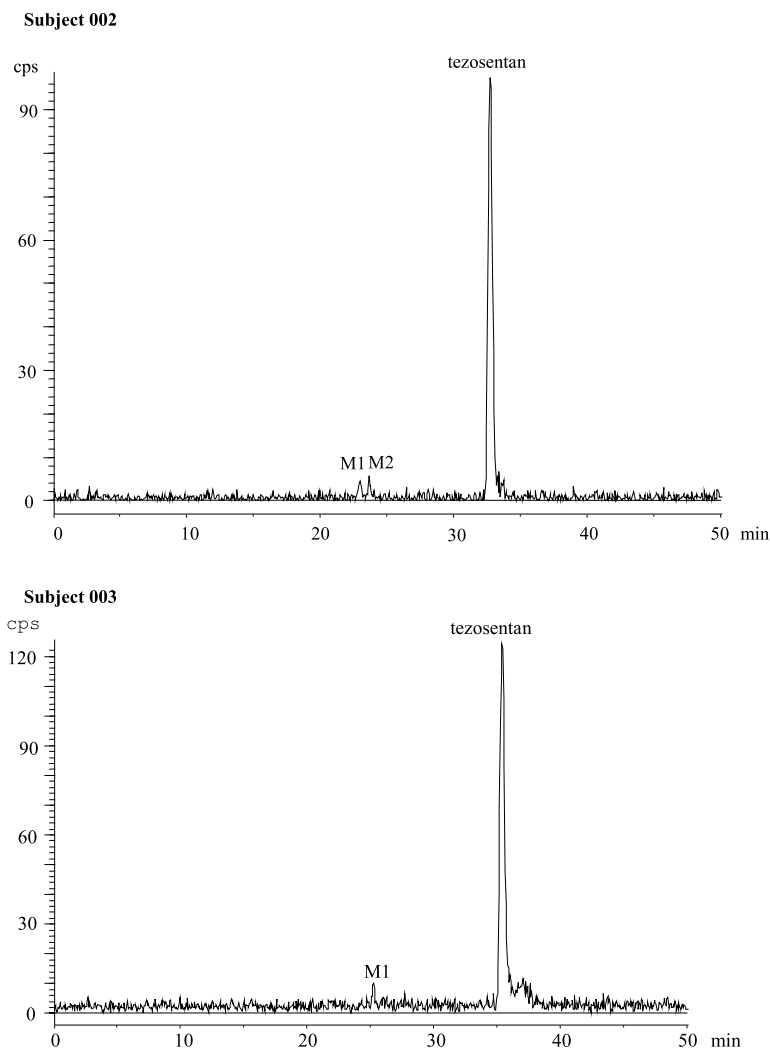


Figure 6. HPLC radiochromatograms of a faeces sample from two different subjects collected after the start of the infusion of ^{14}C -labelled tezosentan.

Metabolite structure identification

The chemical structures of the three metabolites, M1–3, observed *in vivo* and *in vitro* were identified with a combination of biochemical and spectroscopic/spectrometric tools. The chemical nature of M1 was identified by means of ^1H -NMR spectroscopy after isolation from an incubation with human hepatocytes. The ^1H -NMR spectrum of M1 exhibited the complete set of 10 aromatic protons also present in the spectrum of tezosentan. In contrast, all signals characteristic for the isopropyl group, i.e., the doublet at $\delta 1.06$ and the septet at $\delta 2.88$, had disappeared. Instead, a singlet at $\delta 1.39$ was observed integrating for six protons and suggesting hydroxylation at the α -position of the isopropyl group. The proposed chemical structure of M1 was confirmed by comparison with an independently synthesized reference sample. The structure of M2 was tentatively elucidated based on mass spectrometry data. Figures 7 and 8 show the proposed

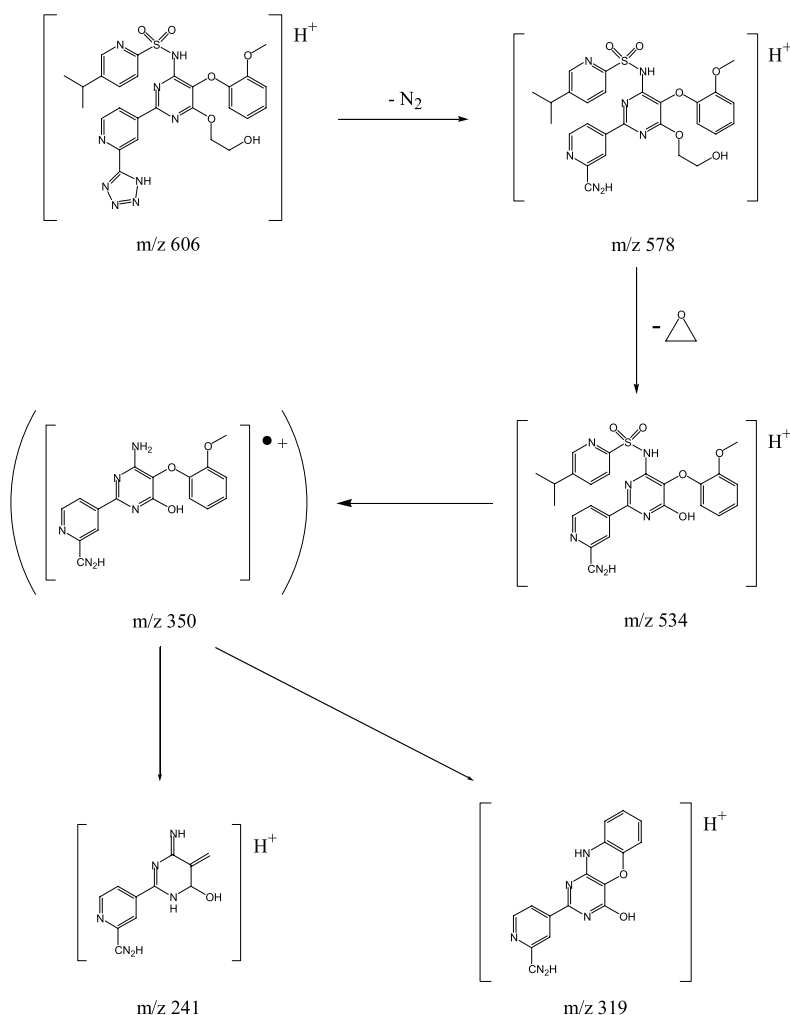


Figure 7. Proposed fragmentation mechanism of tezosentan.

fragmentation pattern of tezosentan and the authentic reference sample of M1, respectively. The fragmentation pattern of tezosentan was suggested based on a detailed mechanistic study with the structural analogue bosentan involving a sequence of cleavage and complex rearrangement steps (Hopfgartner *et al.* 1996). Key steps in the fragmentation of tezosentan involve the loss of molecular nitrogen ($m/z\ 578$) and ethylene oxide ($m/z\ 534$) finally leading to the rearranged fragments $m/z\ 319$ and 241 . The fragment with $m/z\ 350$ formed after cleavage of the sulfonamide bond, and postulated in analogy to bosentan, was not observed in the mass spectrum of tezosentan. However, its formation as an intermediate is plausible in view of the occurrence of the rearranged fragments with $m/z\ 319$ and 241 . The fragmentation pattern of M1 was more complex. Sequential loss of water and/or molecular nitrogen led to fragments $m/z\ 604$, 594 and 576 followed by the loss of ethylene oxide to yield $m/z\ 550$ from $m/z\ 594$. Cleavage of the sulfonamide bond would then yield $m/z\ 350$, which again was not observed. As described for tezosentan, further fragmentation and rearrangement led to the fragments at m/z

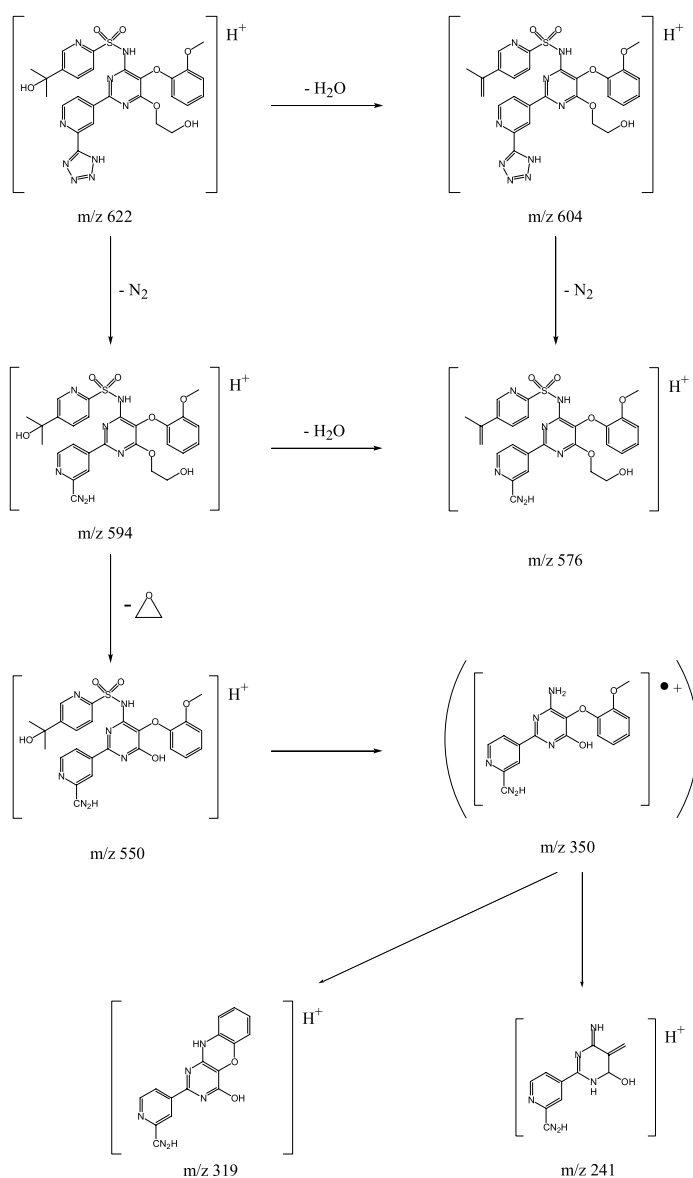


Figure 8. Proposed fragmentation mechanism of the authentic reference of M1.

319 and 241. The mass spectra of M1 and M2 extracted from human faeces samples were very similar and exhibited the same characteristic fragmentation pattern as seen with the authentic reference sample of M1. Alternative sites of oxidation in the pyridyl sulfonamide moiety of M2 leading to an identical molecular ion peak are the β -positions of the isopropyl group, the aromatic hydroxylation of the pyridine ring or the oxidation of the aromatic nitrogen atom with formation of a pyridine N-oxide. Since hydroxylation in an aromatic ring position is not expected to result in a facile loss of water upon fragmentation, and fragmentation of pyridine N-oxides is rather characterized by the loss of molecular oxygen (Tong *et al.* 2001), hydroxylation at the β -position of the isopropyl group

was considered as most consistent with the experimental data. The glucuronide conjugate M3 was identified by means of enzymatic cleavage in the presence of a glucuronidase/sulphatase enzyme mixture (Vane *et al.* 1990) and by mass spectrometry. M3, isolated from an incubation of radiolabelled tezosentan with human hepatocytes, exhibited a parent ion of m/z 784 and two characteristic fragments at m/z 608 and 580, indicative for the glucuronide adduct of ^{14}C -labelled tezosentan, the molecular ion of ^{14}C -tezosentan itself and the fragment after loss of molecular nitrogen.

Blood-to-plasma partitioning

The mean blood-to-plasma distribution ratio in freshly drawn blood samples from two individuals was 0.60 (0.57–0.63) and constant over the entire concentration range tested. The value of 0.60 is close to the theoretical minimum blood-to-plasma distribution ratio which is defined as (1-haematocrit), the mean of the latter being determined as 0.40. It can therefore be concluded that tezosentan does not significantly penetrate into human red blood cells and resides almost exclusively in plasma.

Plasma protein binding

Tezosentan was highly bound to plasma proteins with a free fraction of 0.74–0.96%. Albumin was shown to represent the major binding component whereas no binding to α_1 -acid glycoprotein was observed. Saturation of plasma protein binding was only seen at concentrations above 40–50 $\mu\text{g ml}^{-1}$ (data not shown).

Inhibition of human CYP enzymes

The effects of tezosentan on the activities of the main human CYP enzymes are summarized in table 2. With the exception of CYP2C9, no inhibition was observed of any of the CYP forms up to a tezosentan concentration of 100 μM . The effect on CYP2C9 was weak with a reduction to 55% of the initial activity at the highest concentration of 100 μM .

Table 2. IC_{50} values for tezosentan and enzyme-specific reference inhibitors for the inhibition experiments with recombinant human CYPs.

CYP isoform	Marker assay	IC_{50} (tezosentan) (μM)	IC_{50} (reference) (μM)
1A2	CEC- <i>O</i> -deethylation ¹	> 100	2.8 (furaphylline)
2C9	MFC- <i>O</i> -demethylation ²	> 100 ³	0.8 (sulfaphenazole)
2C19	CEC- <i>O</i> -deethylation	> 100	2.0 (tranylcypromine)
2D6	AMMC- <i>O</i> -demethylation ⁴	> 100	0.003 (quinidine)
3A4	BzRes- <i>O</i> -debenzylation ⁵	> 100	0.02 (ketoconazole)
	BFC- <i>O</i> -debenzylation ⁶	> 100	0.06 (ketoconazole)

¹ CEC, 3-cyano-7-ethoxycoumarin; ² MFC, 7-methoxy-4-trifluoromethylcoumarin; ³ enzymatic activity was reduced to 55% of initial activity at the highest tezosentan concentration of 100 μM ; ⁴ AMMC, 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin; ⁵ BzRes, benzyloxyresorufin; ⁶ BFC, 7-benzyloxy-4-trifluoromethylcoumarin.

Discussion

The three concentration–time profiles of tezosentan and total radioactivity in plasma and blood shown in figure 2 indicate that maximum concentrations were attained between 0.5 and 1.0 h after start of the infusion. After termination of the infusion, the concentrations declined in a biphasic manner. The initial, very rapid decline was characterized by a half-life of 3–5 min, depending on whether tezosentan itself or total radioactivity was assayed. Owing to the lower sensitivity of radioactivity detection, the second disposition phase with a half-life of about 4 h was only well characterized when tezosentan was quantified by LC-MS/MS. The systemic plasma clearance was 341 h^{-1} , classifying tezosentan as an intermediate to high clearance drug. The volume of distribution at steady state was 20 litres, i.e., close to the volume of extracellular body water (Rowland and Tozer 1995) indicating limited distribution of drug into tissues. Pharmacokinetic parameters determined with total radioactivity were very similar to those of tezosentan itself indicating that the latter compound constituted the major drug-derived component in plasma. In line with this assumption was the metabolic pattern recorded from plasma samples taken 1 h after start of the infusion in which unchanged tezosentan accounted for the majority of radioactivity. The pharmacokinetic parameters determined with both methods are in good agreement with results published earlier (Dingemanse *et al.* 2002a, b, van Giersbergen *et al.* 2002). Peak plasma concentrations, C_{max} , and AUC values for total radioactivity in blood were 30–40% smaller than the respective values in plasma indicating that tezosentan poorly binds to or penetrates into erythrocytes. This correlated well with the mean blood-to-plasma distribution ratio of 0.60 determined *in vitro* which was close to the minimum possible value defined by the haematocrit of the blood sample.

Excretion of total radioactivity was virtually complete within 4 days with most of the radioactivity excreted into faeces. Urinary excretion accounted for less than 5% of the dose and no radioactivity was detectable in expired air. The latter finding was expected since the ^{14}C -radiolabel was intentionally incorporated into the tetrazole ring which was considered as metabolically stable based on prior experience gained with the structural analogue bosentan (Weber *et al.* 1999). The rapid recovery most likely reflects the short disposition half-life and is in good agreement with preclinical findings in rats showing complete excretion of the radiolabel within 4 days and lack of any radiolabel retention in tissue in a whole-body autoradiography study (Actelion Pharmaceuticals, data on file).

Most of the radioactive material in faeces was unchanged drug, indicating that tezosentan, without prior metabolism, is mainly excreted into the bile. Under physiological conditions, tezosentan exists as a highly water-soluble dianion with a $\log D_{7.4}$ of 0.015. The low conversion rates in the incubations with human liver microsomes and hepatocytes as well as the lack of significant inhibition of any of the major human CYP enzymes are most likely reflections of this hydrophilic character. Taking into account the molecular weight of 605 Dalton, which is well in excess of the minimum 500 Daltons generally considered as threshold for efficient biliary excretion in humans, the observed excretion pattern was well within the predictions made from preclinical data.

The metabolism of tezosentan in humans is shown in figure 9. Two metabolites were observed in human faeces samples and identified as isomeric hydroxylation products at the isopropyl group of the pyridyl sulfonamide substructure. It is interesting to note that only M1 was observed in all four subjects whereas the

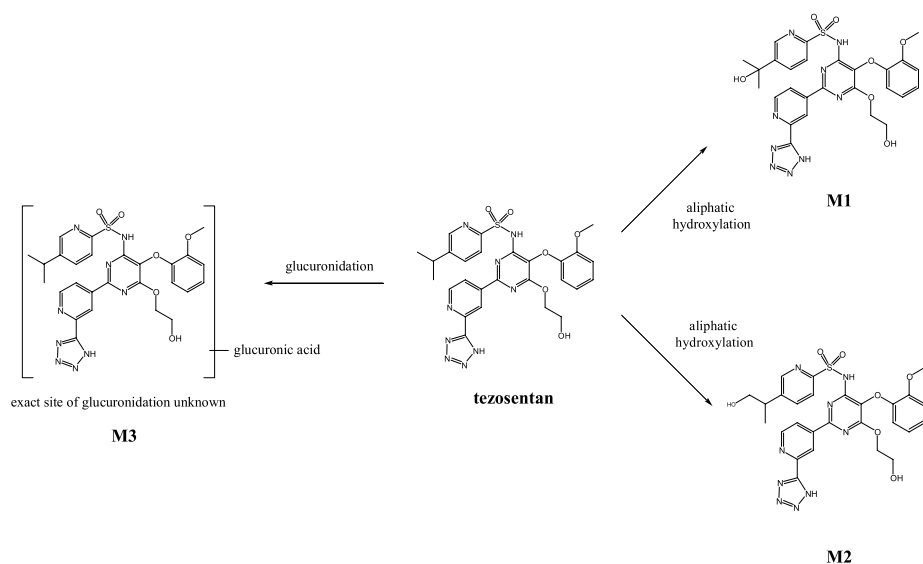


Figure 9. Metabolism of tezosentan in humans.

extent of M2 formation was variable. In preclinical studies, only formation of M1 was observed with the batch of human hepatocytes used for the recording of metabolic profiles and subsequent isolation of metabolites. The fact that tezosentan exhibited a weak inhibition of CYP2C9-mediated activity and that the hydroxylation of the structural analogue bosentan at the *t*-butyl group is also catalysed by CYP2C9 might be taken as indirect indications that this enzyme is involved in the formation of M2. CYP2C9 is known to be polymorphic (Brockmoller *et al.* 2000, Redman 2001) and the observed variability in the formation of M2 might be rationalized in terms of a CYP2C9-mediated metabolism.

Apart from hydroxylation at the isopropyl group, tezosentan underwent glucuronidation in the presence of human hepatocytes to form M3. With respect to the chemical structure of tezosentan, conjugation might occur either at the ethylene glycol moiety or the tetrazole ring and examples are known for both types of reactions (Perrier *et al.* 1994, Krieter *et al.* 1995, Fisher *et al.* 2001). Again, in analogy to bosentan that bears the same ethylene glycol moiety but does not undergo glucuronidation, conjugation at the tetrazole ring appears as the more likely alternative despite the lack of definitive structural data. *In vivo*, however, M3 was not observed, which is most likely the result of enzymatic digestion of the glucuronide conjugate, if indeed formed in the liver, by the gut microflora (Chang *et al.* 1994).

Tezosentan has been tested for its inhibition of the main five human CYP enzymes, i.e. CYP1A2, 2C9, 2C19, 2D6 and 3A4. No effect was observed up to a tezosentan concentration of 100 μ M with the exception of a small inhibitory effect on CYP2C9 activity. With respect to the plasma concentrations observed in clinical trials and the fact that tezosentan is excreted almost exclusively as unchanged drug, tezosentan pharmacokinetics are neither expected to be modified in the presence of CYP inhibitors nor is there an obvious potential for CYP-mediated interactions on concomitantly administered drugs.

In conclusion, the disposition profile of the dual endothelin receptor antagonist tezosentan in humans after intravenous dosing is characterized by a biphasic decline of plasma concentrations with half-lives of 3–5 min and 4 h, respectively, high systemic plasma clearance and limited distribution into tissues. Tezosentan is highly bound to plasma proteins, mainly albumin, and does not penetrate into red blood cells. Excretion into faeces as unchanged drug constitutes the major elimination pathway, whereas renal excretion is negligible. Small amounts of two isomeric hydroxylated metabolites were detected in faeces representing at most 5% of the administered dose. Based on *in vitro* results, the potential of tezosentan to be involved in CYP-mediated drug–drug interactions appears to be small.

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Pharmacokinetics, metabolism and excretion of the glycine antagonist GV150526A in rat and dog

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Received 7 October 2002

1. The pharmacokinetics, metabolic fate and excretion of 3-[2-(phenylcarbamoyl) ethenyl]-4,6-dichloroindole-2-carboxylic acid (GV150526), a novel glycine antagonist for stroke, in rat and dog following intravenous administration of [¹⁴C]-GV150526A were investigated.

2. Studies were also performed in bile duct-cannulated animals to confirm the route of elimination and to obtain more information on metabolite identity.

3. Metabolites in plasma, urine and bile were identified by HPLC-MS/MS and NMR spectroscopy.

4. GV150526A was predominantly excreted in the faeces via the bile, with only trace metabolites of radioactivity in urine (< 5%). Radioactivity in rat bile was predominantly due to metabolites, whereas approximately 50% of the radioactivity in dog bile was due to parent GV150526.

5. The principal metabolites in bile were identified as glucuronide conjugates of the carboxylic acid, whereas in rat urine the main metabolite was a sulphate conjugate of an aromatic oxidation metabolite. Multiple glucuronide peaks were observed and identified as isomeric glucuronides and their anomers arising from acyl migration and mutarotation.

Introduction

GV150526A, (3-[2-(phenylaminocarbonyl) ethenyl]-4,6-dichloroindole-2-carboxylic acid as the sodium salt), is a novel, potent and selective antagonist of the modulatory glycine site of the *N*-methyl-D-aspartate (NMDA) receptor. Neural cell damage after stroke is thought to occur (Zivin 1997) from a rapid increased release of glutamate and a subsequent over stimulation of the NMDA receptor which results in a massive influx of Ca²⁺ into the post-synaptic neurones and ultimately cell death. As glutamate can only activate the NMDA receptor in the presence of the co-agonist glycine, a glycine antagonist blocking the modulatory effect of the glycine-binding site is an attractive target for controlling brain damage after stroke onset. GV150526A was shown to be highly efficacious in animal models of stroke and was selected for development for the treatment of ischaemic stroke (Di Fabio *et al.* 1997, 1998, Gaviraghi *et al.* 1997).

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Previous studies have described the pharmacokinetics (Hoke *et al.* 2000) and the *in vitro* and *in vivo* metabolic fate of GV150526 (Gilissen *et al.* 2000) in humans. The studies described here were performed in the rat and dog using both radiolabelled and non-radiolabelled material to obtain information about the pharmacokinetics, metabolic fate and excretion routes of GV150526A in these species. Comparisons were made with the previously published data on human *in vitro* and *in vivo* metabolism.

Materials and methods

Chemicals and materials

GV150526A was labelled at the side chain double bond carbon as shown in figure 1 and was synthesized by the Radiosynthesis Laboratory, Department of Chemical Development, GlaxoSmithKline S.p.A, Verona, Italy. Specific activity was 4.14 MBq mg^{-1} ($112 \mu\text{Ci mg}^{-1}$) and radiochemical and chemical purities were both $> 99\%$ as measured by HPLC and TLC, respectively. GV150526A and GV217053 (MET-2), equivalent to GV150526 hydroxylated on the *para*-position, GV154679 *ortho*-isomer and GV218787 *meta*-isomer on the phenyl ring with chemical purities $> 99\%$ were synthesized by the Medicinal Chemistry Department, GlaxoSmithKline, Verona. Liquid scintillation reagents (Soluene-350, Pico-Fluor 40 and Hionic-Fluor) were purchased from Packard Bioscience (Milan, Italy). All the other reagents were of analytical or HPLC grade. β -glucuronidase (Type B-1, CE 3.2.1.31, sulphatase-free, from bovine liver) and sulphatase (Type H-1, EC 3.1.6.1 from *Helix pomatia*) were obtained from Sigma-Aldrich (Milan, Italy).

Animal experimentation

The research complied with national legislation and with the company policy on the Care and Use of Animals and with related codes of practice.

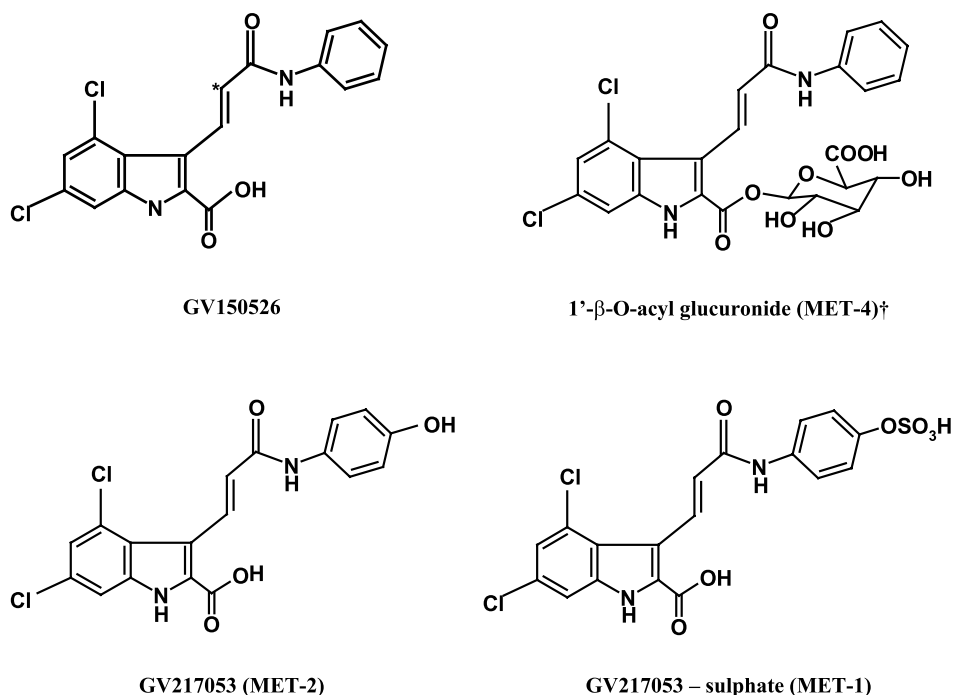


Figure 1. Structures of GV150526 and its principal metabolites in rat and dog. †MET-3ab and MET-5ab are isomers of MET-4 arising from acyl migration. *Position of the radiolabel.

Rat studies. Male and female Wistar rats (140–255 g) were purchased from Iffa Credo (Monticello, Italy) and maintained on '4RF 18' diet (Mucedola srl, Milan, Italy) with water provided *ad libitum*. Food was withdrawn 16 h before compound administration and restored 4 h after dosing. For all studies, animals were housed in standard metabolic cages suitable for the collection of urine and faeces.

For the mass balance study, rats were dosed via an intravenous bolus with 10 mg kg^{-1} (about $50 \mu\text{Ci kg}^{-1}$) GV150526A (free acid equivalent) dissolved in 5% dextrose with a dose volume of 5 ml kg^{-1} . Four male and four female animals were killed at 5, 15, 30 min, 1, 2, 4, 6, 8, 24, 48, 72 and 96 h following dosing. Blood was collected by cardiac puncture under light ether anaesthesia and placed into heparinized containers at 4°C . After liquid scintillation counting of a suitable aliquot, the remaining blood sample was centrifuged at 4°C and the harvested plasma counted for radioactivity. Residual plasma was subsequently stored at -80°C . Urine and faeces were collected from four separate male and four female rats at the intervals 0–6, 6–24, 24–48, 48–72, 72–96, 96–120, 120–168 and 168–192 h after dosing. Urine samples were collected in glass containers cooled to approximately -20°C . All samples were stored at -80°C immediately after collection.

Two biliary excretion studies were performed in the rat. In the first, five male and three female Wistar rats had their bile ducts cannulated towards the liver. The tubing was exteriorized at the back of the neck and connected to a swivel joint, allowing free movement about the cage. After surgery, animals were allowed to recover in individual cages for at least 18 h. During that time, bile was not returned to the duodenum and the drinking water was supplemented with 0.9% sodium chloride, 0.05% potassium chloride and 5% glucose. Animals were administered with 10 mg kg^{-1} ^{14}C GV150526 A (as the free acid) as above. Food was allowed 4 h after administration of ^{14}C GV150526 A and water and saline solutions were available *ad libitum* following surgery. Bile was collected in plastic vials maintained at approximately 4°C at intervals after dosing of 0–1, 1–2, 2–3, 3–4, 4–6, 6–8 and 8–24 h. The bile flow was observed to be constant up to 24 h. Urine was collected in glass containers cooled to approximately -20°C at 0–6 and 6–24 h after dosing. Faeces and cage washings were collected at 24 h after dosing. All samples were stored at -80°C immediately after completion of the collection.

A second study was performed using non-radiolabelled GV150526 at a higher dose, which provided bile samples for principal metabolite identification by NMR. Three Han Wistar male rats were cannulated as above and 60 mg kg^{-1} GV150526A was administered intravenously (as the free acid). The formulation used was 30% glycofurol, 2% poloxamer 188 in injectable water, which had previously been noted not to alter GV150526 pharmacokinetics after intravenous administration in rats (unpublished data). A single bile sample was collected for each animal into glass vials, which were maintained at about -20°C . After completion of the collection the vials were stored at -80°C .

Dog studies. Male and female Beagle dogs (> 6 months, 8–10 kg) were provided by F Morini breeding kennels (S. Polo d'Enza, Italy). The dogs were fed a diet coded 6TH 20 GLP-top certificate, produced by Mucedola srl (Milan, Italy). For all studies, animals were housed in standard metabolic cages suitable for the collection of urine and faeces. Food was withdrawn at least 16 h before dosing and allowed 4 h after dosing.

For the mass balance study, four male and four female dogs were dosed via an intravenous fast infusion with 10 mg kg^{-1} (about $20 \mu\text{Ci kg}^{-1}$) GV150526A (free acid equivalent) dissolved in 5% dextrose. The dose volume was 4 ml kg^{-1} and the rate of infusion was 8 ml min^{-1} , resulting in a 4–5-min infusion. Blood samples were taken at the following times: predose, 5, 15, 30, 45 min, 1, 2, 4, 6, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h after the end of the infusion. Blood was collected via the jugular vein and placed into heparinized containers at 4°C . After liquid scintillation counting of a suitable aliquot, the remaining blood sample was centrifuged at 4°C and harvested plasma counted for radioactivity. Urine and faeces were collected from the same dogs at 24-hourly intervals up to 10 days after dosing. An additional 0–6-h collection was made for urine. Urine samples were collected at 4°C and all samples stored at -80°C immediately after completion of the collection.

For the biliary excretion study, one male Beagle dog (11.4 kg, supplied by Bioscience Support, Glaxo-Wellcome, Ware, UK) possessing a chronically implanted biliary fistula dosed with 10 mg kg^{-1} ^{14}C GV150526 A (as acid) dissolved in 5% dextrose was used. The dose volume and rate of administration was as above. Bile was collected hourly up to 8 h at room temperature and then subsequently redirected into the gut lumen. Further collections were made at approximately 24, 48 and 72 h after dosing. Blood samples were collected from the jugular vein at 1, 4, 8 and 24 h after dosing. Urine and faeces were collected at 0–24, 24–48 and 48–72 h after dosing. All samples were stored at -80°C immediately after completion of the collection.

Determination of radioactivity

Radioactivity was determined by liquid scintillation counting using models LS3801, LS6000 (Beckman, Milan, Italy) and Tricarb1900, 2200CA, or 2500CA (Packard Bioscience) with quench correction by external standardization. Background values were subtracted from each counting. The limit of quantification was defined as twice the mean background counts per min for each type of sample. Plasma, urine and bile samples were assayed for radioactivity by dilution with suitable volumes

of scintillation cocktail. Faecal samples were homogenized in a suitable volume of distilled water to ensure easy handling. Suitable aliquots were weighed and radioactivity was determined after solubilization and bleaching. For dog faeces samples from the biliary excretion study, the sample was combusted by a Packard Bioscience 307 Sample Oxidizer before liquid scintillation counting.

Determination of GV150526

In plasma, GV150526 (free acid) was determined using a validated column switching HPLC method. Briefly plasma (150 μ l) was diluted with water (125 μ l) and 99% formic acid (25 μ l) and injected onto a C1 precolumn. After suitable washing, the sample was back flushed onto a Hypersil ODS analytical column (10 $\times$ 0.5 cm, 5 μ m) with mobile phase (methanol:25 mM phosphate buffer, pH 7.5, 60:40). At a UV detector wavelength of 343 nm, the limit of quantification was 10 ng ml⁻¹. GV150526 was determined in urine and bile by direct injection after suitable dilution with either mobile phase (1:10 for urine) or water (1:1 for bile). Chromatographic conditions were the same as for plasma. The complete specificity for GV150526 in all matrices was confirmed by LC-MS/MS.

Metabolite profiling

Separation of radioactive components in biological extracts was performed by gradient HPLC with on-line radiochemical (Flo-One, Packard Bioscience) and UV detection. Urine and bile samples were diluted 1:1 with water and injected directly onto column. Plasma samples (50 μ l) were deproteinized with methanol (150 μ l), centrifuged and the supernatant diluted further with water (100 μ l) before injection. HPLC profiling was performed employing a Hypersil ODS (25 $\times$ 0.46 cm, 5 μ m) column and a mobile phase containing ammonium acetate (25 mM, pH 8.0) (solvent A) and methanol (solvent B). Elution was achieved by starting with 40% B and over 5 min changing to 50% A. For the next 15 min the composition of B changed from 50 to 60% B, followed by a more rapid ramp to 85% B in the next 15 min. Conditions were returned to the starting mobile phase composition over the next 6 min and left to equilibrate for a further 12 min before the next analysis. Column temperature was maintained at 35 °C and the flow rate was 1 ml min⁻¹. UV detection was at 343 nm. Radioactivity was detected in a 0.5 ml flow cell with a scintillator flow of 3 ml min⁻¹. LC-MS/MS analysis confirmed that no metabolites co-eluted with GV150526.

Metabolite isolation under neutral conditions

Semipreparative chromatography (Hypersil ODS 25 $\times$ 1 cm, 5 μ m) was used to isolate and purify metabolites from rat and dog bile and rat urine for characterization by MS. The studies were performed using radioactive compound. Samples were injected directly after diluting 1:1 with distilled water. The HPLC mobile phase containing methanol and the gradient system as described above was used with a flow rate of 3 ml min⁻¹. Collected fractions were concentrated by vacuum centrifugation and reconstituted in a small volume of acetonitrile:water (50:50) before MS analysis.

Metabolite isolation under acidic conditions

As it became evident from initial studies that unstable glucuronide metabolites were present, rat bile samples from the subsequent non-radiolabelled biliary excretion study were purified under acidic conditions. A pooled sample (1.5 ml) from three animals was diluted to 5 ml and used to prepare samples for metabolite identification and injected on to a semipreparative column (Inertsil ODS-3 10 cm $\times$ 10 mm). An acetonitrile:water gradient (0.01% TFA, pH 2.5) increasing from 0 to 100% organic over 30 min at flow rate of 4 ml min⁻¹ was used. Collected fractions were concentrated by vacuum centrifugation and reconstituted in a 50:50 mixture of ACN/D₂O before analysis by NMR.

Enzymatic hydrolysis

Bile (rat and dog) and urine (rat) samples from the studies using radiolabelled GV150526A were hydrolysed enzymatically to obtain some preliminary qualitative information on the likely presence of conjugates. The enzymatic procedure involved incubation of aliquots (100 μ l) with a solution containing 2500 units β -glucuronidase or 100 units sulphatase dissolved in 0.2 M sodium acetate buffer, pH 5.0. Control incubations were also performed for bile and urine, using buffer as enzyme control (2 ml). All samples were incubated at 37 °C for 24 h for enzymatic hydrolysis. Aliquots of 50 μ l of each sample were injected into the HPLC system and analysed using the gradient system used for metabolite profiling, as described above.

Mass spectrometry

Mass spectrometry detection of GV150526 and metabolites was aided by the presence of two chlorine atoms, which provided a useful fingerprint from the natural isotopic abundance of chlorine. Fast-ion bombardment mass spectrometry (FAB-MS) and FAB-MS/MS were performed on a triple

quadropole instrument (VG Quattro, Manchester, UK) using 3-nitrobenzyl alcohol (NBA) as the solid probe matrix.

NMR

Isolated rat bile metabolite fractions were dissolved in a 50:50 mixture of acetonitrile and D₂O with the pH adjusted to 3.3 using formic acid and placed into 5-mm NMR tubes and analysed by a Bruker (Coventry, UK) DRX600 NMR spectrometer operating at 600.13 MHz. ¹H-NMR spectra were acquired using a 1D NOESY presat sequence to perform dual solvent suppression and the chemical shifts referenced in ppm with respect to the residual acetonitrile signal at 2.00. The 1'-β-O-acyl glucuronide metabolite fraction was freeze-dried and redissolved in phosphate buffer (pH 7.4, D₂O/ACN 1:1) to induce the formation of the acyl migrated isomers and ¹H-NMR spectra acquired over 24 h.

Results

Pharmacokinetics

The pharmacokinetic parameters of GV150526 are shown in table 1. As no differences between sexes were observed, all data were averaged. Standard deviations for rat pharmacokinetics could not be calculated because they were derived from plasma profiles originating from one rat per time point. The mean plasma AUC_{0-∞} ratio of GV150526/total radioactivity was 0.86 and 0.79 in rat and dog, respectively, indicating that plasma metabolite levels were very low and plasma radioactivity was mostly due to GV150526. The estimated elimination half-lives of the total radiolabelled drug-related material in both rat and dog were longer than that of GV150526, suggesting a greater retention of metabolites in the body compared with GV150526 or possibly enterohepatic recycling of metabolites.

Stability of glucuronide conjugates in blood and plasma

Studies in rat and dog blood (data not shown) indicated that instability (mainly of MET-4) was evident at room temperature even after only 10 min. However, stability of all glucuronide isomers was good for up to at least 3 h when maintained at 4 °C. Stability of GV150526 and the glucuronide conjugates in rat and dog plasma samples, obtained after GV150526 administration, was good at -80 °C for up to 6 months. These sample-handling precautions ensure the integrity of the plasma samples and therefore the plasma concentration data and derived pharmacokinetic parameters from the present study are not influenced by potential *ex vivo* conversion of glucuronide metabolites to GV150526. No stability studies of the glucuronide conjugates were performed in urine and bile, therefore precise quantitative information on relative metabolite levels cannot be given.

Table 1. Single-dose pharmacokinetics of GV150526 in rat and dog following intravenous administration at 10 mg mg⁻¹.

Species	Analyte	C _{max} (μg ml ⁻¹)	Vd _{area} (l kg ⁻¹)	AUC _{0-∞} (μg h ml ⁻¹)	t _{1/2} (h)	Cl _R (ml min ⁻¹ kg ⁻¹)
Dog (n = 4 M/F)	GV150526	85.7 (8.6)	0.5 (0.1)	72.8 (10.5)	2.6 (0.3)	2.1 (0.3)
	TRDM	84.6 (10.0)	—	91.8 (10.6)	—	—
Rat (n = 4) M/F)	GV150526	109 (8.5)	0.70	137	6.6	1.22
	TRDM	112 (8.2)	—	159	—	—

TRDM, total radiolabelled drug-related material.
Values in parentheses are SD.

Mass balance studies

Mean recoveries of radioactivity in male and female rats are shown in table 2. The majority of administered radioactivity was excreted in the faeces (79 and 88% of the dose in males and females, respectively), suggesting that significant biliary excretion occurs. There was a slight sex difference in the route and extent of excretion with males excreting more in the urine and less in bile than females (3.93 and 1.59% of the dose, respectively, in urine). Analysis of parent GV150526 in urine indicated that only approximately 10% of the radioactivity present unchanged GV150526. The mean total recovery of radioactivity over 196 h was 83 and 90% for male and female rats, respectively. There was no evidence for any accumulation of the dose, with < 0.2% of the dose remaining in the carcass 196 h after administration. Radioactivity eliminated as carbon dioxide was negligible. In addition, a whole-body radiography study did not reveal substantial retention of radioactive material (all unpublished data).

In dog, the situation was very similar with a mean of 91% of the radioactivity being excreted in faeces and only 3.9% in urine. No sex differences in elimination were noted. Elimination of radioactivity was rapid, with the majority of the dose excreted in the first 48 h following dosing, and essentially completed by 72 h after dosing. Approximately 40% of radioactivity in urine was attributed to GV150526. Radioactivity recovery was high at 95%.

Biliary excretion studies and metabolite profiling using radiolabelled GV150526A

Both rat and dog biliary excretion studies confirmed that the majority of the dose was eliminated via bile into faeces. Only negligible amounts (< 2%) were eliminated by direct secretion into the intestine. The bile collected in these studies was used to obtain metabolite profiles. Metabolites were denoted by ‘MET’ and a number selected depending upon their chromatographic retention time. Where there was an indication of two metabolites co-eluting (shoulder on peak), they were identified by MET followed by the elution order number but with the addition of ‘ab’. Variation in the absolute retention times was due to changes in column characteristics with time. Representative chromatograms of rat and dog bile and also rat plasma are shown in figure 2. The chromatograms indicated the possibility of up to seven metabolites being present in rat and dog bile. In rat, the majority of the radioactivity was present in the form of two metabolites, MET-4 and MET-5ab and GV150526 accounted for less than approximately 10% of the dose. In dog, MET-4 and MET-5ab were also present as the major metabolites, but more than

Table 2. Mean percentage recovery of radioactive dose in rat and dog 196 h following intravenous administration of ¹⁴C-GV150526 at 10 mg kg⁻¹.

Excreta	Rat		Dog	
	Male	Female	Male	Female
Urine	3.9 (0.7)	1.6 (0.2)	3.2 (1.6)	4.5 (1.6)
Faeces	78.5 (2.5)	87.7 (6.7)	90.7 (1.8)	91.4 (1.2)
Total	82.4 (28)	89.3 (6.6)	93.9 (0.7)	95.9 (1.3)

Values in parentheses are SD.

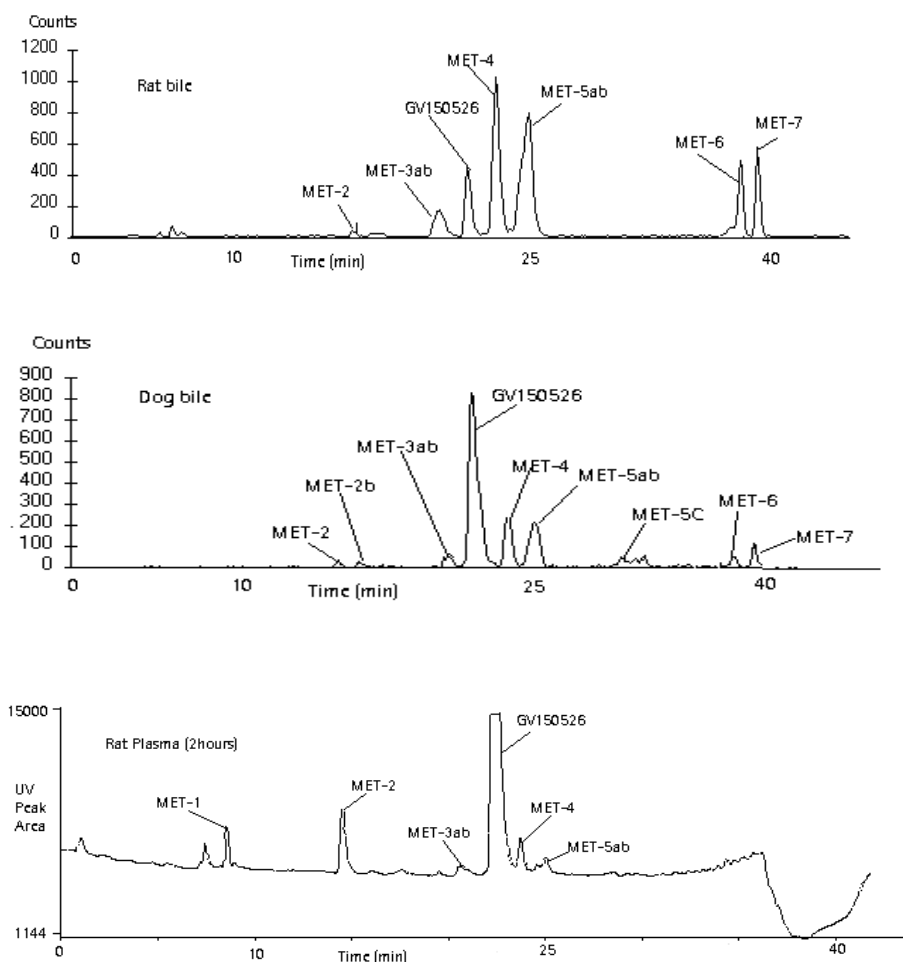


Figure 2. Typical HPLC radio or UV chromatograms of GV150526 and its metabolites in rat and dog bile and rat plasma. Radioactivity units are dpm. UV wavelength is 343 nm. HPLC conditions: Hypersil ODS (25×0.46 cm, $5 \mu\text{m}$) column. Mobile phase ammonium acetate (25 mM, pH 8.0) (solvent A) and methanol (solvent B). Gradient elution was as follows: 0–5 min 40 $\rightarrow$ 50% B, 5–20 min 50 $\rightarrow$ 60% B, 20–35 min 60 $\rightarrow$ 85% B, 35–41 min 85 $\rightarrow$ 40% B.

approximately 50% of the radioactivity eliminated in bile appeared to be due to GV150526. Accurate quantitative information cannot be given here because of the possibility of some acyl migration and hydrolysis *ex vivo*.

Metabolite identification

Enzymatic hydrolysis of bile and urine. After incubation of rat bile and dog bile samples with β -glucuronidase at pH 5.0, HPLC analysis indicated qualitatively that MET-4 was hydrolysed and converted to GV150526, but that MET-5ab was relatively stable to hydrolysis (figure 3). The levels of MET-3 were too low to determine stability. The results suggest that the metabolites are glucuronide conjugates of which only MET-4 is substantially cleavable by glucuronidase.

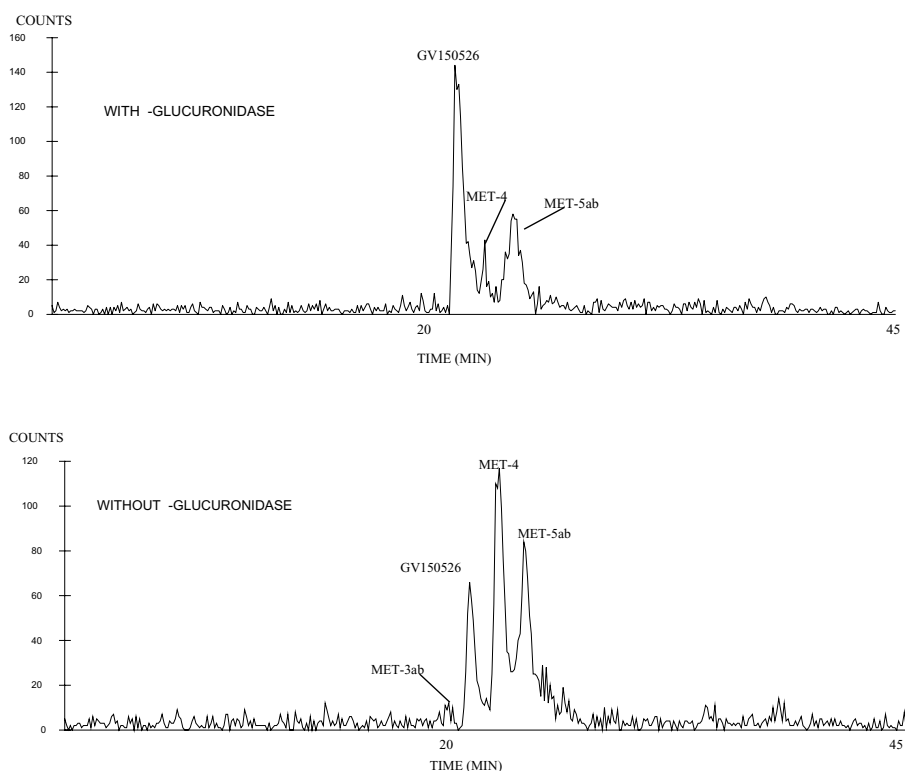


Figure 3. Typical HPLC chromatograms obtained from male rat bile samples with and without β -glucuronidase treatment taken 6–8 h after intravenous administration of ^{14}C GV150526A. Radioactivity units are dpm. HPLC conditions are as for figure 2.

The HPLC profile of urine samples and HPLC fraction MET-1 after hydrolysis with sulphatase show that MET-1 was converted to MET-2 (figure 4), indicating that MET-1 is probably a sulphate conjugate of MET-2.

Analysis of plasma, bile and urine samples by mass spectrometry. The FAB mass spectrum of the HPLC fraction containing the major metabolite from rat urine (MET-1, figure 4), and suspected from enzymological studies to be a sulphate conjugate, gave a molecular ion at m/z 471 corresponding to the expected protonated sulphate conjugate with hydroxylated GV150526A $[\text{MH} + \text{SO}_4]^+$. This was confirmed by performing FAB/MS/MS of the m/z 471, which gave a number of product ions at mass to charge ratios, m/z 453 $[\text{MH} + \text{SO}_4 - \text{H}_2\text{O}]^+$, 391 $[\text{MH} + \text{O}]^+$, and 282 $[\text{MH} - (\text{NH}_2 - \text{C}_6\text{H}_5)]^+$ (table 3). FAB mass spectrum of the HPLC fraction from rat urine (MET-2) was dominated by m/z 391 corresponding to GV150526A with one hydroxy group. The HPLC retention time of the peak from this fraction was identical to a pure standard of the *para*-hydroxy substituted compound (GV217053), whereas the *ortho*- and *meta*-substituted compounds eluted at different times, indicating that the OH is in the *para*-position of the side chain aromatic ring.

The positive FAB mass spectrum of the three HPLC fractions (MET-3ab, MET-4 and MET-5ab) from rat bile all contained a strong signal at m/z 551

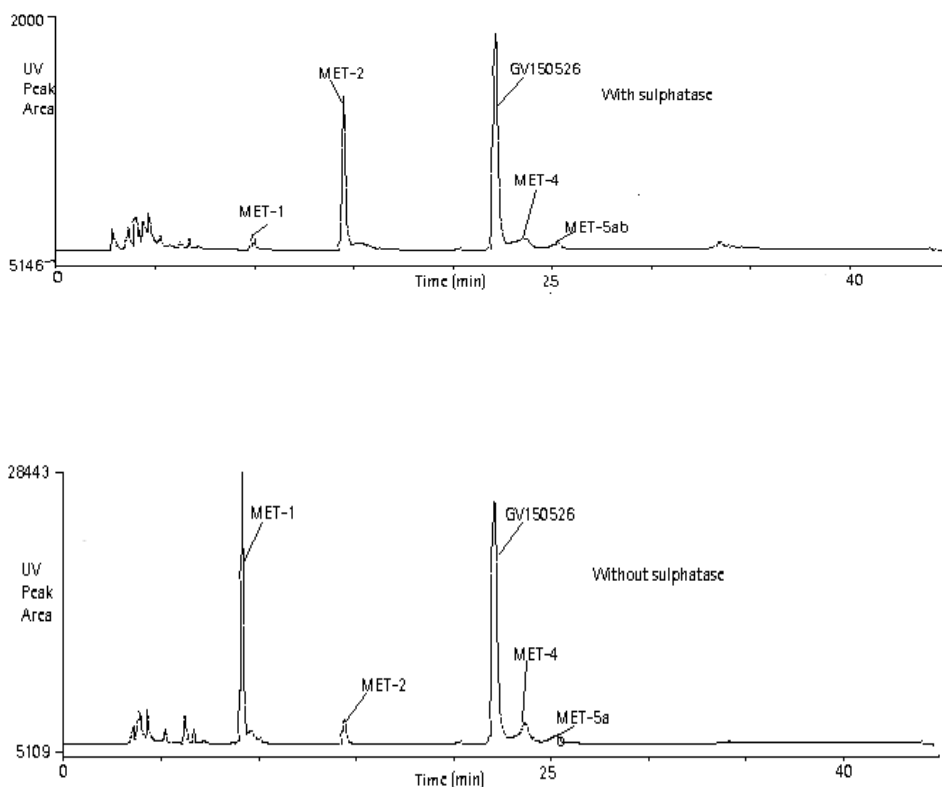


Figure 4. Typical HPLC chromatograms obtained from male rat urine with and without treatment with sulphatase taken 0–24 h after intravenous administration of ^{14}C GV150526A. UV wavelength is 343 nm. HPLC conditions are as for figure 2.

corresponding to GV150526 plus 176 mass units. This ion was tentatively attributed to a glucuronide conjugate of GV150526. This deduction was supported by the FAB/MS-MS spectrum of m/z 551 for MET-3ab, MET-4 and MET-5ab, which all gave the product ions 533 $[\text{M} + \text{C}_6\text{H}_9\text{O}_6] - \text{H}_2\text{O}]^+$ and 375 $[\text{MH}]^+$ (protonated GV150526) (table 3).

These data together with the results of glucuronidase hydrolysis strongly indicate that MET-3ab, MET-4 and MET-5ab are glucuronides of GV150526. As the molecular ion of GV150526 is a product ion of the glucuronide MS mass spectrum, it is highly probable that GV150526 is conjugated at the carboxylic acid. No specific studies on metabolite identification in rat and dog plasma were performed due to the very low percentage present. The retention times in the UV chromatogram (figure 2) suggested the presence of all the metabolites identified in rat urine and bile. No spectra could be obtained for trace metabolites MET-5c (dog bile only), MET-6 and MET-7 with any of the aforementioned techniques. Figure 1 shows the structures of the metabolites identified.

Analysis of rat and dog bile metabolites by NMR

^1H -NMR spectra of the rat bile metabolites from the non-radiolabelled study after isolation using the acidic pH method described above indicated primarily the

Table 3. Protonated molecular and product ions observed in the FAB/MS-MS spectra of HPLC fractions from rat bile and urine.

Metabolites (MET')	Protonated molecular (m/z)	Ion structure	Fragment ion ion (m/z)	Fragment ion assignment
1	471	MH ⁺ + SO ₄	453	(MH ⁺ + SO ₄)-(H ₂ O)
			391	MH ⁺ + O
			282	MH ⁺ -(C ₆ H ₅ -NH + H)
2	391	MH ⁺ + O	241	MH ⁺ -(C ₆ H ₅ -NH-CO-CH + H)
3ab	551	M ⁺ + C ₆ H ₉ O ₆	533	(M ⁺ + C ₆ H ₉ O ₆)-(H ₂ O)
			443	(M ⁺ + C ₆ H ₉ O ₆)-(CH ₅ -NH-O)
			375	MH ⁺
4	551	M ⁺ + C ₆ H ₉ O ₆	533	(M ⁺ + C ₆ H ₉ O ₆)-(H ₂ O)
			443	(M ⁺ + C ₆ H ₉ O ₆)-(C ₆ H ₅ -NH-O)
			375	MH ⁺
5ab	551	M ⁺ + C ₆ H ₉ O ₆	533	(M ⁺ + C ₆ H ₉ O ₆)-(H ₂ O)
			443	(M ⁺ + C ₆ H ₉ O ₆)-(C ₆ H ₅ -NH-O)
			375	MH ⁺
GV150526	375	MH ⁺		

presence of the 1'-β-O-glucuronide acid conjugate, which coincided with the retention of MET-4. Comparison of the ¹H-NMR spectrum of GV150526A with that of the 1'-β-O-glucuronide acid conjugate clearly indicates that the point of conjugation is the carboxylic acid moiety of GV150526A. This is evident from the effect on the proton signal of the double bond seen at 8.51 ppm in GV150526A (figure 5a), which moves to 8.31 ppm following conjugation (figure 5b). Additional signals seen in the NMR spectrum at 5.7 and between 3.5 and 3.8 ppm arise from the attached glucuronic acid moiety. Despite the optimization of the isolation procedures to stabilize the glucuronide conjugates from acyl migration and muta-

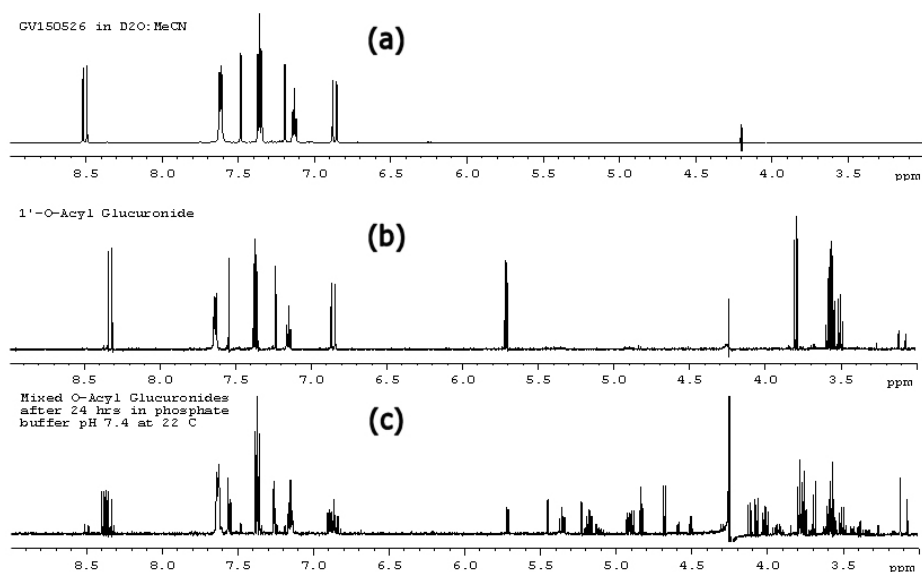


Figure 5. (a) ¹H-NMR spectrum of GV150526A, (b) the 1-β-O-acyl glucuronide conjugate isolated from rat bile under acidic conditions and (c) the 1-β-O-acyl glucuronide (pH 7.4) conjugate after 24 h.

Table 4. ^1H -NMR chemical shifts and coupling constants of the positional isomers following acyl migration from $1'\text{-}\beta\text{-O}$ -acyl glucuronide of GV150526.

	Glucuronic acid protons				
	1'	2'	3'	4'	5'
1'-O-acyl (β -anomer)	5.72 (7.7)	3.51	3.59	3.56 (2×9.5)	3.79 (9.9)
2'-O-acyl (α -anomer)	5.45 (3.7)	4.92 (3.7, 9.9)	4.02 (9.2, 9.9)	3.59	4.07 (10.3)
2'-O-acyl (β -anomer)	4.83 (8.1)	4.90 (8.1, 9.5)	3.77	3.57	3.69 (9.9)
3'-O-acyl (α -anomer)	5.23 (3.7)	3.78	5.46 (2×9.5)	3.78	4.12 (10.3)
3'-O-acyl (β -anomer)	4.68 (7.7)	3.51 (7.7, 9.5)	5.18 (9.2, 9.5)	3.79*	3.75* (9.9)
4'-O-acyl (α -anomer)	5.20 (4.0)	3.62 (4.0, 9.2)	3.91* (2×9.5)	5.10	4.30 (10.2)
4'-O-acyl (β -anomer)	4.59 (8.1)	3.34 (8.1, 9.5)	3.71 (2×9.5)	5.12	3.93* (9.9)
Glucuronic acid (α -anomer)	5.20 (4.0)	3.43 (4.0, 9.9)			3.95 (10.3)
Glucuronic acid (β -anomer)	4.51 (8.1)				

*Tentative assignments.

Chemical shifts determined in the phosphate buffer (pH 7.4): MeCN (1:1) spectrum after 24 h and are quoted in ppm from acetonitrile.

Coupling constants are quoted in parentheses (Hz).

rotation, the fractions examined showed the presence of minor amounts of the $2'\alpha$ and $2'\beta$ -O-isomers.

Repeated measurement of the ^1H -NMR spectrum of the isolated $1'\text{-}\beta\text{-O}$ -glucuronide conjugate redissolved in phosphate buffer (pH 7.4, $\text{D}_2\text{O}/\text{ACN}$ 1:1) over a few hours indicated the formation of the 2α - and β -, $3'\alpha$ - and β - and $4'\alpha$ - and β -isomers at the expense of the $1'\text{-O}$ -isomer. The ^1H -NMR spectrum measured after 24 h shows the presence of all the isomers (figure 5c). The chemical shifts and coupling constants of all the isomers have been assigned and are given in table 4. After 6 days no $1'\text{-}\beta\text{-O}$ -glucuronide conjugate could be detected in the sample and only an equilibrium mixture of glucuronide conjugate isomers could be observed (figure 6) as well as free glucuronic acid, presumably resulting from the hydrolysis of the conjugated metabolites. These assignments are also given in table 4. Previous ^1H -NMR analysis of the principal metabolite fraction (MET-4) from dog bile, using neutral mobile phase conditions, also confirmed that MET-4 in dog bile is the $1'\text{-}\beta\text{-O}$ -glucuronide as obtained above for rat bile.

Discussion

The pharmacokinetics of GV150526A in rat and dog indicate a low clearance and low volume of distribution. These parameters are strongly influenced by extensive protein binding (Iavarone *et al.* 1999), which effectively limits tissue distribution and limits clearance. These characteristics are also observed in man (Hoke *et al.* 2000). The similarity of radioactivity and GV150526 AUCs indicated very low levels of metabolites circulating in plasma. However, further studies on bile duct-cannulated rats and dog revealed that the elimination was in fact mainly

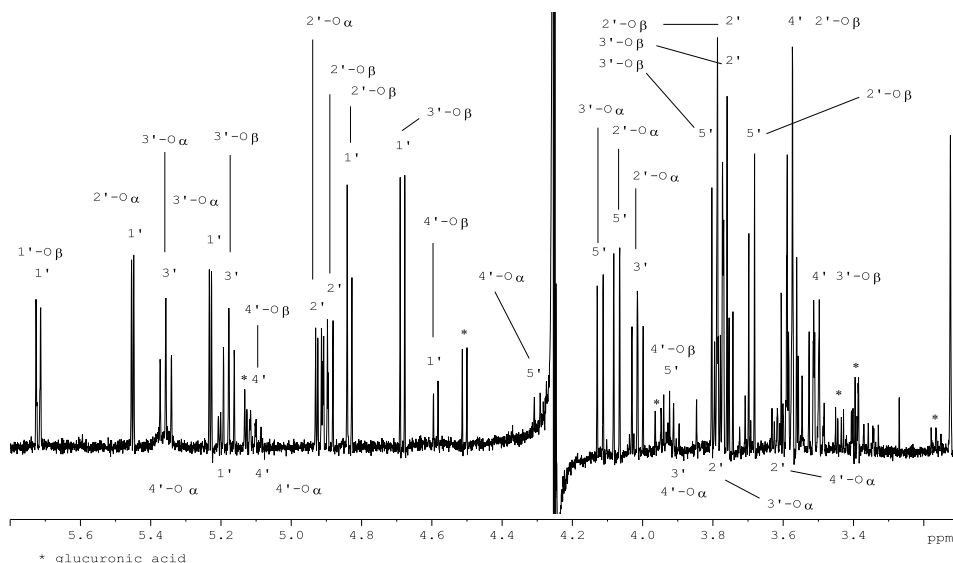


Figure 6. ^1H -NMR spectrum of the glucuronide region (3.3–5.6 ppm) of the equilibrium mixture of isomers.

metabolic in nature, especially for the rat. Clearly, once formed in the liver, metabolites are efficiently cleared into the bile duct rather than transported back into the systemic circulation.

The mass balance and biliary excretion studies have revealed that following intravenous administration GV150526 is predominantly eliminated in the faeces via bile. A combination of enzymatic studies, LC-MS/MS and NMR have been used to confirm that the majority of metabolites in rat and dog bile are glucuronide conjugates of the carboxylic acid group. ^1H -NMR analysis confirmed that the major drug-related metabolite in rat and dog bile is the 1'- β -O-glucuronide conjugate (MET-4) of the carboxylic acid group of GV150526. Although the 1'- β -O-glucuronide conjugate is initially formed *in vivo* in both rat and dog as the major metabolite, migration of the acyl group onto the different available hydroxyl groups of the glucuronic acid moiety, produces a series of positional isomers and anomers. This can occur *in vivo* and *ex vivo*. The enzymatic studies confirmed, at least for MET-5ab, that the glucuronide isomers formed from acylmigration are stable to β -glucuronidase (Hasegawa *et al.* 1982). The propensity of ester glucuronide conjugates to undergo isomerization and mutarotation by means of an internal rearrangement reaction is well known (Faed 1984, Spahn-Langguth and Benet 1992). There are three possible positional isomers (Lenz *et al.* 1996, Sidelmann *et al.* 1996), each may exist in the α - or β -forms thereby giving in theory a further six species in addition to the 1-O-acyl that can only exist in the β -form. Although the acyl migration reaction occurs spontaneously in solution, the rate of reaction is dependent on the pH as well as the nature of the aglycone. At pH 5–7 and above, acyl migration and hydrolysis to the aglycone begins to occur. Therefore, precautions have to be taken in the collection and storage of samples in studies where the formation of ester glucuronic acid conjugates is suspected so that acyl migration and hydrolysis in samples *ex vivo* is avoided. Stability is ensured by acidifying and cooling the samples as soon as possible after sampling (Spahn-

Langguth and Benet 1992). When the excretion balance and biliary excretion studies, with radiolabelled GV150526A, were initially performed, the nature of its metabolism was unknown and therefore these precautions were not taken. Although the blood stability of GV150526 and the glucuronide conjugates was subsequently observed to be satisfactory in the sampling-handling conditions used in this study, controlled stability studies at 4 and -80°C in bile and urine spiked samples were not performed. Therefore, accurate quantitative information on relative glucuronide conjugate concentrations cannot be given in this study. Preparative HPLC purification under acidic conditions followed by NMR enabled the confirmation of the identity of the principal biliary rat metabolite as MET-4 and induction of acyl migration of this same sample enabled the unambiguous characterization of all its subsequent acyl migrated isomers.

Analyses of rat urine by enzymatic techniques and LC-MS/MS revealed the presence of a *para*-hydroxylated derivative of GV150526 (MET-2) and its sulphate conjugate (MET-1), the latter being the major metabolite in rat urine. In rat and dog bile, MET-2 was only observed in trace quantities, whilst MET-1 was not detected at all. The qualitative metabolic profile of GV150526 in human plasma (Gilissen *et al.* 2000) is the same as that observed in rat and dog. Parent GV150526 is by the far the major component and both multiple glucuronide conjugates and the hydroxylated sulphate conjugate were also observed in human plasma (Gilissen *et al.* 2000).

For MET-6 and MET-7, no mass spectra could be obtained. However, it was clear from the HPLC retention time of these peaks that they were considerably more lipophilic than GV150526 or its glucuronide metabolites, and it suggested that the acid group was no longer present. It was strongly suspected that the peaks were analytical artefacts produced by the utilization of methanol in the mobile phase. Salmon *et al.* (1974) and Hasegawa *et al.* (1982) reported that the presence of methanol in samples containing labile acyl glucuronides could produce analytical artefacts by the methanolysis of either the acidic group of the aglycone or the acidic group of the glucuronic acid moiety. In fact, in subsequent studies, methanol was replaced by acetonitrile and these peaks were subsequently no longer detected.

The propensity for reactive acyl glucuronides to bind covalently to proteins potentially producing a toxic or anaphylactic response has been well documented. In particular, the non-steroidal anti-inflammatory drugs, e.g. benoxaprofen (Qiu *et al.* 1997), mefenamic acid (McGurk *et al.* 1996) and diflunizal (Wang and Dickinson 1997) amongst others, all of which form reactive glucuronide metabolites, have demonstrated this characteristic. This possibility was considered also for GV150526. However, at no stage in the preclinical or clinical development of GV150526 was there any evidence of toxicity that could be associated with protein adduct formation.

In summary, the pharmacokinetics and metabolism of GV150526 in rat and dog have been studied. Low clearance and volume of distribution were dominant features in both species. Whereas metabolite levels in plasma were very low, in both species GV150526 was eliminated preferentially in bile as glucuronide metabolites of the parent compound. A less preferential metabolic route was the *para*-hydroxylation of GV150526 and subsequent conjugation by sulphation and which was the major metabolite in rat urine. Evidence of acyl migration was obtained by HPLC-MS/MS and NMR. However, no evidence of toxicity relating

to potential protein adduct formation with the isomeric glucuronides was observed either in animals or man. The qualitative metabolic profile in rat and dog was identical to that in human plasma.

Acknowledgements

The authors thank the following personnel from GlaxoSmithKline Verona for valuable contributions to this work: Dr Mario Almi of Chemical Development, for the preparation of the radiolabelled GV150526A, Dr M. Hamdan for performing the FAB/MS-MS analysis, Dr Scott Harrison (GlaxoSmithKline, Ware) for providing expert guidance during the dog biliary excretion study and Mr Alberto Ruffo for excellent technical assistance.

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Hepatotoxicity of ketoconazole in Sprague–Dawley rats: glutathione depletion, flavin-containing monooxygenases-mediated bioactivation and hepatic covalent binding

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Received 5 September 2002

1. This study has examined ketoconazole (KT)-induced hepatotoxicity *in vivo* and *in vitro*, using male Sprague–Dawley rats with [³H]KT (1.5 µCi mg⁻¹) at 40 and 90 mg KT kg⁻¹ doses. Blood and liver samples were collected from 0 to 24 h for alanine aminotransaminase (ALT), glutathione (GSH) and covalent binding analyses.

2. Covalent binding occurred as early as 0.5 h, peaked at 2 h (0.026 ± 0.01 nmol KT mg⁻¹ protein) and 8 h (0.088 ± 0.04 nmol KT mg⁻¹ protein) for 40 and 90 mg KT kg⁻¹ doses, respectively. ALT levels increased at 0.5 h for the 40 and 90 mg KT kg⁻¹ doses (44.3 and 56.4 U ml⁻¹, respectively) relative to control, 22.7 U ml⁻¹. At 24 h, the 90 mg KT kg⁻¹ dose reduced hepatic GSH levels from 9.92 ± 1.1 to 4.76 ± 0.3 nmol GSH mg⁻¹ protein.

3. The role of the flavin-containing monooxygenases (FMO) utilized Sprague–Dawley microsomes with 1, 10 and 100 µM [³H]KT. Maximum covalent binding occurring at 100 µM KT. Heat inactivation of microsomal FMO significantly decreased covalent binding by 75%, whereas 1 mM GSH significantly reduced covalent binding by 65%.

4. Thus, KT-induced hepatotoxicity is dose- and time-dependent and appears to be FMO mediated, in part, to metabolites that may react with protein and, possibly, GSH.

Introduction

Ketoconazole (KT), an effective broad-spectrum oral anti fungal, has been used in immune-compromised patients since the late 1970s and in recent years has exhibited reliable effectiveness against advanced prostate cancer (Bok and Small 1999). Classified as an azole compound due to the presence of a pyrrole ring within the structure, KT has been shown to inhibit cytochrome P450 (CYP) 3A4 (Back *et al.* 1992), CYP11A (Kurokohchi *et al.* 1992) and is a weak inhibitor of CYP1A and CYP2B (Rodrigues *et al.* 1987a, b). In fungi, KT blocks the conversion of lanosterol to ergosterol by inhibiting the CYP lanosterol 14 alpha-demethylase responsible for the oxidative removal of the [¹⁴C] methyl group from lanosterol resulting in a loss of membrane integrity (Jacobs and Nall 1988, Vanden Bossche *et al.* 1988). KT has also been associated with fatalities (Duarte *et al.* 1984, Janssen 1995) and numerous cases of hepatitis, resulting in increases in serum liver enzymes indicating possible hepatic damage (MacNair *et al.* 1981, Svejgaard and Ranek 1982, Lewis *et al.* 1984, Stricker *et al.* 1986, Benson *et al.* 1988, Knight *et al.*

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1991, Chien *et al.* 1997). Other symptomatic problems include malaise, dark urine, jaundice (Van Parys *et al.* 1987, Cauwenbergh 1989), headache, dizziness, drowsiness and papilloedema (Bok and Small 1999).

The therapeutic concentrations, dosages and duration of treatment for KT vary depending upon the medical diagnosis. The mean peak serum concentrations in humans range from 8 to 19 μM after a 200 mg day⁻¹ dose (Heel *et al.* 1982, Huang *et al.* 1986) and from 13 to 26 μM following a 400 mg day⁻¹ dose (Baxter *et al.* 1986); however, plasma concentrations have been reported to be as high as 94 μM (Brass *et al.* 1982, Sugar *et al.* 1987). In rats, plasma levels of 24 and 62 μM KT resulted from 10 and 20 mg kg⁻¹ doses, respectively (Gascoigne *et al.* 1981). After oral administration in rats, KT and/or its metabolites have shown a higher concentration in the liver than in the plasma (Gascoigne *et al.* 1981, Whitehouse *et al.* 1990).

Specific tissue binding of KT to rat hepatic microsomal fractions was reported to be 89% (Daneshmend and Warnock 1988), indicating possible inhibition of monooxygenase metabolism with the binding of KT to rat liver *in vivo* and *in vitro*. The half-life of KT increases with increasing dose (Gascoigne *et al.* 1981, Daneshmend *et al.* 1983, Huang *et al.* 1986), thus, long-term administration will result in auto-inhibition of metabolism and a potential problem for drug accumulation (Heel *et al.* 1982).

KT-induced hepatitis was first reported in 1981 (MacNair *et al.* 1981) and the first fatality occurred in 1982 due to hepatic coma (Duarte *et al.* 1984). Sudden hepatitis has led to several deaths even though the patients underwent frequent laboratory and clinical evaluations (Duarte *et al.* 1984, Lewis *et al.* 1984, Bercoff *et al.* 1985, Stricker *et al.* 1986). Two other patients that suffered KT-induced hepatitis survived due to liver transplants (Brusko and Marten 1991, Knight *et al.* 1991). An autopsy of the patient who died of KT treatment revealed massive hepatocellular necrosis (Lewis *et al.* 1984). Moreover, a European trial of high dose KT (1200 mg day⁻¹) for prostate cancer resulted in 11 deaths out of 350 participants within 2 weeks of starting the high-dose therapy (Janssen 1995).

Prostate cancer is the second leading cause of cancer death in American men and one in five men will develop prostate cancer during their lifetime (IIT 1999). KT has been found to have inhibitory effects on the precursors of testosterone by inhibiting 17,20-lyase that specifically blocks the synthesis of dehydroepiandrosterone and androstenedione (Bok and Small 1999). High doses of KT of 400 mg/8 h or 600 mg/12 h for 6–9 months result in significant deprivation of androgen, a major factor in prostate cancer (De Coster *et al.* 1996, Bok and Small 1999). The hepatotoxic symptoms that can be mild to severe may develop within 2 days or up to 1 year after the initiation of therapy (Knight *et al.* 1991). The hepatotoxicity appears to be reversible with discontinued use of KT (Janssen and Symoens 1983, Lewis *et al.* 1984, Benson *et al.* 1988, Chien *et al.* 1997).

Challenging problems persist with drug-induced liver injuries such as acute hepatitis. Mechanistic evaluation in the future could aid structural analysis of an entire chemical family such as the azoles. Clinical symptoms, such as increases in serum bilirubin and alanine transaminase (Van Parys *et al.* 1987, Cauwenbergh 1989), indicate cellular damage, but the toxic mechanistic profile of KT is still unclear in humans (Bok and Small 1999) as well as in animals. It is suspected that toxicity responses are not due to an immunoallergic mechanism (Bok and Small 1999) but as the result of a reaction of either KT and/or its metabolites (Lewis *et al.*

1984, Bercoff *et al.* 1985, Stricker *et al.* 1986, Gradon and Sepkowitz 1990, Brusko and Marten 1991). An *in vitro* study using cultured rat hepatocytes has reported that the primary metabolite of KT, *N*-deacetyl ketoconazole (DAK), is more hepatotoxic than KT (Rodriguez and Acosta 1997a). Continued efforts in our laboratory have established that the primary metabolite, DAK, undergoes flavin-containing monooxygenase (FMO)-mediated metabolism to the *N*-deacetyl-*N*-hydroxy ketoconazole (*N*-hydroxy-DAK) metabolite (Rodriguez and Acosta 1997b, Rodriguez *et al.* 1999). The *N*-hydroxy-DAK metabolite is a secondary hydroxylamine, which is susceptible to further FMO-mediated oxidative metabolism, which could produce toxic and reactive metabolites that could be responsible, in part, for the toxicity of KT.

To date, studies investigating the hepatotoxicity associated with KT *in vivo* have not been conducted. The current study evaluates the possible metabolic bioactivation pathways, CYP or FMO, which could be responsible for producing toxic metabolites resulting in hepatic injury. *In vivo* studies have been used to evaluate the comparative changes in serum alanine aminotransaminase (ALT), hepatic glutathione (GSH) and hepatic covalent binding with one another to establish if a relationship exists between metabolism, toxicity and covalent binding. *In vitro* studies will address the involvement of CYP and FMO and GSH on the covalent binding of KT with hepatic microsomes.

Materials and methods

Chemicals and supplies

KT was a generous gift from Janssen Pharmaceuticals (Olen, Belgium), ^3H -KT (5 Ci mmole^{-1} , 98% purity) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). Reduced glutathione (GSH), oxidized glutathione (GSSH) reductase, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, trichloroacetic acid (TCA), sodium hydroxide, Trizma, ethylenediaminetetraacetic acid (EDTA), sucrose, glycerol, glucose-6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺ were purchased from Sigma (St Louis, MO, USA). Alanine aminotransaminase (ALT) analysis was conducted with a Sigma Diagnostics Transaminases kit (ALT/GPT), No. 505-P. HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and CytoScint was purchased from ICN (Costa Mesa, CA, USA). Sprague-Dawley (SD) rats were purchased from Simonsen Laboratories, Inc. (Gilroy, CA, USA).

Animal treatment and sample collection

Male SD rats (200–220 g) were treated intraperitoneally (i.p.) with ^3H -KT ($1.5\text{ }\mu\text{Ci mg}^{-1}$) at doses of 40 and 90 mg KT kg^{-1} . Control rats were treated with 0.9% normal saline. Blood and liver samples were collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h for ALT, GSH and covalent binding analyses. The blood samples and approximately 1.1 g liver were placed on ice for ALT, GSH, covalent binding and protein determination. The remaining liver samples were snap-frozen and placed in a -80°C freezer for metabolite isolation, purification and identification studies. All experiments were performed in triplicate.

In vivo covalent binding studies

After the male SD rats were administered ^3H -KT ($1.5\text{ }\mu\text{Ci mg}^{-1}$) at doses of 40 and 90 mg KT kg^{-1} , approximately 1 g from each of the liver samples was homogenized with Trizma buffer (100 mM Trizma, 1 mM EDTA, 250 mM sucrose, pH 7.5). One ml of the homogenate was mixed with 2 ml ice-cold 0.9 M TCA and centrifuged at $8000g$ at 4°C for 10 min to precipitate the protein. The pellet was then washed three times with ice-cold 0.6 M TCA (2 ml). The protein pellet was then washed six times with ice-cold 80% methanol (2 ml) until the supernatant fraction of the wash achieved background levels of radioactivity. The final protein pellet was dissolved in 1 ml 1 M NaOH at 60°C for 1 h and a 0.3-ml aliquot was placed into scintillation vials containing 2.75 ml scintillation fluid with 1.5% (v/v) glacial acetic acid. Radioactivity was measured in dpms by a Beckman LS6500 scintillation counter (Fullerton,

CA, USA) and normalized to nmol KT mg⁻¹ protein. Protein was determined using the Coomassie PlusTM Assay from Pierce (Rockford, IL, USA). All studies were performed in triplicate.

In vivo glutathione (GSH) studies

Collection of liver samples occurred between 08:30 and 11:30 hours to avoid diurnal pattern variations (Jaeger *et al.* 1973). The method of Roberts and Francetic (1993) was used for GSH analysis. In brief, a 50-mg liver sample was immediately removed from the rat, blotted dry, weighed and placed in 5 ml 5% sulfosalicylic acid (SSA w/v in water). The liver was then homogenized and centrifuged at 3000g for 10 min at 4 °C. The resulting supernatant was diluted 10-fold with sample buffer (100 mM potassium phosphate-buffer, 5 mM EDTA, pH 7.5) and placed on ice. A 300- μ l aliquot of the sample was combined in a vial with 450- μ l sample buffer, 100 μ l GSSG reductase (5 units ml⁻¹ in sample buffer) and 50 μ l DTNB. This sample mixture was then vortexed and 100 μ l was placed into a 96-well plate in duplicate. After a 1-min incubation in the 96-well plate, 100 μ l NADPH (2.4 mM, 2 mg ml⁻¹ in sample buffer) was added to the sample mixture. The 96-well plate was placed in a SpectraMax spectrophotometer, mixed and the absorbance was monitored at 412 nm at time zero and every 30 s for 1.5 min. The change in absorbance per min was determined and converted to μ mol GSH g⁻¹ protein using a calibration curve with known standards. The results are expressed in μ mol GSH g⁻¹ protein.

Alanine aminotransaminase (ALT) assays

For ALT serum determinations, blood samples were collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h. The blood samples were immediately placed on ice and allowed to coagulate before centrifugation at 4 °C and 8000g. The serum was collected and kept on ice. The ALT was assayed using a modified procedure from Sigma Diagnostic Kit #505-P for small sample volumes. Alanine alpha-ketoglutarate (0.25 ml) was placed into a test tube into a 37 °C water bath. Five microliters of serum and 45 μ l normal saline were added to the test tube containing alanine alpha-ketoglutarate. The sample mixture was gently mixed and placed back into the 37 °C water bath to incubate for 30 min. After the incubation, 0.25 ml of the Sigma color reagent (No. 505-2) were added to the incubation mixture, vortexed (low speed) and left at room temperature (25 °C) for 20 min. A total of 2.5 ml 0.4 N NaOH was then added to the mixture and vortexed. After 5 min, 200 μ l of the sample mixture was placed into a 96-well plate and the absorbance was measured at 505 nm on a SpectraMax 190 spectrophotometer (Sunnyvale, CA, USA). A calibration curve was performed to determine the ALT activity in units ml⁻¹ from the serum samples. ALT catalyses conversion of alpha-ketoglutarate and alanine to glutamate and pyruvate. Comparisons were made with the control rats. All experiments were performed in triplicate.

Microsomal preparations

Liver tissues were removed from male SD rats (200–220 g), immediately placed on ice and homogenized with 0.25 M sucrose, 100 mM Tris and 1 mM EDTA, pH 7.5. Microsomes were prepared by standard differential centrifugation and stored in 10 mM Tris, 1 mM EDTA and 20% glycerol, pH 7.5. The microsomes were frozen in liquid nitrogen and stored at –80 °C. Protein concentration was determined using the Coomassie PlusTM Assay from Pierce (Rockford, IL, USA).

In vitro covalent binding studies

Covalent binding of KT and/or its metabolites to hepatic microsomal protein and protein-dependency analyses were performed to determine linearity. The microsomal incubation mixture consisted of 0.25, 0.5 and 1.0 mg ml⁻¹ microsomal protein, incubation buffer (100 mM glycine-25 mM pyrophosphate buffer, pH 8.8), 1, 10 or 100 μ M KT with ³H-KT (1 μ Ci ml⁻¹), and an NADPH-generating system (10 mM glucose-6-phosphate, 1.0 unit ml⁻¹ glucose-6-phosphate dehydrogenase, 1 mM NADP⁺) at 37 °C in a total volume of 0.5 ml. A negative control without the NADPH-generating system was performed with each experiment. The microsomal incubations were terminated at 0, 0.5, 1, 2, 3, 4 and 8 h with 0.5 ml ice-cold 0.9 M TCA. The protein was precipitated, washed and the radioactivity was determined as described in the *in vivo* covalent binding studies section. All experiments were performed in triplicate.

To evaluate the affect of GSH on the covalent binding of KT and/or its metabolites, co-incubations of 100 μ M ³H-KT (1 μ Ci ml⁻¹) and 1 mM GSH using the microsomal incubations described above were performed in triplicate at 0, 1, 4 and 8 h. To evaluate the effect of FMO on the covalent binding of KT and/or its metabolites, microsomal FMO activity (but not CYP activity) was inhibited by heat inactivation (Ziegler 1980). The microsomes were heated at 50 °C for 90 s before adding them to the incubation mixture containing 100 μ M ³H-KT (1 μ Ci ml⁻¹), 1 mg ml⁻¹ microsomal protein and incubation buffer for a total volume of 0.5 ml. The microsomal incubation was terminated at 0, 1, 4 and 8 h with 0.5 ml 0.9 M TCA. Heat inactivation and incubation with 1 mM GSH was performed with 100 μ M ³H-KT (1 μ Ci ml⁻¹), 1 mg ml⁻¹ microsomal protein and incubation buffer for a total of 0.5 ml

was performed at 0, 1, 4 and 8 h. Comparisons of GSH incubations and heat-inactivated experiments were compared with the normal experimental conditions.

Statistical analysis

Statistical analysis of the ANOVA was performed by SAS statistical program, version 8.01, from SAS Institute, Inc. (Cary, NC, USA) to determine the significance of *in vivo* covalent binding. Tukey's multiple comparison was performed for the *in vivo* ALT study and Dunnett's multiple comparison was performed for the *in vivo* GSH studies. ANOVA analyses were performed for the *in vitro* dose- and time-response studies. Probabilities ≤ 0.05 were considered as statistically significant.

Results

In vivo Studies

Figure 1 shows the 'covalent' or tight binding of [^3H]KT-related material to hepatic proteins from SD rats treated i.p. with KT (40 or 90 mg kg $^{-1}$; 1.5 μCi mg $^{-1}$). The radioactivity was normalized to nmol KT mg $^{-1}$ hepatic protein. The 40 mg kg $^{-1}$ dose represents a low dose that would be expected to produce KT plasma concentrations in the 10–60 μM range, whereas the 90 mg kg $^{-1}$ represents a high-dose treatment for prostate cancer that would produce KT concentrations in the 75–100 μM range. There was a significant difference between KT-derived [^3H] binding to hepatic protein over the 24 h period using ANOVA ($p = 0.014$, $n = 3$). Covalent binding occurred as early as 0.5 h after treatment for both doses. The 40 mg KT kg $^{-1}$ dose peaked at 2 h (0.03 ± 0.01 nmol KT mg $^{-1}$ protein) with a steady decline thereafter. The 90 mg KT kg $^{-1}$ dose peaked at 8 h (0.09 ± 0.04 nmol KT mg $^{-1}$ protein), and then there was a steady decline to 24 h (0.04 ± 0.02 nmol KT mg $^{-1}$ protein).

Figure 2 shows the effect of i.p. administration of KT, 40 or 90 mg kg $^{-1}$, on serum ALT activity in SD rats. Interestingly, there was an immediate increase in ALT levels as early as 0.5 h after treatment for both doses (44.2 and 56.4 U ml $^{-1}$

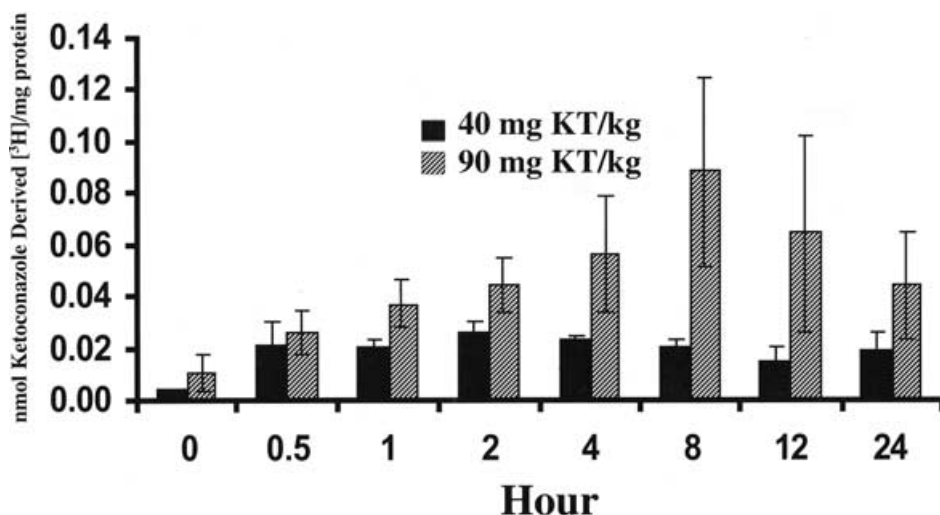


Figure 1. Covalent binding of ketoconazole (KT) to hepatic proteins from Sprague-Dawley rats treated i.p. with ^3H -KT (40 or 90 mg kg $^{-1}$; 1.5 μCi mg $^{-1}$). The radioactivity was normalized to nmol ketoconazole-derived [^3H] mg $^{-1}$ hepatic protein. There was a significant difference between the 40 and 90 mg kg $^{-1}$ treatments over time using ANOVA ($p = 0.014$, $n = 3$).

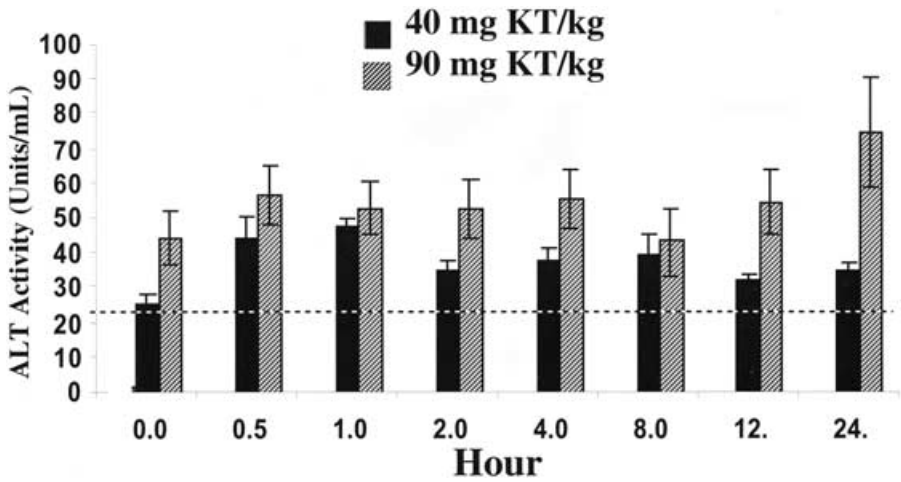


Figure 2. Effect of i.p. administration of ketoconazole (KT) (40 or 90 mg kg⁻¹) on serum alanine aminotransaminase (ALT) activity in Sprague–Dawley rats. The dashed line represents the ALT control value of saline-treated rats, 22.7 ± 9.7 U ml⁻¹. Tukey's multiple comparison indicates significant differences between the 90 mg KT kg⁻¹ dose and control and between the 40 and 90 mg KT kg⁻¹ doses (*p* < 0.05). Data are the mean ± SEM from three determinations.

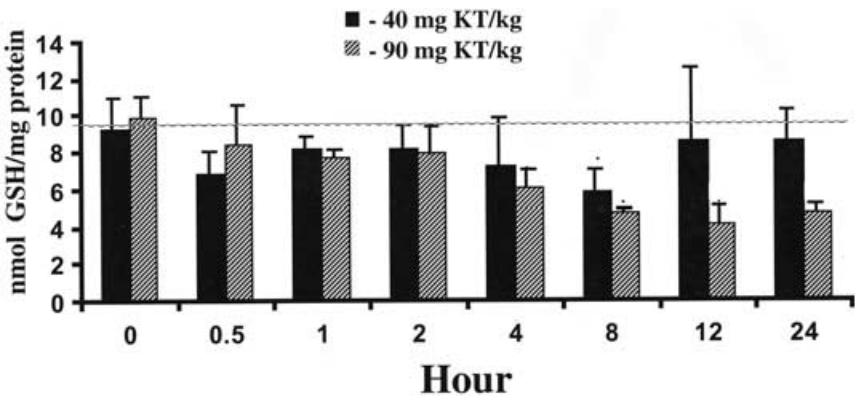


Figure 3. Effect of i.p. administration of ketoconazole (KT) (40 or 90 mg kg⁻¹) on hepatic glutathione (GSH) levels in Sprague–Dawley rats. The dashed line represents the GSH control value of saline-treated rats, 9.66 ± 1.51 nmol GSH mg⁻¹ protein. Dunnett's multiple comparison indicated a significant difference between 40 or 90 mg KT kg⁻¹ dose and control (*p* < 0.05). Data are the mean SEM from six determinations.

for the 40 and 90 mg kg⁻¹ doses, respectively). The control rats treated with normal saline had an ALT of 22.7 ± 9.7 U ml⁻¹. Also, there was an apparent and steady increase in ALT levels from 8 h to 24 h for the 90 mg KT kg⁻¹ dose. There was a significant difference between 90 mg KT kg⁻¹ dose and control using Tukey's multiple comparison (*p* < 0.05). There was also a significant difference between the 40 and 90 mg kg⁻¹ doses at 12 and 24 h after KT administration using Tukey's multiple comparison (*p* < 0.05).

Figure 3 demonstrates the effect of KT on hepatic GSH levels in SD rats. Owing to diurnal variation concerns about GSH levels, all doses were conducted to coordinate extractions between 08:30 and 11:30 hours, when GSH levels would be

at the highest level. The control rats had hepatic GSH levels of 9.66 ± 1.51 nmol GSH mg^{-1} protein. There was a significant difference between the 40 mg KT mg^{-1} and the control rats at 0.5 and 8 h where the GSH levels were 6.89 ± 1.1 nmol GSH mg^{-1} protein and 5.87 ± 1.1 nmol GSH mg^{-1} protein, respectively ($p < 0.05$). Afterwards, the GSH levels increased back to control values. The 90 mg KT kg^{-1} was significantly different from the control rats at 4, 8, 12 and 24 h where the GSH levels were 6.1 ± 0.91 , 4.7 ± 0.19 , 4.17 ± 0.9 and 4.76 ± 0.34 nmol GSH mg^{-1} protein, respectively, using the Dunnett's multiple comparison ($p < 0.05$). At 24 h, there was a significant difference between the 40 and 90 mg kg^{-1} doses, 8.55 ± 1.64 nmol GSH mg^{-1} protein and 4.76 ± 0.34 nmol GSH mg^{-1} protein, respectively ($p < 0.05$).

In vitro studies

Figure 4 shows the covalent binding of ^3H -KT (1, 10 and 100 μM ; 1 $\mu\text{Ci ml}^{-1}$) to hepatic microsomes from SD rats in an *in vitro* NADPH-generating system containing 1 mg ml^{-1} hepatic microsomal protein. Controls without NADPH were used for comparison for each concentration studied. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. All three KT incubation concentrations, 1, 10 and 100 μM , resulted in significant differences with control incubation conditions using an ANOVA ($p \leq 0.01$). As the concentration of KT increased, so did the covalent binding to hepatic protein. In addition, there appeared to be an

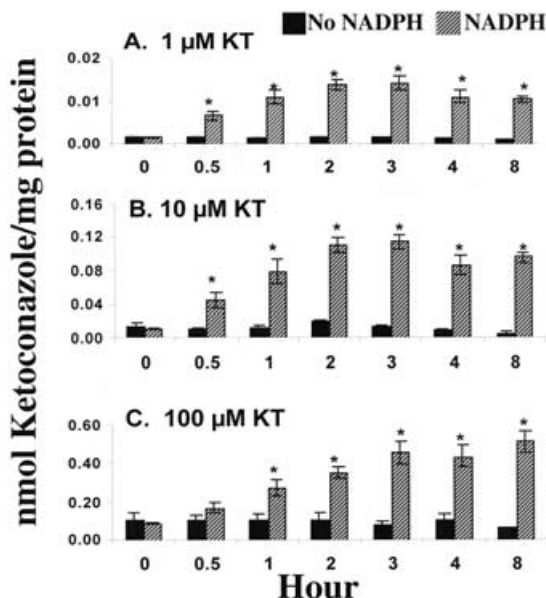


Figure 4. *In vitro* covalent binding of ^3H -ketoconazole (KT) (1 $\mu\text{Ci ml}^{-1}$) to hepatic microsomes from Sprague-Dawley rats in an NADPH-generating system. Controls without NADPH were used for comparison for each concentration. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. In (A), an asterisk indicates a significant difference between the 1 μM KT and control using ANOVA ($p < 0.05$, $n = 6$). In (B), an asterisk indicates a significant difference between the 10 μM KT and control using ANOVA ($p \leq 0.05$, $n = 6$). (C) * Significant difference between 100 μM KT and control using ANOVA ($p \leq 0.05$, $n = 6$).

increase in covalent binding with increasing incubation periods for the 10 and 100 μM KT. The 100 μM KT incubation had the highest covalent binding at 8 h (0.51 ± 0.06 nmol KT mg^{-1} protein). The control levels at 8 h were approximately 10-fold lower (0.06 ± 0.01 nmol KT mg^{-1} protein); thus, indicating an NADPH-dependent mechanism of covalent binding of *KT-derived activity*. The 10 μM KT incubation appears to reach a plateau at 2 h. Thus, there is a dose proportionality between 1 and 10 μM KT and saturation at 100 μM KT that is NADPH dependent. These studies were also performed with protein concentrations of 0.25, 0.5 and 1 mg ml^{-1} and ^3H -KT (10 and 100 μM). There was a linear increase over time (0–8 h) in the covalent binding of KT to the hepatic protein with 0.25 mg ml^{-1} microsomal proteins having a plateau at 2 h (data not shown).

Owing to a decrease in *in vivo* hepatic GSH levels, *in vitro* comparative analyses using microsomal incubations fortified with 1 mM GSH (*in vivo* GSH concentrations) were conducted to determine whether a reduction of covalent binding to hepatic proteins would occur. The *in vitro* covalent binding of 100 μM ^3H -KT (1 $\mu\text{Ci ml}^{-1}$) to hepatic microsomes (1 mg ml^{-1}) from SD rats in an NADPH-generating system with 1 mM GSH is shown in figure 5A. Control incubations without GSH were used for comparison for each of the time points evaluated. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. There was a significant reduction of covalent binding to hepatic protein

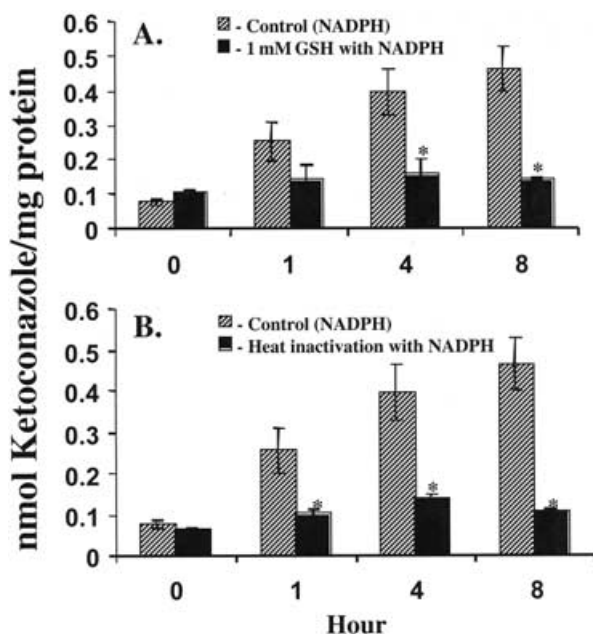


Figure 5. (A) Effect of glutathione (GSH, 1 mM) on *in vitro* covalent binding of ^3H -ketoconazole (KT) (100 μM ; 1 $\mu\text{Ci ml}^{-1}$) to hepatic microsomes from Sprague–Dawley rats in an NADPH-generating system. Controls without GSH were used for comparison. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. *Significant difference using ANOVA ($p \leq 0.05$, $n = 6$). (B) Effect of heat inactivation (50 $^{\circ}\text{C}$ for 90 s) on *in vitro* covalent binding of ^3H -ketoconazole (KT) (100 μM ; 1 $\mu\text{Ci ml}^{-1}$) to rat hepatic microsomes from Sprague–Dawley rats in an NADPH-generating system. Controls without heat inactivation were used for comparison. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. *Significant difference using ANOVA ($p \leq 0.05$, $n = 6$).

at 4 and 8 h using ANOVA ($p \leq 0.01$); even as early as 1 h after treatment, metabolic activation appears to occur, thus suggesting that KT might also bind to hepatic GSH.

To evaluate the role of microsomal FMO bioactivation of KT to reactive metabolite(s) that may bind to hepatic protein, heat inactivation of microsomal FMO was performed. Microsomal FMO activity was inhibited by heat-inactivation of the microsomes at 50 °C for 90 s, which is an established method to differentiate between FMO and CYP microsomal activities (Ziegler 1980). Figure 5B shows the effect of heat inactivation on *in vitro* covalent binding of 100 μM ^3H -KT (1 $\mu\text{Ci ml}^{-1}$) to SD rat hepatic microsomes in an NADPH-generating system containing 1 mg ml^{-1} microsomal protein. Control incubations without heat inactivation treatment were used for comparison at each time point evaluated. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. Interestingly, heat inactivation significantly reduced the covalent binding of KT-derived radioactivity to hepatic protein at the 1, 4 and 8 h using ANOVA ($p < 0.05$). The 8 h incubation had a greater than four-fold decrease from $0.46 \pm 0.06 \text{ nmol KT mg}^{-1} \text{ protein}$ to $0.11 \pm 0.01 \text{ nmol KT mg}^{-1} \text{ protein}$. Co-incubation of heat-inactivated microsomal FMO and 1 mM GSH resulted in similar reduction of covalent binding of KT and/or its metabolite(s) to hepatic protein and/or GSH as seen with the heat inactivation studies from 0.46 ± 0.06 to $0.09 \pm 0.01 \text{ nmol KT mg}^{-1} \text{ protein}$ (data not shown).

Discussion

This study is the first to investigate the hepatotoxicity associated with KT *in vivo*. All of the *in vivo* parameters evaluated, ALT levels, hepatic GSH levels and hepatic covalent binding suggest that KT produce hepatic injury as indicated by increases in ALT levels after KT administration (figure 2). This hepatic injury occurred as early as 0.5 h with both the 40 and 90 mg kg^{-1} doses. The 90- mg KT kg^{-1} dose is representative of high-dose KT treatment seen in patients being treated for prostate cancer. The high-dose KT therapy for prostate cancer ranges from 400 mg KT/8 h or 600 mg KT/12 h for a total daily dose of 1200 mg for 6–9 months (De Coster *et al.* 1986, Bok and Small 1999). As shown in figure 2, there is an initial and significant increase in ALT levels for the 40 and 90 mg KT kg^{-1} dose. The 90 mg KT kg^{-1} declines after 4 h and then increases at 12 h and 24 h. In the past, hepatic injury was suspected as being a reaction of KT or its metabolite(s) and not due to an immunoallergic response (Lewis *et al.* 1984, Stricker *et al.* 1986, Bok and Small 1999). Thus, the early release of ALT may be due to the parent compound, KT, whereas the later increase of ALT at 12 and 24 h may be due to reactive metabolite(s).

The present study also investigated the possibility that KT may covalently bind to hepatic macromolecules and/or to hepatic GSH in a dose- and time-dependent response. Figure 1 demonstrates that there was a definite dose-response in KT covalent binding to hepatic SD rat protein. The 90 mg KT kg^{-1} dose produced higher levels of $\text{nmol KT-derived } [^3\text{H}] \text{ mg}^{-1} \text{ protein}$ in comparison with the 40 mg KT kg^{-1} dose over the 24 h time period. Figure 3 indicates that hepatic GSH levels were significantly decreased after a 90 mg KT kg^{-1} dose in a time-dependent manner. The 90 mg KT kg^{-1} dose resulted in lower hepatic GSH levels than the 40 mg KT kg^{-1} dose at 4, 8, 12 and 24 h with the most difference between

the doses being at 24 h. In the 40 mg KT kg⁻¹ dose, the GSH levels appear to decrease from 0 to 8 h, then increase back to control GSH levels at 12 h and 24 h. It is likely the liver was able to recover from low-dose treatment and return to normal hepatic GSH levels. On the other hand, the 90 mg KT kg⁻¹ treatment continued to deplete hepatic GSH at 12 h and 24 h. When comparing the *in vivo* hepatic GSH levels with the covalent binding studies of KT to hepatic protein and the ALT studies, no significant correlations were made between the *in vivo* alterations in GSH and ALT levels (figures 2 and 3, respectively) to the covalent binding (figure 1) that occurred.

It is possible that as KT undergoes oxidative metabolism such that the metabolite(s) of KT may be reacting with GSH because the decrease in GSH occurs hours after exposure to KT rather than earlier. Thus, the parent compound, KT, may be directly binding to hepatic protein whereas the metabolite(s) bind to hepatic protein and hepatic GSH. Earlier studies indicate that the primary metabolite of KT is *N*-deacetylketonazole (DAK) in mice (Whitehouse *et al.* 1990) and rat (Rodriguez and Acosta 1997b, Rodriguez *et al.* 1999) and DAK is more hepatotoxic in an *in vitro* rat hepatocyte culture system (Rodriguez and Acosta 1997a). Furthermore, DAK is further metabolized by FMO to the *N*-deacetyl-*N*-hydroxyketonazole, a secondary hydroxylamine (*N*-hydroxy-DAK), metabolite (Rodriguez and Acosta 1997b, Rodriguez *et al.* 1999). *N*-hydroxy-DAK is susceptible to further FMO-mediated oxidative metabolism, where it has been speculated that toxic and reactive metabolites such as the nitrene of KT could be responsible, in part, for the toxicity of KT. Soft nucleophilic sulphur, selenium, nitrogen or phosphorus pharmaceuticals can undergo oxidative metabolism catalysed by FMOs (Ziegler 1988). Hydroxylamines may directly interact with biomacromolecules to impair cellular function (Cashman *et al.* 1999). Therefore, a reduction of GSH would be expected if bioactivation of an electrophilic metabolite such as DAK and *N*-hydroxy-DAK occurred due to the conjugating nature of this cellular scavenger to preserve the integrity of the cell (Hogberg and Kristoferson 1977). Another possibility is DAK undergoes Phase II metabolism by sulfation and/or glucuronide conjugation which would provide good leaving groups resulting in a nitrenium cation that could possibly bind to protein and DNA leading to adduct formation.

The *in vitro* covalent binding studies of KT and/or its metabolite(s) to SD rat hepatic microsomal protein was NADPH-dependent and inhibited by co-incubation with GSH (figures 4 and 5A). Figure 5A demonstrates that the addition of GSH in the microsomal incubation significantly reduced the covalent binding of KT and/or its metabolites to hepatic protein. The 1 mM GSH *in vitro* incubation with KT would prevent the formation of protein- and/or DNA-KT adducts, thereby possibly forming GSH-KT adducts. To differentiate between CYP- or FMO-mediated bioactivation to potential reactive metabolite(s), heat inactivation of microsomal-FMO fractions inactivated FMO activity, but CYP activity remained unaltered (Ziegler 1980). Earlier studies of DAK employed SKF-525A, an inhibitor of CYP activity (Ziegler and Mitchell 1972), and octylamine, a known positive effector for FMO and an inhibitor of CYP (Cashman and Ziegler 1986, Rodriguez and Acosta 1997), resulted in unaltered FMO-mediated metabolism. Figure 5B demonstrates that with NADPH present, a profound and significant reduction in covalent binding of KT and/or its metabolite(s) to hepatic protein by FMO heat inactivation of the rat liver microsomes. Because FMO-mediated

metabolism was inhibited, it is speculated that the metabolite(s) of KT is primarily responsible for the covalent binding to hepatic proteins. Continued efforts are being made to identify whether KT and/or its metabolite(s) bind to hepatic protein and to identify the nature of the protein adduct. Further studies are also being conducted to determine or verify if the binding of KT or its metabolites to GSH is mediated by liver microsomal FMOs or by individual FMO forms. If a GSH adduct is formed, attempts will be made to characterize its structure by mass spectrometry and NMR methods.

In summary, these studies demonstrate that KT-induced hepatotoxicity is dose- and time-dependent and appears to be dependent, in part, upon its bioactivation by FMO to metabolite(s) that may react with protein and, possibly, GSH. Also, GSH appears to be important in the detoxification pathway of KT by the probable conjugation of DAK and the *N*-hydroxy-DAK. Thus, our combined *in vitro* and *in vivo* studies point to potential mechanisms of KT-induced hepatotoxicity that merit further investigation.

Acknowledgements

The authors are greatly appreciative for the statistical assistance of Mr Jan-Shiang Taur and for Mr Sokkan Kimpau's technical assistance with the *in vivo* studies. Mr Kimpau was supported by the NIEHS minority training Grant No. 5 T35-ES07316-04. This research was supported by the 1999–2000 Pharmaceutical Manufacturers of America Foundation Research Starter Grant and C. J. B. was supported, in part, by the University Club Foundation of Oregon. As always, thanks to Dr Cristobal Miranda for his constructive review of the research and manuscript.

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Effects of 1,2-dibromoethane on haematopoiesis in the chick embryo

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Received 5 September 2002

1. Chick embryo *in ovo* was used to investigate the effects of 1,2-dibromoethane (DBE) on haematopoiesis at a developmental stage where the primitive erythroid cells divide and differentiate in circulation.

2. Early after DBE treatment on embryonic day 3, annexin V/propidium iodide labelling showed acute cell death of erythroid elements, which was subsequently compensated for by the release of immature cells into the circulation. Simultaneously, the comet assay indicated increased DNA damage in DBE-exposed blood cells when compared with controls.

3. After embryonic day 5, there was no indication for ongoing prominent cell death in the DBE-treated group. However, the DNA damage assessed by the comet assay persisted until embryonic day 10 in the peripheral blood cells, and for even longer in cells from thymus and bursa.

4. The kinetics of DNA fragmentation in both erythroid and lymphoid cells implied genotoxic damage by DBE to the stem cells of the definitive elements and transmission of this damage through the successive cell generations.

5. The early chick embryo provides a suitable alternative to mammalian models for investigation of long-term effects of xenobiotics on haematopoiesis.

Introduction

1,2-Dibromoethane (DBE; synonym: ethylene dibromide) has been widely used as a soil fumigant, an additive to leaded gasoline and an industrial solvent (Fishbain 1980, Alexeeff *et al.* 1990). People exposed for a long time to low concentrations of DBE suffered from bronchitis, headache, depression and impaired spermatogenesis, whereas intoxication with high doses results in severe damage to the liver and kidney, frequently with fatal consequences (Letz *et al.* 1984, IARC 1999, Prakash *et al.* 1999, Singh *et al.* 2000). Any route of exposure (inhalation, oral or dermal) leads to rapid distribution of DBE within the organism via the blood stream, and no effective antidote is as yet known (Humphreys *et al.* 1999). In experimental animals, the liver and kidney also appear to be major targets for DBE action (IARC 1999). Inhalation exposure of rats is associated with injury to the tracheal mucosa vessels and intraluminal haemorrhage (Koptagel and Bulut 1998). Damage to the vascular system seems to be a more general phenomenon because chick embryos treated intra-amniotically with DBE also bled from intra- and extra-embryonic vessels (Dušek *et al.* 2001). Other studies have

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described decreased fertility in treated animals, behavioural defects in their progeny (IARC 1999) and impaired development of directly exposed embryos (Mitra *et al.* 1992, Brown-Woodman *et al.* 1998, Dušek *et al.* 2001). Genotoxic properties of DBE have been demonstrated in many test systems (reviewed in IARC 1999). The long-term effects of DBE include carcinogenicity in experimental animals, but direct epidemiological evidence in man is still lacking (Weisburger 1977, Wong *et al.* 1982, Alavanja *et al.* 1990). According to the International Agency for Research on Cancer (IARC 1999), dibromoethane is classified as probably carcinogenic to humans – Group 2A.

The harmful effects of DBE are usually attributed to products of its bioactivation in the organism (e.g. Brem *et al.* 1974, Tezuka *et al.* 1980), although the teratogenic effects of DBE itself have also been reported (Brown-Woodman *et al.* 1998). Dibromoethane is metabolized by two main routes. Microsomal oxidation mediated by cytochrome P4502E1 represents the first pathway (Wormhoudt *et al.* 1996), leading to the formation of bromoacetaldehyde capable of binding to proteins and non-protein thiols (Hill *et al.* 1978). The second pathway gives rise to a highly reactive episulfonium ion via a glutathione-*S*-transferase (GST)-mediated conjugation of DBE with cellular glutathione. This episulfonium ion reacts with DNA and forms N^7 -guanine adducts (Ozawa and Guengerich 1983, Inskeep *et al.* 1986, Koga *et al.* 1986) and, to a lesser extent, N^1 -adenine adducts (Ballering *et al.* 1993). Treatment of isolated rat liver mitochondria with DBE induces formation of DNA adducts, even in mitochondrial DNA, and the process is accompanied by glutathione depletion, decreased ATP levels and inhibition of respiratory enzymes (Thomas *et al.* 2001).

Owing to the numerous adverse effects of DBE in the organism, the US Environmental Protection Agency (EPA) recommended to stop its use in agriculture more than 25 years ago. However, this prohibition has not been obeyed on a world-wide scale (Immig 1995, Singh *et al.* 2000) and industrial applications of DBE still lead to the exposure of a vast number of workers, e.g. in Central and Eastern Europe (Fabianová *et al.* 1999) or in Spain (Gonzales and Agudo 1999). In addition, DBE contaminates soil and groundwater (Xia and Rice 2001) and it has recently been shown in Minnesota, USA, that its concentration in the environment is higher than previously assumed based on mathematical modelling (Pratt *et al.* 2000).

The haematopoietic system appears to be highly sensitive to the action of genotoxicants. On the basis of large epidemiological studies, it is assumed at present that environmental and occupational exposure to pesticides, organic chemicals and heavy metals are risk factors for the development of heterogeneous group of clonal haematopoietic disorders – myelodysplastic syndromes (Rosenfeld and List 2000). The lack of experimental data on the effects of DBE on haematopoiesis encouraged us to study this in detail, using the well-characterized and complex model of the chick embryo *in ovo*. This model fits well with the present requirements to reduce the numbers of animals for testing purposes as no maternal organisms have to be killed in order to examine the embryos. It has been demonstrated that the immune system of the chick functions in a similar way to that of a human (Péault *et al.* 1987, Toivanen and Toivanen 1987). Therefore, the chick has become a commonly used tool for studies intent on the ontogeny of haematopoietic and lymphoid elements and has contributed substantially to the understanding of the fundamental concepts of immunology (Vainio and Imhof

1995). Erythroid cells of the early chick embryo differentiate in circulation (Lemež 1964, Bruns and Ingram 1973) and peripheral blood thus resembles, in part, the bone marrow of adult organisms including humans. Moreover, the first population of primitive erythrocytes is easily discernible from the following waves of definitive erythrocytes and during the time of thymus and bursa colonization, blood also contains T- and B-cell progenitors, which are distributed by the bloodstream to lymphoid organs (Dunon *et al.* 1999). In contrast to rodent embryos, chick embryos *in ovo* are capable of biotransformations from the early stages of development (Heinrich-Hirsch *et al.* 1990). Although the embryonic liver starts functioning after embryonic day (ED) 5, the activity of, for example, the cytochrome P450 monooxygenase system is detected in the endoderm of the yolk sac as early as ED 2 (Gamett and Klein 1984), thus enabling the detection of indirectly acting compounds (Novotná and Jelínek 1986, 1990). All these advantages have already been employed to introduce the so-called 'hen's egg test for micronucleus induction (HET-MN)', where the formation of micronuclei in the erythrocytes of the peripheral blood of incubated hen's eggs is used as a measure of genotoxicity (Wolf and Luepke 1997, Wolf *et al.* 2002).

In the present study, we have addressed the relationship between the acute and long-term effects of 1,2-dibromoethane on proliferation, maturation, death and genotoxic damage of haematopoietic cells of the chick embryo.

Materials and methods

Treatment of embryos and collection of blood samples

Fertilized eggs of outbred Grey Leghorns (Koleč farm, Czech Republic) were incubated in a horizontal position at 37.0–37.5 °C and relative air humidity of 50% in a thermostatically controlled oven. On ED 3, embryos of appropriate developmental stages (HH 18–20) were selected according to Hamburger and Hamilton (1951) and intra-amniotic administration of DBE (CAS 106-93-4, Sigma-Aldrich, Prague, Czech Republic) was performed through a cut-out window in the shell (Dušek *et al.* 2001). Because of the volatility of DBE, the treatment doses were prepared in Wheaton glass serum bottles (10 ml volume, Sigma-Aldrich) sealed by aluminium caps (tear-out centre disk and teflon-faced rubber septum, Sigma-Aldrich). Administration was performed by a gas-tight Hamilton syringe with cemented needle (Sigma-Aldrich). Embryos were treated with 100 or 300 µg DBE in 5 µl 6% ethanol (Lachema, Brno, Czech Republic), the controls received 5 µl 6% ethanol. According to our previous results, the lower dose exhibited only a weak embryotoxic effect while the higher one represented approximately LD₅₀ (Dušek *et al.* 2001). The treated eggs were returned to the incubator. At different intervals after the treatment, blood samples were collected from the aorta dorsalis (ED 3–5) or from the vitelline veins (ED 10, 12 and 14) using a calibrated glass micropipette. At each interval, blood was collected from five embryos/group. These individuals were excluded from further experiments.

On ED 12 and 14, thymus and bursa Fabricii were also removed from the embryos, torn with pins in chick saline (0.12 M NaCl) (Romanoff 1967) and the suspension was filtered through a nylon mesh, using an insulin syringe.

Assessment of DBE concentrations in embryonic and extra-embryonic compartments

Partition coefficients for different egg compartments and solvents were assessed using the air phase as the reference medium. At each interval, five embryos/group were collected. To determine the amount of DBE (1) in embryonic tissues, the embryos being removed from the amnion and rinsed with chick saline; (2) in the full amniotic sac, the amniotic sacs with embryos were removed from the eggs and rinsed with chick saline; and (3) in the full amniotic sac including its surface; the amniotic sacs containing embryos were removed from the egg without subsequent rinsing. To obtain information on the rate of DBE-diffusion through the egg yolk at early intervals after treatment, the yolk was either sampled near the embryo ('sample of yolk') or taken as a whole ('total yolk'). The concentrations of DBE in embryos, yolk and white were analysed by head-space gas chromatograph (GC) as described by Beneš *et al.* (1992). Briefly, the collected tissue was placed into 5 ml head-space air-tight vials. The next day, the vials were incubated at 37 °C for 120 min, 1 ml of the air phase was injected into GC (10% OV-17 column, FID detector, column temperature 130 °C, sampler temperature 140 °C, N₂ flow rate

44 ml min⁻¹, H₂ and air flows 40 and 500 ml min⁻¹, respectively). Calibration standards (2 µl DBE in 5 ml vial) were processed simultaneously with tissue samples.

Haematological analysis

Blood smears were left to dry at room temperature and, 1 week later, stained according to Pappenheim (May-Gruenwald and Giemsa-Romanovsky; NYCOM, Prague, Czech Republic). The blood cell types were analysed in 500 cells/embryo. To assess the proliferation activity, mitotic indices were calculated from 3000 cells/embryo as a percentage of metaphase and anaphase figures from all analysed cells.

Nucleolar test

The nucleoli of cells in blood smears were visualized according to Smetana *et al.* (1969). Briefly, the slides were stained for 10 min in 0.02% toluidine blue (Chroma-Gesellschaft, Berlin, Germany) buffered with McIlvain's buffer. The percentage of the three main nucleolar types (figure 1) was evaluated under a light microscope in 500 cells/embryo. Large and compact nucleoli represented active nucleolar types with ongoing RNA synthesis, ring-shaped nucleoli were considered as reversibly inactive and micro-nucleoli (or cells with no nucleoli at all) as irreversibly inactive (Busch and Smetana 1970, Schwarzscher and Wachtler 1993).

Cytogenetic analysis

Cytogenetic slides were prepared as described by Novotná and Jelínek (1986), i.e. blood cells in minimal essential medium (E-MEM, Sevapharma a.s., Praha, Czech Republic) with 0.1% colcemid (Ciba, Basel, Switzerland) were incubated for 1.5 h at 37 °C, hypotonized with 0.075 M KCl, fixed with methanol-acetic acid (3:1), dropped on slides, flame-dried and stained with 1% Giemsa solution (Merck, Darmstadt, Germany) for 10 min. For chromosome aberration analysis (i.e. chromatid and chromosome breaks and rearrangements), 100 mitoses/embryo were scored. Only the first six pairs of macrochromosomes (from 78 chicken chromosomes), including the sex-chromosome Z, were evaluated because these can be clearly discerned according to their size and centromere position and contain > 50% of genomic DNA (Cormier and Bloom 1973).

Comet assay for DNA damage

The blood, bursa and thymus cells were diluted with chick saline to a final concentration of 10⁶ cells ml⁻¹ and processed by an alkaline version of the comet assay (Singh *et al.* 1988, Miyamae *et al.* 1997) to detect double- and single-strand breaks as well as alkali-labile sites. The cells were mixed with 0.75% low melting point (LMP) agarose and spread on microscope slides precoated with 0.75% normal melting point (NMP) agarose (both agaroses were from AMRESCO, Solon, USA). Slides were submersed for 1 h in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.16 M DMSO, 0.016 mM Triton X-100; all Sigma Chemical Co., St Louis, MO, USA) at pH 10. After lysis, the slides were equilibrated for 20 min in alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH 13) to allow the DNA to unwind. Following this, the slides were electrophoresed for 20 min in fresh alkaline buffer (1 V cm⁻¹, 300 mA). The microgels were then neutralized in 0.4 M Tris, pH 7.5, fixed in methanol (15 min), allowed to dry at room temperature and stored. Before analysis, the slides were submersed for 2 h in distilled water. DNA was stained for 10–15 min with 0.005% ethidium bromide (Sigma) and examined using an Olympus VANOX BHS fluorescence microscope (Prague, Czech Republic). The extent of DNA damage was quantified with a LUCIA 3.52a software (Laboratory Imaging, Prague, Czech

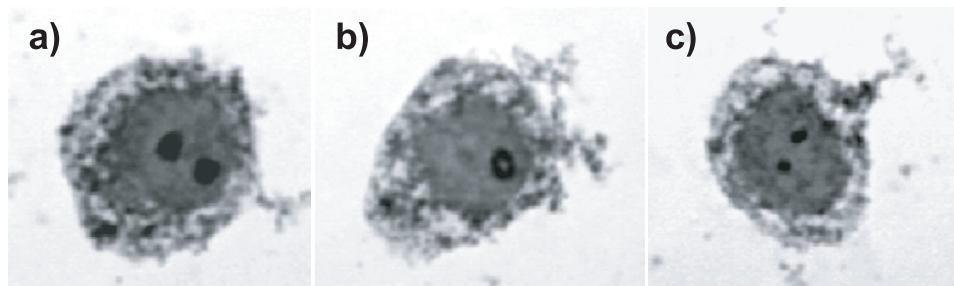


Figure 1. Blood cell with (a) two active nucleoli, (b) reversibly inactive (ring-shaped) nucleolus and (c) two inactive nucleoli.

Republic) in 100 cells/embryo. The results were expressed as 'Tail DNA', representing the proportion of damaged DNA from the total nuclear DNA.

Flow cytometry

Blood samples were diluted with chick saline to a concentration of 10^6 cells mL^{-1} and stained with annexin-V-FLUOS Staining Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The extent of cell death was measured using an argon laser fluorescence activated cell analyser (FACSort) and the CELLQuest software version 1.2.2 (all H&D, Becton Dickinson, San Jose, CA). A minimum of 16 000 events were acquired for each sample.

Statistical analysis

The differences between the DBE-treated and control embryos were analysed using a two-tailed Student's *t*-test.

Results

Kinetics of DBE distribution

The partition coefficient (α) of DBE was assessed as 30.6 for embryonic tissues, as 24.8 for the blood, as 650.1 for the yolk, as 64.1 for the albumen, as 111.1 for ethanol and as 6.1 for water. The non-polar media (ethanol and yolk) exhibited the highest partition coefficients, e.g. the affinity of DBE to the egg yolk exceeded more than 20 times the affinity of DBE to the embryo itself.

The highest concentration of DBE in the embryonic tissues proper was detected immediately after the administration of DBE into the amniotic sac (interval 0) and decreased continuously with time (table 1). A high amount of DBE at the surface of the amniotic sac was observed at the first interval (see the values of DBE concentration in amniotic sac including the embryo without and after rinsing). Similarly, the concentration of DBE in the sample of yolk collected from the region contiguous to the amniotic sac was at its maximum immediately after treatment, then decreased and reached steady-state after 6 h. In the whole yolk, however, the concentration of DBE increased with time. Whereas at time 0 the total yolk contained about 63% of the administered dose, during the next 6 h

Table 1. Kinetics of DBE distribution in the embryo and extraembryonic compartments after the intra-amniotic administration of 300 μg DBE on incubation day 3

Time after treatment (h)	Concentration of DBE (mgL^{-1})					
	Amniotic sac with embryo	Rinsed amniotic sac with embryo	Rinsed embryo	Sample of yolk	Sample of albumen	Total yolk
0	1913.4 ± 2107.0	194.6 ± 189.6	39.4 ± 29.7	5.5 ± 6.8	0	3.5 ± 0.95
0.5	ND	114 ± 111	11.7 ± 8.4	ND	ND	ND
1	213.1 ± 170.9	48.1 ± 36.0	9.9 ± 9.0	3.29 ± 0.45	0.44 ± 0.36	ND
2	ND	ND	1.76 ± 1.43	1.51^a	0.31^a	ND
3	ND	6.2 ± 3.9	1.70 ± 0.49	ND	ND	ND
6	8.8 ± 5.0	5.3 ± 2.9	1.35 ± 1.29	0.42^a	2.0 ± 2.5	4.93 ± 1.85
12	ND	1.7 ± 0.9	0.90 ± 0.37	ND	ND	ND
24	ND	0.37 ± 0.05	0.71 ± 0.35	0.50 ± 0.59	0.26 ± 0.26	ND
48	ND	0.51 ± 0.26	0.57 ± 0.08	0.43 ± 0.54	0.87 ± 0.27	ND

** Values are mean $\pm$ SD.

The SD was derived from five determinations.

^a Only one assessment available.

ND, not determined.

Table 2. Types of erythroid cells in the blood of chick embryos during development.

Time after treatment on ED 3	Dose of DBE (µg)	Number of cells (%)						
		Basophilic EB I	Chromatophilic EB I	E I	Pro-EB II	Basophilic EB II	Chromatophilic EB II	E II
6 h	0	80.17 ± 3.33	19.83 ± 4.22	0	0	0	0	0
	300	78.46 ± 2.87	21.54 ± 2.50	0	0	0	0	0
24 h	0	2.23 ± 0.50	97.77 ± 0.92	0	0	0	0	0
	300	26.98 ± 4.02**	73.01 ± 4.32**	0	0	0	0	0
48 h	0	0	58.81 ± 3.27	39.31 ± 3.97	0.72 ± 0.43	1.16 ± 0.24	0	0
	300	0	64.44 ± 3.33*	29.90 ± 4.76**	2.38 ± 1.28*	3.28 ± 1.32*	0	0
7 days	0	0	0	7.61 ± 2.36	0	6.44 ± 1.19	24.97 ± 2.25	59.40 ± 4.99
	300	0	0	5.48 ± 1.88	0	4.38 ± 1.86	25.50 ± 4.73	64.63 ± 3.40
9 days	0	0	0	5.58 ± 1.14	0	2.19 ± 0.90	25.84 ± 1.72	64.96 ± 2.38
	300	0	0	5.03 ± 0.54	0	2.49 ± 0.62	26.19 ± 2.22	66.29 ± 2.58
11 days	300	0	0	2.87 ± 0.14	0	1.49 ± 0.31	19.96 ± 0.88	75.68 ± 1.35

* $p < 0.05$, ** $p < 0.01$ with respect to the control group.
Values are near ± SD. The SD was derived from five determinations.
EB, erythroblasts; pro-EB, proerythroblasts; E, erythrocytes; I, primitive series; II, definitive series.

this amount reached 80%. For comparison, the unrinsed amniotic sac including embryo contained only 30% of the administered dose just after administration and 6 h later this decreased substantially to 0.1%.

Differentiation of erythroid cells

Only the immature forms of primitive erythrocytes (basophilic and chromatophilic erythroblasts I) were present in circulation between ED 3 and 4 (table 2). The mature primitive erythrocytes appeared 1 day later, simultaneously with the first pro-erythroblasts and basophilic erythroblasts of the definitive series. Subsequently, the number of primitive erythrocytes continuously decreased in favour of definitive erythrocytes. From the earliest stages of development, blood also contained 3–4% of thrombocytes. The first granulocytes appeared on ED 12, and on ED 14 they still represented only about 0.5% of the total blood cell population.

Within 6 h after administration of DBE, the blood picture of exposed embryos did not differ from controls. After 24 h, however, a significant shift towards less mature cells was observed in DBE-treated embryos and persisted until the next day. Later, no differences in the types of erythroid cell were observed between the DBE-treated and control group.

Table 3 summarizes the results of the nucleolar test. More than 70% of blood cells in both control and DBE-treated embryos contained active nucleoli 6 h after treatment (i.e. on ED 3). With the subsequent maturation of blood elements, the number of cells with active nucleoli gradually diminished, such that on ED 14 (11 days after the treatment) these cells represented < 1% of the total number of blood cells. In comparison with controls, DBE-exposed embryos exhibited a significantly increased percentage of cells with active nucleoli only 24 and 48 h after the treatment. This corresponded well with the results of conventional haematological analysis, i.e. with the higher incidence of less mature blood cells in DBE-treated individuals at 24- and 48-h intervals.

Table 3. Types of nucleoli in the erythroid cells of chick embryos during development.

Time after treatment on ED 3	Dose of DBE (µg)	Number of nucleoli (%)		
		active	reversibly inactive	inactive
6 h	0	70.80 ± 1.66	14.20 ± 1.41	15.00 ± 2.16
	300	71.20 ± 2.17	14.07 ± 5.04	14.73 ± 4.83
24 h	0	58.06 ± 4.85	14.68 ± 2.23	27.26 ± 5.84
	300	67.43 ± 2.27*	12.75 ± 1.96	19.78 ± 3.13
48 h	0	37.64 ± 3.17	12.42 ± 2.32	50.04 ± 2.49
	300	51.72 ± 8.97*	12.04 ± 2.34	36.14 ± 8.76*
7 days	0	5.04 ± 0.29	12.72 ± 0.86	82.24 ± 1.11
	300	4.90 ± 0.46	12.45 ± 0.73	82.65 ± 1.18
9 days	0	1.18 ± 0.68	3.38 ± 0.83	95.45 ± 1.36
	300	1.74 ± 0.31	3.42 ± 0.55	94.84 ± 0.85
11 days	0	0.87 ± 0.09	2.70 ± 0.29	96.43 ± 0.29
	300	0.80 ± 0.14	2.35 ± 0.30	96.85 ± 0.43

* $p < 0.05$ with respect to control group.

Values are mean ± SD. The SD was derived from five determinations.

Table 4. Mitotic activity in blood smears.

Time after treatment	Proportion of mitoses (%)		
	Dose of DBE (µg)		
	0	100	300
6 h	4.33 ± 1.59	3.35 ± 0.85	2.57 ± 0.17*
9 h	2.26 ± 0.83	1.96 ± 0.81	2.12 ± 0.47
24 h	2.18 ± 0.34	2.03 ± 0.78	1.60 ± 0.37
48 h	1.50 ± 0.22	1.36 ± 0.34	1.13 ± 0.26
7 days	0.28 ± 0.20	0.13 ± 0.05	0.17 ± 0.12

* $p = 0.0502$ with respect to the control group.
Values are mean ± SD. The SD was derived from five determinations.

The number of mitoses in blood smears decreased with time, regardless of the treatment dose (table 4). Dose-dependent mitotic inhibition appeared 6 h after administration of DBE. In the next intervals, the embryos from both exposed groups did not differ significantly from the controls although their mitotic indices were permanently bellow the control values.

Chromosome damage

Both doses of DBE induced a significant increase of aberrant cells in the blood as early as 6 h after the treatment (figure 2). The frequency of chromosome aberrations decreased with time, such that at the 12- and 24-h intervals only embryos exposed to the high dose of DBE exceeded significantly the control levels of aberrant cells. Chromatid breaks were the only type of detected chromosome aberrations.

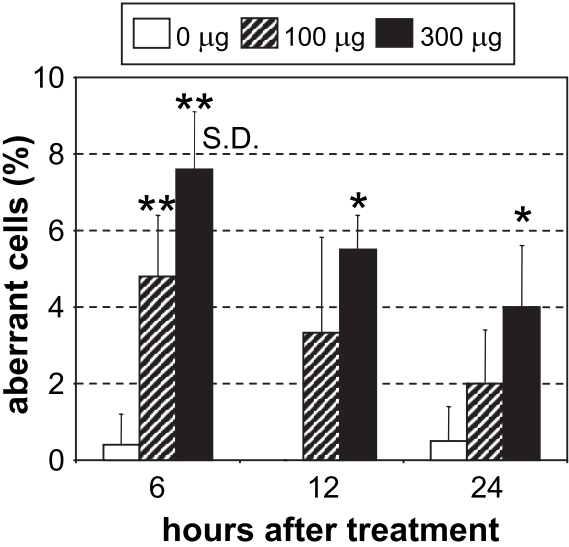


Figure 2. Cytogenetic analysis in blood cells at different intervals after the treatment with DBE. ** $p < 0.01$, * $p < 0.05$ with respect to the control group. Data were derived from five determinations/group.

Table 5. Kinetics of DNA damage assessed by the comet assay in embryonic blood cells.

Time after treatment	Tail DNA (%)		
	Dose of DBE (μg)		
	0	100	300
3 h	16.97 $\pm$ 2.17	19.74 $\pm$ 3.06*	19.77 $\pm$ 3.46**
6 h	7.90 $\pm$ 0.60	11.33 $\pm$ 1.35	15.08 $\pm$ 2.00**
9 h	13.69 $\pm$ 2.02	13.09 $\pm$ 1.71	13.41 $\pm$ 1.65
12 h	13.14 $\pm$ 1.86	16.62 $\pm$ 2.71	18.83 $\pm$ 3.35**
24 h	12.04 $\pm$ 1.45	14.29 $\pm$ 1.96	17.75 $\pm$ 2.78**
48 h	9.46 $\pm$ 0.83	15.12 $\pm$ 2.28**	18.49 $\pm$ 3.36**
7 days	13.42 $\pm$ 1.79	15.27 $\pm$ 2.26	23.66 $\pm$ 5.14**
9 days	17.80 $\pm$ 1.96	ND	15.07 $\pm$ 2.09
11 days	17.34 $\pm$ 2.50	ND	18.89 $\pm$ 3.49

* $p < 0.05$, ** $p < 0.01$ with respect to the control group.

Values are mean $\pm$ SD. The SD was derived from five determinations.

ND, not determined.

Tail DNA is defined as the proportion of damaged DNA from the total nuclear DNA.

DNA damage

Blood cells of embryos treated with 300 μg DBE exhibited an increase in DNA fragmentation in comparison with controls as early as 3 h after treatment (table 5). This effect persisted until ED 10, i.e. 7 days after treatment (with the exception of the 9-h interval). Later, on ED 12 and 14, the treated embryos did not differ from controls.

The kinetics of DNA damage induced by the low dose of DBE displayed a similar trend. However, the differences between the DBE-treated and control embryos were statistically significant only at 3 and 48 h after treatment.

Thymus and bursa cells were analysed 9 and 11 days after the administration of DBE, i.e. on ED 12 and 14. On ED 12, both cell types exhibited a significantly higher DNA fragmentation in DBE-treated embryos than in controls (table 6). This damage persisted until ED 14 only in bursa cells, whereas thymus cells showed no difference between DBE-treated embryos and controls at this interval.

Table 6. DNA damage assessed by the comet assay in thymus and bursa cells.

Time after treatment (days)	Type of cells	Dose of DBE (μg)	Tail DNA (%) (mean $\pm$ SD)
9	bursa	0	29.99 $\pm$ 1.85
		300	34.34 $\pm$ 1.95*
	thymus	0	20.90 $\pm$ 1.03
		300	28.61 $\pm$ 2.41**
11	bursa	0	22.49 $\pm$ 1.21
		300	36.91 $\pm$ 2.43**
	thymus	0	16.70 $\pm$ 0.81
		300	17.22 $\pm$ 0.93

* $p < 0.05$, ** $p < 0.01$ with respect to the control group.

The SD was derived from five determinations.

Tail DNA is defined as the proportion of damaged DNA from the total nuclear DNA.

Cell death

Flow cytometry revealed about 10% dead blood cells 3 h after DBE administration (16.79 versus 6.21% in controls). These cells were either necrotic or late apoptotic because they were labelled with both annexin V and propidium iodide (figure 3). Later, no significant differences between the DBE-treated and control embryos were observed (data not shown).

Discussion

Intra-amniotically administered DBE rapidly reached not only the embryonic tissues, but also the areas of the yolk adjacent to the amniotic sac surface. This demonstrated a rapid diffusion of DBE through the amniotic sac wall, although some release of DBE from the hole remaining in amniotic wall after the injection of DBE could not be excluded. During the first 6 h after treatment, the DBE concentration in the embryo and amniotic sac decreased rapidly. In parallel, the amount of DBE in the sample of yolk close to the amniotic sac also diminished, whereas in the whole yolk it rose, undoubtedly due to the rapid diffusion of DBE to the remote areas of the yolk. According to the partition coefficients, DBE exhibited a much higher affinity for the non-polar environment of the yolk than for the other egg components such as the embryonic tissues, blood and albumen. Hence, the yolk can serve as a reservoir of DBE for a long-term exposure of the embryo.

Our previous study demonstrated a rapid action of DBE in the chick embryo, manifested as serious damage to the extra- and intra-embryonic vascular system within 3 h after DBE administration (Dušek *et al.* 2001). Only at this interval (i.e. 3 h after treatment) did labelling of cells with annexin V/propidium iodide reveal a higher percentage of dying blood cells in DBE-treated than in control embryos. In contrast to annexin V, which binds to the surface of cell membranes from the early stages of apoptosis, propidium iodide binds to DNA only if it has free access through disintegrated membranes. Hence, simultaneous annexin V and propidium iodide labelling of the cells suggested either necrosis or an advanced stage of

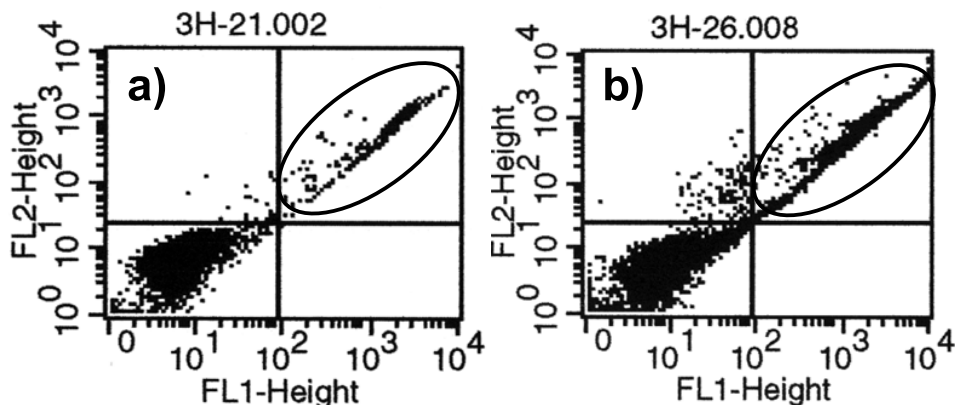


Figure 3. FACS analysis of blood cells labelled with annexin V/propidium iodide at 3-h interval: (a) controls, (b) 300 µg DBE. FL1, annexin-V-FLUOS fluorescence; FL2, propidium iodide fluorescence. Cells in the upper right part are double-labelled, i.e. either necrotic or late apoptotic.

apoptosis. Rapid decrease of the DBE supply in the amniotic sac together with the efficient macrophage-dependent removal of dying cells from the circulation could be the reasons why no significant increase in the number of dying cells was detected at longer intervals after the treatment, although the cytogenetic analysis implied an ongoing cell death. An increased number of aberrant cells were still noted in the blood of DBE-treated individuals 24 h after the administration of the high dose. In comparison with classical chromosome-damaging agents such as cyclophosphamide (Novotná and Jelínek 1986), the DBE-induced aberrations were not frequent (7.6% of aberrant cells at most) and consisted mainly of single chromatid breaks. The loss of an even small acentric fragment during anaphase, however, may result in the loss of genetic material, which is incompatible with further survival of the affected cell. These chromatid breaks could have arisen from incomplete repair of DNA adducts and may thus provide indirect evidence for efficient bioactivation of DBE via the glutathione conjugation pathway in the 3-day-old chick embryos.

In the rat, a single pretreatment with ethanol strongly enhances the cytotoxic effects of DBE due to the inhibition of glutathione-S-transferase by the major metabolite of ethanol, acetaldehyde, which shifted DBE metabolism towards the oxidative route (Aragno *et al.* 1996). In our experiments, we used 6% ethanol as a solvent for DBE. Although the final dose of ethanol was about three orders of magnitude lower in comparison with the adult rats, we cannot quite exclude additive effects of ethanol on DBE cytotoxicity in the chick embryo blood cells, at least at the 3-h interval. In addition, growth retardation and prominent cell death in neural tissue was observed in rat embryos growing for 24 h in a medium with DBE. As early rodent embryos lack metabolic enzymes, these effects were attributed to the direct interaction of the hydrophobic molecule of DBE with membrane lipids, altering reversibly the transport functions of cell membranes (Brown-Woodman *et al.* 1998). In the chick embryonic tissues, we identified DBE as long as 48 h after treatment. Thus, the parental compound itself (besides metabolic intermediates) could influence the survival and proliferation activity of blood cells in DBE-treated embryos.

The changes in the incidence of various types of erythroid cells in the blood of control embryos at different collection intervals illustrate the physiological process of erythrocyte maturation and replacement of the senescent primitive erythrocytes by the successive waves of definitive cells during chick embryo development (Bruns and Ingram 1973, Komárek *et al.* 1982). The shift towards less differentiated immature erythroblasts, observed in DBE-treated embryos 24 and 48 h after the treatment, could be explained most probably by the release of immature cells into the circulation to compensate for the loss arising from continuous cell death, although the delay of the cell cycle (reflected in mild decrease of mitotic activity) could contribute to this effect as well.

The size and distribution of RNA in nucleoli is considered as the morphological expression of nucleolar, i.e. ribosomal, RNA synthesis (Busch and Smetana 1970). Young erythroid cells of the chick embryo contain compact nucleoli, which are thought to be active in the biosynthesis of RNA. With progressing differentiation and maturation of the cells, the compact nucleoli are gradually replaced by ring-shaped nucleoli and eventually by micronucleoli, reflecting the reversible and irreversible inhibition of RNA synthesis, respectively (Likovský *et al.* 1994). The changes in the activity of rRNA synthesis in the early maturation stages of

erythroid cells serve as a very sensitive marker for embryotoxic effects during the first days of embryonic development (Likovský *et al.* 1993). In the case of DBE, the prevalence of the active form of nucleoli was noted in erythroid cells of exposed embryos when compared with controls at 24- and 48-h intervals. This corresponded with the results of haematological analysis, i.e. with the higher incidence of less mature and, hence, more proteosynthetically active cells in the blood of the DBE-treated group.

After ED 5, neither the haematological analysis nor the nucleolar test showed any difference between the control and DBE-exposed embryos. DNA damage, detected by the comet assay in the blood cells within the first 2 days after the administration of DBE, could thus involve DNA cleavage in dying cells. In contrast, the persistence of DNA damage in the blood cells of 10-day-old DBE-exposed embryos could reflect exclusively the presence of long-lived DNA-adducts. It has been reported that DNA-adducts lessen the stability of the N-glycosidic bond between deoxyribose and the modified base and may thus lead to spontaneous hydrolytic reaction resulting in an apurinic site. These apurinic sites represent what is termed 'alkali-labile sites' because in alkaline conditions (e.g. in the comet assay) they are converted into single-strand breaks (Fairbairn *et al.* 1995). According to Inskeep *et al.* (1986), the average half-life of the main DBE-adducts on N^7 -guanine is about 70–100 h in rat liver, kidney and lungs, and even longer in *in vitro* conditions (about 150 h in the calf thymus).

Similarly to the mammals, the chick stem cells of the primitive erythroid population appear in the early blood islands of the yolk sac. Between ED 3 and 4, a second population of stem cells emerges in parallel in the embryo and in the yolk sac. The majority of definitive erythrocytes, however, arise from intra-embryonic stem cells located in intra-aortic clusters, ventrolateral to the aortic wall. At ED 6–8, the dorsal mesentery close to the aorta was identified as a source of haematopoietic stem cells (Vainio and Imhof 1995). So far, it has not been satisfactorily resolved whether these stem cells are derived from initial intra-amniotic clusters. Besides that, mesonephros also seemed to play an important role in production of chick haematopoietic progenitors (Kudo *et al.* 1989) before the main centres of haematopoiesis move to the bone marrow and spleen. Recently, the para-aortic splanchnopleura/aorta-gonads-mesonephros (PAS/AGM) region has been confirmed as the first embryonic site with complex haematopoietic activity in the mouse embryo (Sainio and Raatikainen-Ahokas 1999, de Bruijn *et al.* 2000, 2002, Ling and Dzierzak 2002).

Considering the course of embryonic haematopoiesis, the absence of DNA damage in the blood cells of 12- and 14-day-old DBE-treated embryos suggests that only primitive erythroid cells and the progenitors of the first population of the definitive erythroid cells were sufficiently exposed to DBE. On ED 12, these cells or their progeny in major part disappeared from the circulation and were replaced by a new wave of definitive erythrocytes.

Blood-borne haematopoietic stem cells are distributed by the blood stream also into the bursal and thymic rudiments. Thymus is colonized in three discrete waves, beginning on days 6, 13 and 18, respectively, which are separated by non-receptive intervals. Progenitors of the first wave originate from para-aortic foci, whereas progenitors of the second and the third waves are derived from the bone marrow (Péault *et al.* 1987, Dunon *et al.* 1999). In thymus, the cells undergo rapid division and maturation and then they migrate to peripheral organs to make space

for the next wave of stem cells (Toivanen and Toivanen 1987). In contrast, the bursa is seeded in one continuous inflow of stem cells between ED 8 and 14 (Péault *et al.* 1987). Whereas the definitive determination to the T-cell lineage occurs in the thymus, the B-cell lineage is determined very early before the colonization of bursa Fabricii and B-cell development in the bursa proceeds by a discrete series of ontogenetically restricted stages (Houssaint *et al.* 1991, Vainio and Imhof 1995). Cells expressing surface IgM were detected first in the bursa on ED 12 and specific antigen-binding cells after ED 16 (Lydyard *et al.* 1976, Ratcliffe *et al.* 1987).

In DBE-treated embryos, both thymus and bursa cells exhibited higher levels of DNA fragmentation in comparison with controls on ED 12, i.e. 9 days after the administration of DBE. Two days later, however, only bursa cells still differed from controls. This finding could be explained by the differences in bursa and thymus homing. Whereas on ED 12 both organs contained the progeny of stem cells exposed to DBE during the early stages of development, on ED 14 this cell population was already replaced in the thymus by the second influx of progenitor cells. Whether the observed DNA damage reflected simply the presence of alkali-labile lesions or involved the apoptotic cleavage of DNA in cells with defective maturation remains to be clarified.

In conclusion, our results not only document the rapid deleterious effects of 1,2-dibromoethane on the haematopoietic cells of the developing organism leading to prominent cell death, but also show the persistence of DNA damage in otherwise seemingly normal individuals long after DBE exposure. This persistence is explicable only by the transmission of DBE-induced DNA lesions through consecutive generations of the initially exposed haematopoietic cells.

Owing to the easy experimental access and well-described development of the lymphohaematopoietic system, the chick embryo *in ovo*, in combination with such sensitive methods as the comet assay, provides an alternative to mammalian models for studies aimed at evaluating the impact of xenobiotics on haematopoiesis.

Acknowledgements

The study was supported by Grants 304/98/1613 (GAČR) and AV0Z5039906 (IEM). The authors express their sincere thanks to Ms Jolana Vaňková and Ms Dominika Krčmová for excellent and enthusiastic technical support.

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Prediction of *in vivo* hepatic clearance from *in vitro* data using cryopreserved human hepatocytes

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Received 1 August 2002

1. Cryopreserved human hepatocytes were used to predict *in vivo* hepatic clearance (CL_{hepatic}) from estimates of *in vitro* intrinsic clearance (CL'_{int}).
2. CL'_{int} was estimated for phenytoin, valproic acid, carbamazepine, theophylline, quinidine and procainamide after their addition to hepatocytes suspended either in human serum or in serum-free media. CL'_{int} was estimated from *in vitro* concentration versus time data fitted to a monoexponential decay model. CL'_{int} was estimated from concentrations measured at four time points and from just two-point measures, namely the initial concentration (C_0) and the final concentration measurement (C_{last}).
3. Predicted CL_{hepatic} was within twofold of reported *in vivo* values of CL_{hepatic} for all substrates. Moreover, predictions were not significantly different whether derived from hepatocytes suspended in serum or in serum-free medium.
4. Two-point estimates of CL'_{int} were just as accurate in predicting CL_{hepatic} as were multipoint estimates of CL'_{int} .
5. Although the data set was limited, the findings suggest that the measurement of the disappearance of xenobiotics from serum or serum-free media in which primary human hepatocytes have been suspended provides a physiologically relevant estimate of hepatic clearance that can be employed early in the drug development process to eliminate xenobiotics with unacceptable clearances.

Introduction

In the era of combinatorial chemistry and high throughput (HTP) and ultrahigh throughput (UHTP) screening, there is a pressing need to characterize the likely dispositional characteristics such as absorption, distribution, metabolism and excretion (i.e. ADME) of new chemical entities (NCEs) in humans as early as possible in the drug development process (Bachmann and Ghosh 2001). Before 1985, approximately 80% of NCEs were withdrawn from further drug development due to unfavourable pharmacokinetic (PK) parameters (Prentis *et al.* 1988). Currently, fewer than 40% of NCEs survive preclinical testing, and fewer than 10% of those that make their way into clinical trials actually become marketed products (Borchardt 2001). It has been argued that in the near future the assessment of these 'drugability' characteristics of NCEs will precede rather than follow pharmacodynamic assessment, i.e. molecular target identification (Lipinski 2000). *In vitro* models for evaluating likely human metabolic stability, metabolic pathways, drug-drug interactions and the prediction of *in vivo* PK parameters encompass the use of recombinant enzymes that are stably expressed in a variety of expression systems,

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subcellular fractions, usually microsomes, liver slices and hepatocytes (Ekins *et al.* 2000). Both the successes of these systems and their attendant problems as they relate to the prediction of *in vivo* PK parameters, namely metabolic clearance, have been summarized by Bachmann and Ghosh (2001).

Although the development of *in vitro* screening technologies for predicting *in vivo* PK parameters (e.g. metabolic clearance) are generally modelled after the successful single-mechanism molecular target screens (Ekins *et al.* 2000, Lipinski 2000), there is some evidence that the use of multimechanism systems, e.g. pooled microsomes, may be highly predictive (Obach 1999). In the current study, we have evaluated the *in vitro* metabolic clearances of six xenobiotics from a suspension of cryopreserved primary human hepatocytes with a view of determining the accuracy with which the *in vivo* human hepatic clearance (CL_{hepatic}) of each could be determined. These xenobiotics were selected because they are substrates for different CYP enzymes, they are chemically diverse, they exhibit a wide range of clearances, pK_a 's and protein binding, and are principally eliminated by hepatic metabolism. A secondary objective of this investigation was to determine whether *in vivo* CL_{hepatic} for a given xenobiotic could be accurately predicted from an *in vitro* system with as few as two measurements.

Materials and methods

Materials

Serum-free hepatocyte incubation media (HIM) and cryopreserved human hepatocytes were purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA). Hepatocyte Lot #104 was used for phenytoin, and hepatocyte Lot #102 was used for all other substrates. Details about each lot can be found at: <http://www.invitrotech.com> but the key features of the donors were as follows: Lot #102 was from a 48-year-old male Caucasian who died of anoxia and who had no history of smoking or substance use, and a medication history including only diuretics. Lot #104 was from a 53-year-old Asian male who died of head trauma, who had no history of smoking or substance use apart from marijuana. His medical history included hypertension and non-insulin-dependent diabetes mellitus.

Phenytoin, valproic acid, carbamazepine, theophylline, procainamide, quinidine, (-NADH and pyruvic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Fluorescence polarization immunoassay (fpia) assay kits for phenytoin (free and total), valproic acid (free and total), carbamazepine (free and total), theophylline, procainamide and quinidine were purchased as TD_X kits from Abbott Laboratories (Abbott Park, IL, USA). Acetonitrile, methanol and ethanol were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Cryopreserved human hepatocytes

The cryopreserved hepatocytes were thawed according to the protocol described by In Vitro Technologies. After adding the xenobiotic to thawed cell suspensions in HIM and serum, the suspensions were subsequently divided into 500- μ l aliquots containing 1.0×10^6 cells each in round-bottomed tubes. The targeted initial concentrations of each of the drugs in HIM and serum were less than the reported K_m 's for unbound drug or total drug, respectively. Tubes were incubated in a shaking water bath (96 oscillations min^{-1}) at 37°C. One sample (i.e. one tube) was analysed for remaining xenobiotic and for lactate dehydrogenase activity (see below) at 0-, 1-, 2- and 6-h time points.

Fluorescence polarization immunoassays

The depletion of the parent drug from either HIM or serum was measured by a fluorescence polarization immunoassay (fpia) with a TD_X analyser and commercially available assay kits (Abbott Diagnostics). The instrument was calibrated separately for each of the drugs immediately before assaying drug concentrations in HIM or serum. For each analyte, a calibration curve was prepared using six different concentrations of analyte. Details of this assay methodology for each analyte have been published (Anon 1994).

In vitro metabolic clearances

Xenobiotic concentrations in HIM or serum were measured at the following incubation times: 0, 1, 2, 4 and 6 h after addition to human hepatocytes. A simple monoexponential decay model described the disappearance of the xenobiotic from the incubation media:

$$[C_t] = [C_0]e^{-kt}, \quad (1)$$

where k is the rate constant for elimination from the medium, $[C_t]$ is the measured concentration at any time, t , and $[C_0]$ is the concentration at time zero. The elimination rate constant, k , was estimated by non-linear regression (Kaleidagraph, Synergy Software, Reading, PA, USA) of the concentration versus time curve using equation (1). In addition, two-point estimates of k were estimated from the initial concentration measurement and the 6-h concentration measurement as follows:

$$k = \frac{\ln[C_0] - \ln[C_6]}{6}, \quad (2)$$

where $[C_6]$ was the xenobiotic concentration measured at 6 h.

In vitro intrinsic clearance (CL'_{int}) was calculated as follows:

$$CL'_{\text{int}} = k \cdot V, \quad (3)$$

where V is the volume of the *in vitro* cell suspension. For cells suspended in serum-free HIM, the measured CL'_{int} was factored by the fraction of unbound drug.

Scale-up to in vivo hepatic clearance, CL_{hepatic}

Multi- and two-point estimations of *in vitro* CL'_{int} 's were scaled to *in vivo* predictions of intrinsic clearance, CL_{int} , based on the number of cells in the intact liver:

$$CL_{\text{int}} = \frac{N_{\text{H}}}{N_{\text{S}}} CL'_{\text{int}}, \quad (4)$$

where N_{S} is the number of hepatocytes used in the *in vitro* suspensions and N_{H} is the number of hepatocytes found in an average liver. Iwatsubo *et al.* (1997) estimated that there were approximately 120×10^6 cells g^{-1} liver, with the mass of an average liver being 1.5 kg. Overall hepatic clearance, CL_{hepatic} , was then calculated as follows:

$$CL_{\text{hepatic}} = \frac{Q \cdot CL_{\text{int}}}{Q + CL_{\text{int}}}, \quad (5)$$

where Q is the hepatic blood flow rate set at 901 h^{-1} .

Lactate dehydrogenase assay

Determination of cell viability was assessed using a lactate dehydrogenase (LDH) assay. The assay procedure for determining LDH was based on that described by Moss *et al.* (1986) with the modifications specified by Hassoun *et al.* (1993). LDH leakage from cells was determined by diluting 50 μl media to 2.6 ml with phosphate buffer (0.1 M, pH 7.4) in a cuvette. After warming to 37°C , the reaction was initiated in the cuvette by adding 100 μl NADH (125 μM , final concentration) and 100 μl sodium pyruvate (300 μM , final concentration) to the diluted media. Absorbance of the mixtures were recorded at 340 nm every 30 s over 3 min using a Spectronic-20 spectrophotometer (Spectronic Instruments, Rochester, NY, USA). The lamp of the spectrophotometer was allowed to stabilize for 30 min before taking the readings. Slopes of optical density (OD) change versus time were calculated, and after applying correction factors for temperature and dilution of the media, were expressed as measures of LDH activity.

Statistics

The differences between predictions of CL_{hepatic} based on cells suspended in HIM versus cells suspended in serum were tested by a paired *t*-test. The difference between predictions of CL_{hepatic} based on cells suspended in HIM versus actual CL_{hepatic} was determined by a Wilcoxon signed rank test, and the difference between predictions of CL_{hepatic} based on cells suspended in serum versus actual CL_{hepatic} was determined by a paired *t*-test. Differences between multisample predictions of CL_{hepatic} and two-point predictions of CL_{hepatic} were evaluated with a Wilcoxon signed rank test. Application of parametric or non-parametric tests was based on the outcome of the Ryan-Joiner normality test in MINITAB® software (MINITAB, Inc., State College, PA, USA), which was used for the statistical analysis.

Results

Immunoassays

Limits of detection for each analyte are given in table 1. All xenobiotics were detectable throughout the 6-h incubation except for phenytoin and quinidine in HIM, neither of which could be detected after 4 h.

Cellular viability

The vendor reported cell viability of 81% by trypan blue exclusion. The comparative viability of the hepatocytes at every time point was determined by an LDH assay in which untreated cells served as controls. The relative percentage of viable cells determined in this manner was used as a correction factor to normalize measured drug concentrations. This correction was only applied to the 6-h measurement of procainamide in HIM. No other correction was applied to the data.

Table 1. Limits of detection for fluorescence polarization assays of test xenobiotics.

Xenobiotic	Limit of detection (mg l ⁻¹)	Coefficient of variation (%)
Carbamazepine (total)	0.5	< 7
Carbamazepine (unbound)	0.05	< 5
Phenytoin (total)	0.5	< 5
Phenytoin (unbound)	0.02	< 5
Procainamide	0.1	< 6
Quinidine	0.2	< 5
Theophylline	0.9	< 5
Valproic acid (total)	0.7	< 5
Valproic acid (unbound)	0.1	< 5

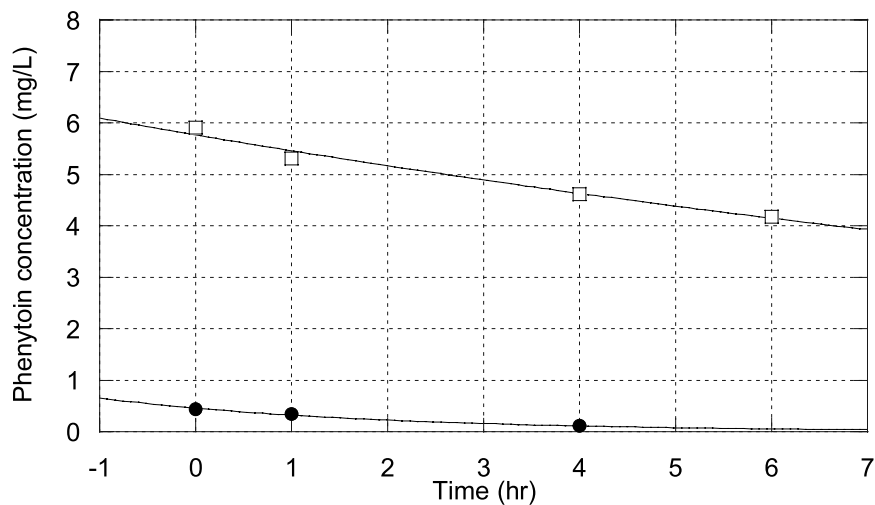


Figure 1. Clearance of phenytoin by human hepatocytes suspended in human serum (squares) or HIM (circles). Rate constants for disappearance are 0.0549 h⁻¹ (serum) and 0.3534 h⁻¹ (HIM). R's are 0.987 and 0.995, respectively. Data were derived from a single determination at each time point.

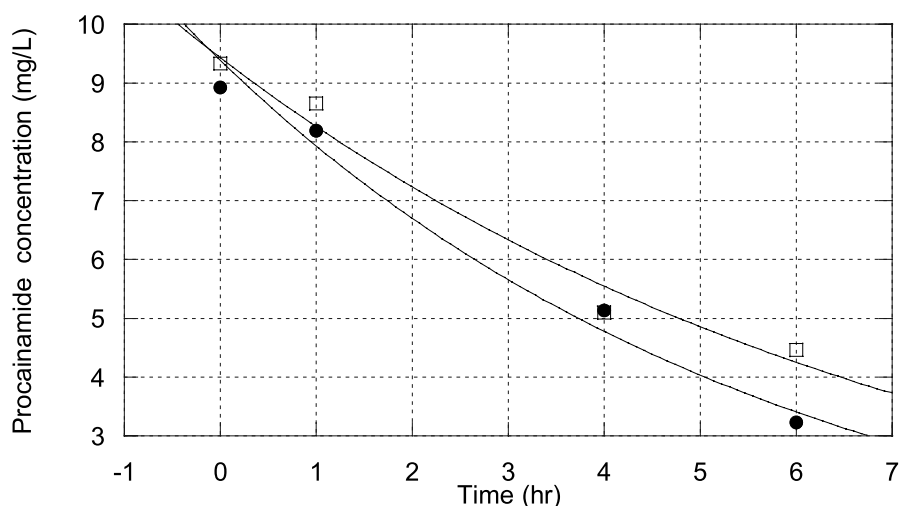


Figure 2. Clearance of procainamide by human hepatocytes suspended in human serum (squares) or HIM (circles). Rate constants for disappearance are 0.1560 h^{-1} (serum) and 0.1689 h^{-1} (HIM). R^2 's are 0.994 and 0.991, respectively. Data were derived from a single determination at each time point.

Table 2. Features of the disappearance of six xenobiotics in suspensions of human hepatocytes in human hepatocyte incubation medium (HIM) or human serum.

Xenobiotic	Mono-exponential equation	r value
Carbamazepine (serum)	$Y = 6.577 e^{-0.5837t}$	0.937
Carbamazepine (HIM)	$Y = 1.956 e^{-0.0964t}$	0.995
Phenytoin (serum)	$Y = 5.767 e^{-0.0549t}$	0.987
Phenytoin (HIM)	$Y = 0.458 e^{-0.3534t}$	0.995
Procainamide (serum)	$Y = 9.6526 e^{-0.1560t}$	0.994
Procainamide (HIM)	$Y = 9.387 e^{-0.1560t}$	0.994
Quinidine (serum)	$Y = 3.209 e^{-0.1437t}$	1.00
Quinidine (HIM)	$Y = 1.514 e^{-0.6606t}$	0.997
Theophylline (serum)	$Y = 7.794 e^{-0.0438t}$	0.994
Theophylline (HIM)	$Y = 3.755 e^{-0.0573t}$	0.988
Valproic acid (serum)*	ND	ND
Valproic acid (HIM)	$Y = 3.993 e^{-0.0589t}$	0.988

*Although the disappearance of valproic acid could be measured from serum-free medium (HIM), the concentration of valproic in hepatocytes suspended in serum remained essentially constant over the entire 6-h incubation period.

ND not determined; r , correlation coefficient.

Human hepatocytes in suspension

With human hepatocytes in suspension, monoexponential decay curves were obtained for phenytoin, carbamazepine, theophylline, quinidine and procainamide in cells suspended either in serum-free HIM or in human serum. Examples for phenytoin, a relatively low clearance substance, and procainamide, a relatively high clearance substance, are shown in figures 1 and 2, respectively.

The disappearance data for all six xenobiotics are listed in table 2.

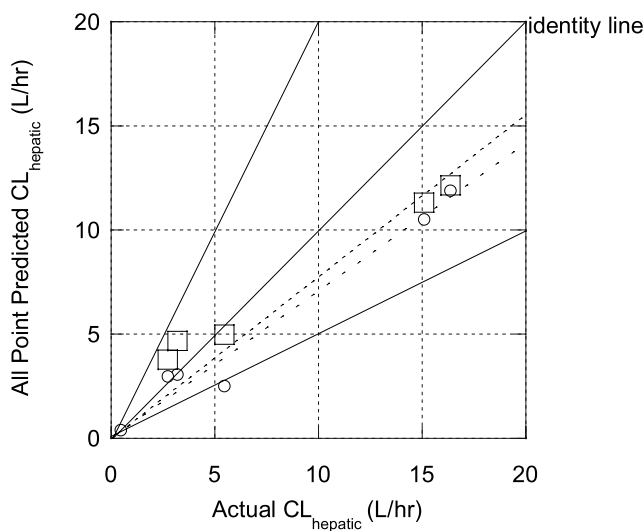


Figure 3. Regression plots of predicted versus actual $CL_{hepatic}$ based on multisample estimates of k using human hepatocytes suspended in serum (squares) or HIM (circles). Outer lines denote twofold deviations from actual clearances. The identity line is also shown. The slope and r^2 's were 0.71 and 0.984 for HIM and 0.776 and 0.995 for serum, respectively.

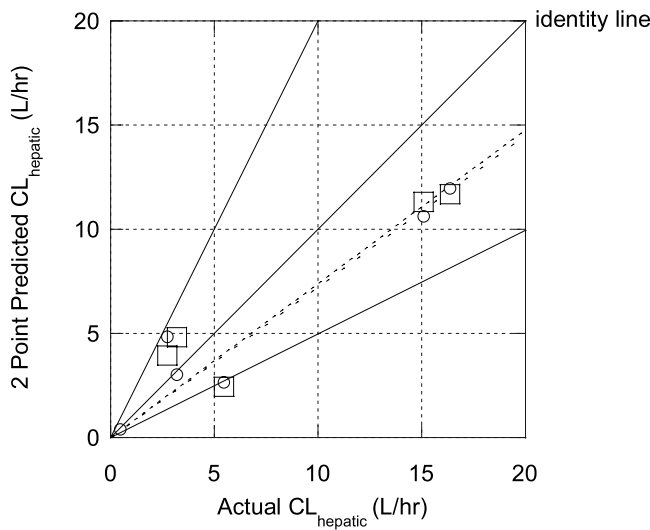


Figure 4. Regression plots of predicted versus actual $CL_{hepatic}$ based on two-sample estimates of k using human hepatocytes suspended in serum (open squares) or HIM (circles). Outer lines denote twofold deviations from actual clearances. The identity line is also shown. The slope and r^2 's were 0.724 and 0.959 for HIM and 0.739 and 0.942 for serum, respectively.

Prediction of in vivo hepatic clearance

The *in vitro* intrinsic clearance (CL'_{int}) was computed as the product of the volume of the incubation system and the elimination rate constant, k , of the disappearance curves of the test drugs from the incubation system. These values were scaled up to predict the *in vivo* hepatic clearance ($CL_{hepatic}$) as described in the Materials and methods.

Table 3. Prediction of *in vivo* hepatic clearance.

Xenobiotic	System	CL _{hepatic} (predicted from all points) (1h ⁻¹)	CL _{hepatic} (predicted from two points) (1h ⁻¹)	Actual CL _{hepatic} (1h ⁻¹)
Carbamazepine	HIM	2.53	2.65	5.46
	serum	4.97	2.45	
Theophylline	HIM	2.99	4.85	2.73
	serum	3.78	3.95	
Phenytoin	HIM	3.07	3.01	3.20
	serum	4.68	4.82	
Procainamide	HIM	11.88	11.97	16.38
	serum	12.15	11.68	
Quinidine	HIM	10.50	10.63	15.79
	serum	11.31	11.32	
Valproic acid	HIM	0.37	0.40	0.46
	serum	ND	ND	

ND, not determined; HIM, hepatocyte incubation medium.

Predicted CL_{hepatic}'s computed both from multisample-based estimates of k and from two-sample estimates of k derived from hepatocytes suspended in HIM or in serum were plotted against CL_{hepatic}'s derived from actual reported total clearances (Thummel and Shen 2001) (figures 3 and 4). Tabulation of CL_{hepatic}'s for all xenobiotics is given in table 3.

The differences between estimates of CL_{hepatic} from cells suspended in HIM or serum were not statistically significant via a paired t -test ($p = 0.257$). Both the paired t -test and Wilcoxon signed rank test failed to detect significant ($p > 0.05$) differences between actual CL_{hepatic} and predicted CL_{hepatic} based on cells suspended in serum ($p = 0.824$) or HIM ($p = 0.163$), respectively. Finally, there was no difference between CL_{hepatic} estimated from multisample measurements versus two-point estimates ($p = 0.398$).

Discussion

Human hepatocytes suspended either in serum-free hepatocyte incubation medium (HIM) or in human serum were used in the estimation of intrinsic hepatic clearance (CL'_{int}) of each of six different xenobiotics encompassing a 35-fold range of CL_{hepatic}'s with reported values ranging from 0.46 to 16.38 1h⁻¹. *In vitro* CL'_{int}'s were subsequently scaled to *in vivo* CL_{hepatic}. A correction for serum protein binding was made for all studies in serum-free HIM, since the measured CL'_{int} in this system was actually unbound CL'_{int} (i.e. CL'_{int,unbound}). Initial xenobiotic concentrations were limited to values less than published K_m 's referenced accordingly to total and to unbound drug. Under these conditions, the disappearance of the xenobiotic from the incubation media would be expected to be monoexponential in character. Xenobiotics were incubated for up to 6 h, and, as expected, the elimination kinetics of all xenobiotics from the incubation media was well characterized by a monoexponential decay model. The rate constant for xenobiotic decay was used to compute CL'_{int}, and CL'_{int}'s were subsequently scaled to predicted CL_{hepatic}. Finally, the predicted CL_{hepatic} were compared with values estimated from previously published total clearances. We found that this simple, *in vitro* system provides good predictions of CL_{hepatic} whether human hepatocytes were suspended in HIM or in human serum. Moreover, two-point

predictions of CL_{hepatic} were as faithful as the multipoint estimates to the actual values of CL_{hepatic} .

The predictions of *in vivo* CL_{hepatic} for these six xenobiotics would be considered good by the twofold standard (Obach *et al.* 1997). However, the underpredictions for carbamazepine, quinidine and procainamide by 54, 31 and 25%, respectively, could result, in theory, from model misspecification. In these cases if xenobiotic decay were polyexponential, it is possible that a faster initial rate constant may have been missed by assuming monoexponential decay. It would be certainly be useful to extend this analysis to a larger number of xenobiotics and over a larger range of CL_{hepatic} 's to see if the current model is adequate or needs to be revised.

We used only two batches of cryopreserved human hepatocytes, and most of the data were collected from a single batch. The advantage to this approach is that variability is minimized, particularly with regard to known xenobiotic exposures. The disadvantage is that extrapolation of these predictions generally must be made with caution because there is no guarantee that hepatocytes from such a limited sample are, in fact, representative of average livers.

Predictions based on hepatocytes suspended in HIM versus serum were equivalent. The advantage of using hepatocytes suspended in serum is that a separate measurement for protein binding is not required (Shibata *et al.* 2000). However, human serum is more costly and somewhat more difficult to work with than serum-free systems. The use of hepatocytes may confer an advantage to the use of subcellular or Phase I expression systems. Observed values of CL_{hepatic} may be a function of several sequential and/or parallel processes (transport of the xenobiotic into the hepatocyte, processing by Phase I/II enzyme systems) of which any can represent the rate-limiting step. Any limits to CL_{hepatic} imposed by xenobiotic influx and/or Phase II processing might well be accounted for automatically when employing intact hepatocytes where all of these processes occur. Disappearance rates based on intact hepatocytes may be more physiologically relevant than rates measured in subcellular expression systems and do not require additional scaling factors to adjust for non-physiological ratios of accessory proteins (Venkatakrishnan *et al.* 2000). Moreover, one does not have to know specific metabolic pathways a priori. The measurement of parent molecule decline rather than the formation of metabolites means that useful PK predictions can be made without the development of assays for and identification of each metabolite.

It must be noted that we have evaluated the predictability of *in vivo* CL_{hepatic} using six xenobiotics whose K_m 's are known. While we do not argue that the prediction of CL_{hepatic} from the decline of xenobiotic from suspensions of human hepatocytes supplants other methods, the application of this strategy for evaluating the PK suitability of NCEs could precede a more detailed analysis of *in vitro* metabolism in which all or most enzymatic pathways and enzyme kinetic parameters must be determined. Moreover, since the estimation of CL_{hepatic} from two-point measurements was as accurate as from multipoint measurements, this strategy is adaptable to HTP technologies. In fact, we found that for five of our six xenobiotics, we could accurately predict CL_{hepatic} from the C_{last} measurement (usually C_6) only. In these cases, C_0 was simply taken as the intended initial concentration. This single sample prediction failed only in the case of theophylline, since the added theophylline concentration was errantly higher than had been

intended. In any case, without prior knowledge of the K_m 's one might add three different concentrations of NCE, with each higher concentration set at one magnitude above the last. Saturation kinetics, if present, would likely be detectable and could be established with as few as three (single-point) measurements or six (i.e. three two-point) measurements.

Putative drug-drug interactions could also be assessed early in drug development using this strategy. CL_{hepatic} would be estimated in the presence and absence of putative inhibitors of interest. Again, prior knowledge of K_i 's would not be required, nor would it be important to know in advance specifically which enzymes were inhibited. The effects on CL_{hepatic} of putative inhibitors of concern added at concentrations approximating their average steady-state plasma concentrations *in vivo* should provide sufficient information for predicting *in vivo* outcomes.

In conclusion, the measurement of the disappearance of xenobiotics from serum or HIM in which primary human hepatocytes have been suspended provides a relatively simple and accurate strategy for predicting *in vivo* human CL_{hepatic} . For the six xenobiotics used — phenytoin, valproic acid, carbamazepine, quinidine, procainamide and theophylline — all predicted values of *in vivo* CL_{hepatic} were within twofold of the actual CL_{hepatic} 's whether CL_{int} was estimated from multisample measurements or from two-point measurements.

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Phase II *in vitro* metabolism of 3-methylindole metabolites in porcine liver

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Received 16 September 2002

1. The Phase II *in vitro* metabolism of 3-methylindole (3MI) metabolites was investigated in pigs to determine the possible relationship between 3MI Phase II metabolism and 3MI accumulation in fat. Sulphation and glucuronidation of five of the seven major metabolites found to be produced by porcine microsomes was investigated using porcine cytosol and microsomes, respectively. The possible formation of glutathione conjugates was also investigated using microsomally activated 3MI intermediate(s).

2. No sulphation or glucuronidation was observed for metabolites 3-hydroxy-3-methyloxindole, 3-methyloxindole, indole-3-carbinol or 2-aminoacetophenone; however, 5-hydroxy-3-methylindole (5-OH-3MI) was conjugated with both sulphate and glucuronic acid.

3. The enzyme responsible for sulphation of 5-OH-3MI was identified as the thermostable form of phenol-sulphotransferase (TS-PST) based on its susceptibility to TS-PST inhibitors and the correlation between sulphation of 5-OH-3MI and sulphation of the prototype substrate *p*-nitrophenol ($r = 0.94$, $p < 0.001$).

4. A 3MI-glutathione adduct was identified in microsomal incubations containing 3MI and glutathione.

5. Sulphation of 5-OH-3MI was high in pigs with low levels of 3MI in fat. No relationship was observed between 3MI levels in fat and either glutathione transferase or glucuronidation activities in liver.

Introduction

Skatole (3-methylindole, 3MI) is a naturally occurring microbial metabolite produced from tryptophan in the gastrointestinal tract of ruminants (Yokoyama and Carlson 1979), humans (Fordtran *et al.* 1964) and pigs (Jensen *et al.* 1995). It is a well-known acute pneumotoxin for cattle and has important implications for pig meat production. Entire male (uncastrated) pigs are used for meat production in several countries owing to a better feed conversion, higher carcass leanness and better composition of fatty acids compared with castrated pigs (reviewed by Babol and Squires 1995). However, 5–10% of male pigs carry the so-called 'boar taint' (a faecal-like odour liberated when the meat is cooked) and 3MI is one of the major contributors to boar taint (Bonneau *et al.* 2000). It is not clear why only a small percentage of a given population of pigs accumulates 3MI to a level that can be detected by humans, but differences in its metabolism have been observed between pigs with high versus low levels of 3MI in fat. In *in vitro* assays, 3MI accumulation in fat has been shown to be significantly and negatively correlated with hepatic

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levels of cytochrome P4502E1 (Squires and Lundström 1997), P4502A6 (Diaz and Squires 2000a), aldehyde oxidase (Diaz and Squires 2000b) and the rate of sulphation of the 3MI metabolite 6-hydroxy-3-methylindole (Babol *et al.* 1998).

Phase II enzymes facilitate the elimination of xenobiotics by conjugation with a highly polar moiety such as sulphuryl, glucuronyl or glutathionyl moieties (Parkinson 2001). Human liver cytosol contains two phenol sulphotransferases (PST) that can be distinguished by their thermal stability and are known as TS-PST (thermally stable) and TL-PST (thermally labile) (Weinshilboum 1990). In humans, 10 sulphotransferase genes have been identified and the TS-PST is now known to reflect the activity of sulphotransferases ST1A2 and ST1A3 (Nagata and Yamazoe 2000). Glucuronidation is quantitatively the major pathway of xenobiotic biotransformation in mammalian species, except for members of the feline family; the uridine diphosphate (UDP) glucuronosyltransferases are enzymes capable of transferring the nucleotide sugar of uridine 5'-diphosphate glucuronic acid (UDP-GA) to endogenous and exogenous compounds having nucleophilic functional groups of oxygen, nitrogen, sulphur and carbon (Clarke and Burchell 1994). The superfamily of glutathione *S*-transferases (GST) catalyses a wide range of reactions in which the glutathione (GSH) thiolate anion participates as a nucleophile (Salinas and Wong 1999).

The Phase I *in vitro* metabolism of 3MI in porcine liver was described by Diaz *et al.* (1999) and Diaz and Squires (2000a, b). It is now known that seven metabolites of 3MI are produced by porcine liver microsomes through reactions catalysed by P4502A6, P4502E1 and the cytosolic enzyme aldehyde oxidase. However, possible conjugation reactions of these metabolites were not investigated and the Phase II metabolism of 3MI in pigs is still not completely understood. The aim of the present study was to characterize further the conjugation reactions of Phase I metabolites of 3MI in porcine liver. The possible relationship between conjugation activity and 3MI levels in fat was also investigated.

Materials and methods

Chemicals

Pentachlorophenol (PCP), 2,6-dichloro-4-nitrophenol (DCNP), 1-chloro-2,4-dinitrobenzene (CDNB), *p*-nitrophenol, adenosine 3'-phosphate 5'-phosphosulphate (PAPS), 3-methylindole (3MI), indole-3-carbinol (I3C), 2-aminoacetophenone, uridine diphosphate glucuronic acid (UDP-GA), glutathione, β -NADPH, type B-1 β -glucuronidase (sulphatase-free) and type H-2 sulphatase from *Helix pomatia* were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Authentic 3-methyloxindole (3MOI), 3-hydroxy-3-methyloxindole (HMOI) and the 3MI-glutathione adduct were graciously provided by Dr G. S. Yost, Department of Pharmacology and Toxicology, University of Utah (Salt Lake City, UT, USA). Authentic 5-hydroxy-3-methylindole (5-OH-3MI) was graciously provided by Jens Hansen Møller of the Danish Meat Research Institute (Roskilde, Denmark).

Preparation of porcine liver microsomes and cytosol

Liver samples were taken from 30 intact male pigs of approximately 108 kg body weight obtained by back-crossing F3 European Wild Pig $\times$ Swedish Yorkshire boars with Swedish Yorkshire sows (Squires and Lundström 1997). These pigs contain a wider range of 3MI levels in fat than commercial breeds and are more suitable for studies where correlations between 3MI levels in fat and other variables are investigated. Liver samples were frozen in liquid nitrogen and stored at -80°C . For the preparation of the microsomal suspension and cytosolic fraction, partially thawed liver samples were finely minced and homogenized with 4 vols 0.05 M Tris-HCl buffer, pH 7.4 (containing 0.15 M KCl, 1 mM EDTA, 0.25 M sucrose), using an Ultra-Turax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 10 000 *g* for 20 min and the resulting supernatant was centrifuged again at 100 000 *g* for 60 min using a Beckman L5-50 Ultracentrifuge (Beckman, Fullerton, CA, USA) in order

to obtain the cytosolic fraction (supernatant) and the microsomal pellet. The pellets were suspended in 0.05 M Tris-HCl buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA and 0.25M sucrose to a final concentration of 20 mg protein ml⁻¹. Cytosolic fractions and microsomal suspensions were stored at -80 °C before analysis. Protein concentrations were determined by the method of Smith *et al.* (1985) using bicinchoninic acid protein assay reagents purchased from Pierce Chemical Co. (Rockford, IL, USA) and bovine serum albumin as standard.

Enzyme assays

Sulphoconjugation. The possible sulphoconjugation of five 3MI metabolites was investigated and preliminary experiments indicated that the metabolites HMOI, 3MOI, I3C and 2-aminoacetophenone were not conjugated with sulphate. The only 3MI metabolite tested that was found to conjugate with sulphate was 5-OH-3MI. Accordingly, sulphotransferase activity was determined in the complete set of samples ($n = 30$) using 5-OH-3MI as substrate; sulphation of *p*-nitrophenol (prototype substrate for TS-PST) was also investigated in order to determine a possible relationship between these two enzymatic activities. For the assay of 5-OH-3MI sulphation, 1 mg cytosolic protein was incubated with 0.01 mM 5-OH-3MI and 1 mM PAPS in 0.05M Tris-HCl, pH 7.4, buffer containing 5 mM MgCl₂ in a total volume of 250 µl. Sulphation of *p*-nitrophenol was conducted using the same buffer and sulphate donor concentration, but 2 mg cytosolic protein and 0.1 mM *p*-nitrophenol were used instead. Sulphation of 5-OH-3MI and *p*-nitrophenol was determined to be linear over a range of 10–40 min and 1–4 mg cytosolic protein. Incubations for 5-OH-3MI and *p*-nitrophenol were carried out in a shaking water bath at 37 °C for 10 and 40 min, respectively; the reaction was stopped with 250 µl ice-cold acetonitrile. After the addition of acetonitrile, the mixture was vortexed and centrifuged at 4000g for 15 min. A 400-µl aliquot of the clear supernatant was diluted with 400 µl water and 100 µl of the mixture was analysed immediately by high-performance liquid chromatography (HPLC) as described below. Dilution with water was necessary in order to avoid 'leading' (an asymmetrical shape) of the chromatographic peaks. The total amount of 5-OH-3MI or *p*-nitrophenol remaining at the end of the incubations was quantitated by using an appropriate external standard of known concentration (0.01 mM 5-OH-3MI and 0.1 mM *p*-nitrophenol, respectively). The rate of conjugation was calculated by subtracting the nanomoles of substrate in a blank run (no cofactor added) from the nanomoles of substrate remaining at the end of the reaction in a test run, using the area under the curve (AUC) of the peaks. Since the nanomoles of substrate in the blank run are known, the nanomoles consumed in the reaction can be calculated using the following equation:

Substrate consumed (nanomole)

$$= \text{substrate in a blank run (nanomole)} - \left[\frac{\text{AUC of substrate in test run}}{\text{AUC of substrate in blank run}} \right] \times \text{concentration of substrate in blank run}.$$

The nanomoles of substrate consumed in the reaction were assumed to be equivalent to the number of nanomoles of conjugate formed in the incubation, and this amount was found to be directly proportional to the AUC of the peak of the conjugate. Using this approach total substrate and conjugate added 100% of the total nanomoles present at the end of each incubation.

Conjugates were identified based on the following criteria: (1) no peaks corresponding to conjugates appeared when cytosol and/or PAPS were excluded from the incubations; (2) no peaks corresponding to conjugates appeared when boiled cytosol was used; and (3) the peaks corresponding to the conjugates disappeared after addition of 10 µl type H-2 sulphatase to the incubation mixture and further incubation for 3 h at 37 °C in a shaking water bath. Sulphation activity was measured as the formation of sulphoconjugate min⁻¹ mg⁻¹ cytosolic protein.

Conjugation with glucuronic acid. The possible conjugation with glucuronic acid of five 3MI metabolites was investigated and preliminary experiments indicated that the metabolites HMOI, 3MOI, I3C and 2-aminoacetophenone were not conjugated. The only 3MI metabolite tested that was found to conjugate with glucuronic acid was 5-OH-3MI. Accordingly, glucuronidation activity was determined in the complete set of samples ($n = 30$) using 5-OH-3MI as substrate. For the assay of 5-OH-3MI glucuronidation, 0.2 mg microsomal protein was incubated with 0.1 mM 5-OH-3MI and 4 mM UDP-GA in 0.05 M Tris-HCl, pH 7.4, buffer containing 5 mM MgCl₂ in a total volume of 250 µl. Glucuronidation of 5-OH-3MI was determined to be linear over a range of 2–10 min and 0.05–0.25 mg protein. Incubations were carried out in a shaking water bath at 37 °C for 3 min and the reaction was stopped with 250 µl ice-cold acetonitrile. After the addition of acetonitrile, the mixture was vortexed and centrifuged at 4000g for 15 min. A 400-µl aliquot of the clear supernatant was diluted with 400 µl water and 100 µl of the mixture was analysed immediately by HPLC as described below. The total amount of 5-OH-3MI remaining at the end of the incubations was quantitated by using an external standard of 0.1 mM 5-OH-3MI. The amount of conjugate formed was calculated as explained above. The 5-OH-3MI glucuronide was identified based on the following criteria: (1) no peak corresponding to a conjugate appeared when microsomes and/or UDP-GA were excluded from the incubations; (2) no peak corresponding to a conjugate appeared when boiled microsomes were used; and (3) the peak

corresponding to the conjugate disappeared after addition of 10 μ l β -glucuronidase (3000 IU) to the incubation mixture and further incubation for 3 h at 37°C in a shaking water bath. Glucuronidation activity was measured as the formation of glucuronide $\text{min}^{-1} \text{mg}^{-1}$ protein.

Glutathione conjugation. Incubations were conducted with pig liver microsomes as described by Diaz *et al.* (1999). The 0.5-ml incubation mixtures contained 2 mg microsomal protein, 0.4 mM 3MI, 2 or 4 mM GSH, and 4 mM NADPH. NADPH and/or glutathione were omitted from appropriate control incubations. After 30 min at 37°C, 0.5 ml ice-cold acetonitrile were added to incubations both to stop the reaction and to precipitate protein. After centrifugation, 100 μ l of clear supernatant were analysed by HPLC as described by Diaz *et al.* (1999). Formation of GSH adducts was also investigated using 100 000g hepatic cytosolic fractions containing glutathione-S-transferase activity. Incubations were carried out with and without previous 3MI incubation using hepatic microsomes in order to generate 3MI metabolites. Cytosolic fractions in the complete set of samples ($n = 30$) were measured for glutathione-S-transferase activity according to the method of Habig *et al.* (1974), using CDNB as substrate. Production of conjugate was determined to be linear with the incubation time and protein concentrations used in the assay.

Sulphotransferase inhibition studies

In order to investigate the effects of the phenolsulphotransferase inhibitors PCP and DCNP on the sulphation of 5-OH-3MI, incubations containing 5-OH-3MI, porcine liver cytosol, PAPS and different concentrations of the inhibitors were conducted. Each incubation was run in duplicate and was performed for three randomly selected cytosol samples. Assay mixtures were run under the conditions described above but included different final concentrations of PCP or DCNP (0.001, 0.01, 0.1, 1, 10, 100 μ M). PCP was dissolved in methanol (final assay concentration 1% v/v) and control incubations for PCP inhibition studies contained 1% methanol. DCNP was dissolved in incubation buffer. Incubations were carried out and terminated under the same conditions described above for 5-OH-3MI sulphation assay.

Chromatographic analysis of sulphate and glucuronic acid conjugates

HPLC was conducted using a Spectra-Physics system (San Jose, CA, USA) consisting of a SP8800 gradient pump, a SP8880 autosampler with a 100- μ l injection loop, a SP Spectra 100 UV detector and a Spectra System FL-2000 fluorescent detector. The HPLC method for the determination of sulphoconjugates and their parent compounds is a modification of a previously reported binary gradient system method (Hernández *et al.* 1991). *p*-Nitrophenol, 5-OH-3MI and their sulphate conjugates were separated using a reverse-phase Prodigy ODS, 5 μ m, 250 $\times$ 4.6 mm column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.05 M ammonium acetate buffer, pH 5.0, at a flow rate of 1.2 ml min^{-1} . A 25-min linear gradient from 10 to 60% acetonitrile was used, with initiation of the gradient 2 min after sample injection. *p*-Nitrophenol and its conjugate were determined by their UV absorbance at 300 nm, whereas 5-OH-3MI and its conjugate were determined by their fluorescence (excitation wavelength 285 nm, emission wavelength 350 nm). Typical retention times for *p*-nitrophenol and its conjugate were 21.1 and 15.2 min, respectively; typical retention times for 5-OH-3MI and its conjugate were 18.5 and 13.4 min, respectively. The HPLC method for the simultaneous determination of the 5-OH-3MI glucuronide and 5-OH-3MI is a modification of a previously reported ion-pair isocratic system method (Hamar-Hansen *et al.* 1986, Di Girolamo *et al.* 1998). The glucuronide and parent compound were separated using the same chromatographic column used for sulphoconjugates, but the mobile phase consisted of water-acetonitrile (76:24) modified with 0.1% trifluoroacetic acid at a flow rate of 1.0 ml min^{-1} . Both 5-OH-3MI and its glucuronide were detected by fluorescence (excitation wavelength 285 nm, emission wavelength 350 nm). With this system, typical retention times for 5-OH-3MI and its glucuronide were 15.7 and 5.4 min, respectively. HPLC analysis after sulphation and glucuronidation were conducted immediately after the addition of acetonitrile, centrifugation of the incubation mixture and dilution of the supernatant with water.

Measurement of 3MI in fat

For the quantitation of the 3MI in fat, a sample of subcutaneous backfat was taken from each of the pigs from which the liver samples were taken ($n = 30$) and each sample was analysed for 3MI using a colorimetric assay (Mortensen and Sørensen 1984). Samples were taken after slaughter at approximately 108 kg live weight and were kept at -20°C until they were analysed. All analyses were done in duplicate.

Statistical analysis

Pearson correlation coefficients, linear regression analysis and one-way ANOVA were computed using the Statistical Analysis System (SAS 1995).

Results

Sulphoconjugation

The only metabolite found to conjugate with sulphate in the presence of PAPS and porcine cytosol was 5-OH-3MI. Figure 1 shows typical chromatograms obtained after the incubation of 5-OH-3MI and cytosol, with and without the addition of PAPS. No 5-OH-3MI sulphoconjugate was formed when cytosol was boiled before the incubation, and when no cytosol and/or PAPS were added to the

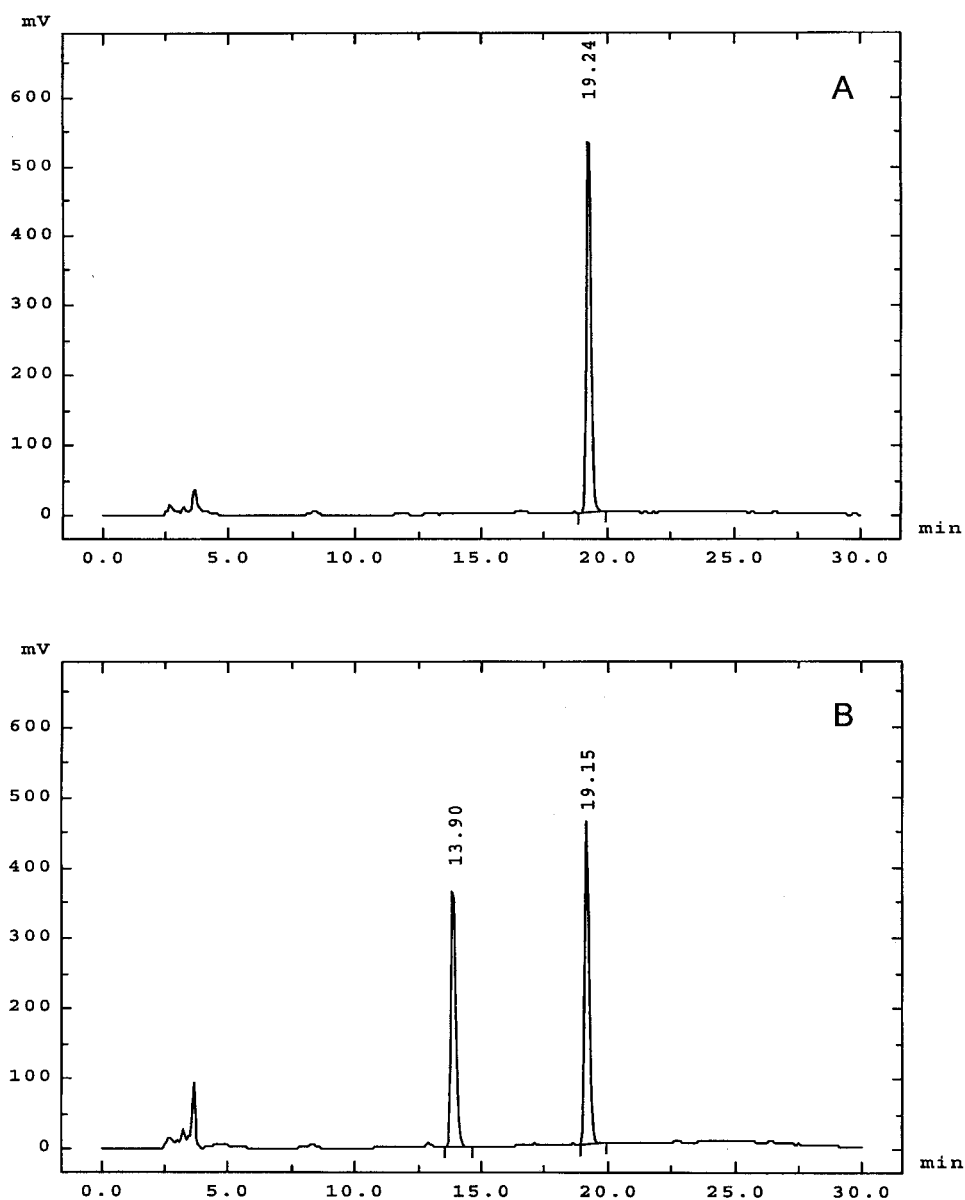


Figure 1. HPLC of sulphation conjugate from 5-OH-3MI following incubation with porcine liver cytosol. (A) Control: 0.01 mM 5-OH-3MI, no PAPS; (B) assay: 0.01 mM 5-OH-3MI and 1 mM PAPS.

incubation mixture (data not shown). The addition of type H-2 sulphatase to the incubation mixture resulted in the complete disappearance of the conjugate peak of 5-OH-3MI (data not shown).

The addition of the PST inhibitors PCP or DCNP to incubation mixtures containing 5-OH-3MI, cytosolic protein and PAPS decreased the formation of conjugate in a concentration-dependent manner (figure 2). When no inhibitor was added, the total amount of 5-OH-3MI sulphoconjugate produced was considered as 100%. At a concentration of 10 μ M DCNP, 13.8% of the control 5-sulphatoxyskatole production was observed, and at 100 μ M 7.1% of the control

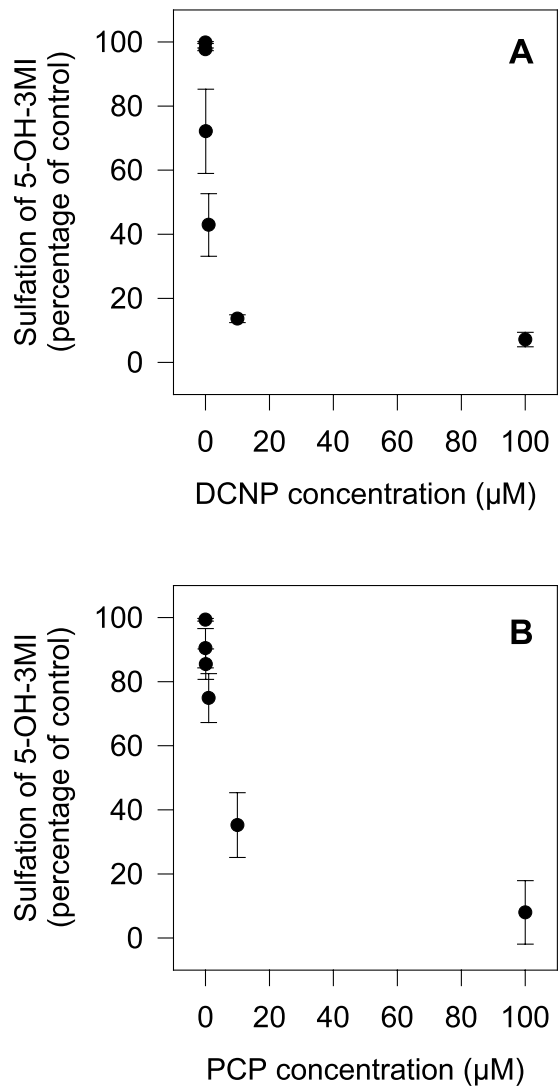


Figure 2. (A) Inhibition of the formation of 5-sulphatoxyskatole from 5-hydroxy-3-methylindole by dichloronitrophenol (DCNP) following incubation with porcine liver cytosol. (B) Inhibition of the formation of 5-sulphatoxyskatole from 5-hydroxy-3-methylindole by pentachlorophenol (PCP) following incubation with porcine liver cytosol. Each data point represents the mean $\pm$ SD of duplicate analysis performed for three pigs.

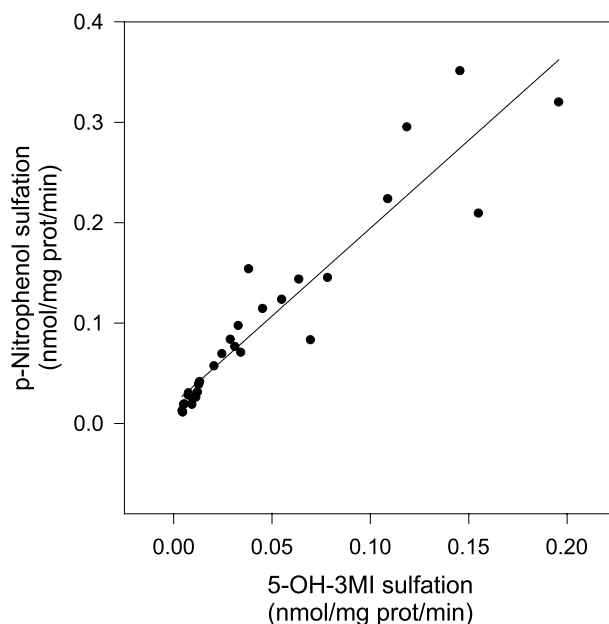


Figure 3. Relationship between 5-OH-3MI sulphation and *p*-nitrophenol sulphation in porcine cytosol ($n = 30$, $r = 0.94$, $p < 0.001$).

sulphatoxyskatole was found (figure 2A). When a concentration of $10\text{ }\mu\text{M}$ PCP was added to the incubations, only 35.3% of the 5-sulphatoxyskatole formed in the absence of PCP was found; when PCP concentration was increased to $100\text{ }\mu\text{M}$, only 8% of 5-sulphatoxyskatole was produced (figure 2B). The relationship between sulfoconjugation of 5-OH-3MI and *p*-nitrophenol, the model substrate for TS-PST, is shown in figure 3. The correlation coefficient between these two variables was 0.94 ($p < 0.001$).

Glucuronidation

The only metabolite found to conjugate with glucuronic acid in the presence of UDP-GA and porcine microsomes was 5-OH-3MI. Figure 4 shows typical chromatograms obtained after the incubation of 5-OH-3MI and microsomes, with and without the addition of UDP-GA. No glucuronide was detected when the microsomal suspension was boiled before the incubation or when microsomes and/or UDP-GA were excluded from the incubation mixture (data not shown). The addition of type B-1 β -glucuronidase to the incubation mixture resulted in the disappearance of the conjugate peak of 5-OH-3MI (data not shown).

Glutathione conjugation

Pig liver microsomal incubations containing 3MI, NADPH and GSH produced a metabolite which was not formed in microsomal control mixtures in which one of these substances was omitted. The metabolite was well separated from other incubation supernatant components by the HPLC conditions used (Diaz *et al.* 1999). The metabolite was detected by its fluorescence (280 nm excitation, 350 nm

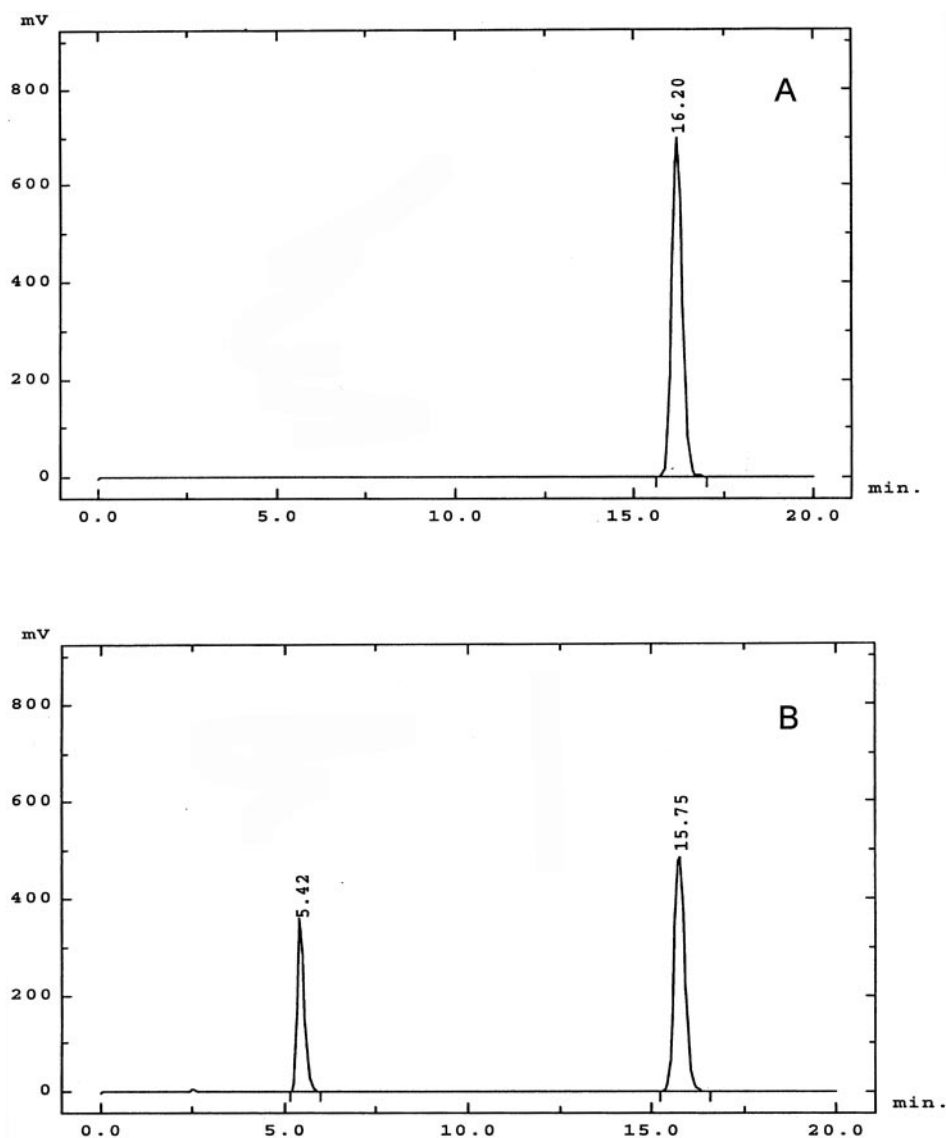


Figure 4. HPLC of glucuronic acid conjugate from 5-OH-3MI following incubation with porcine liver microsomes. (A) Control: 0.1 mM 5-OH-3MI, no UDP-GA; (B) assay: 0.1 mM 5-OH-3MI and 4 mM UDP-GA.

emission wavelengths) at 11.7 min and had the same retention time as the 3MI-GSH adduct 3-[(glutathione-*S*-yl)-methyl]indole. The addition of cytosolic fractions containing glutathione-*S*-transferase activity did not increase GSH adduct formation indicating that the reaction between GSH and 3MI occurs spontaneously in the presence of NADPH. This metabolite was identified as the 3MI-GSH adduct by the following criteria: (1) the metabolite was not produced when NADPH, GSH or both were omitted from incubations; (2) the metabolite had the same retention time as the 3MI-GSH standard; and (3) the metabolite co-eluted with the standard when supernatant incubations were spiked with standard.

Relationship between conjugation activity and 3MI in fat

Figure 5 shows the plots of sulphation of 5-OH-3MI, glucuronidation of 5-OH-3MI and GST activity versus the 3MI levels in fat for the 30 pigs used in the present study. The PST activity, estimated as nmol 5-sulphatoxyskatole produced $\text{min}^{-1} \text{mg}^{-1}$ cytosolic protein, was not significantly correlated with 3MI levels in

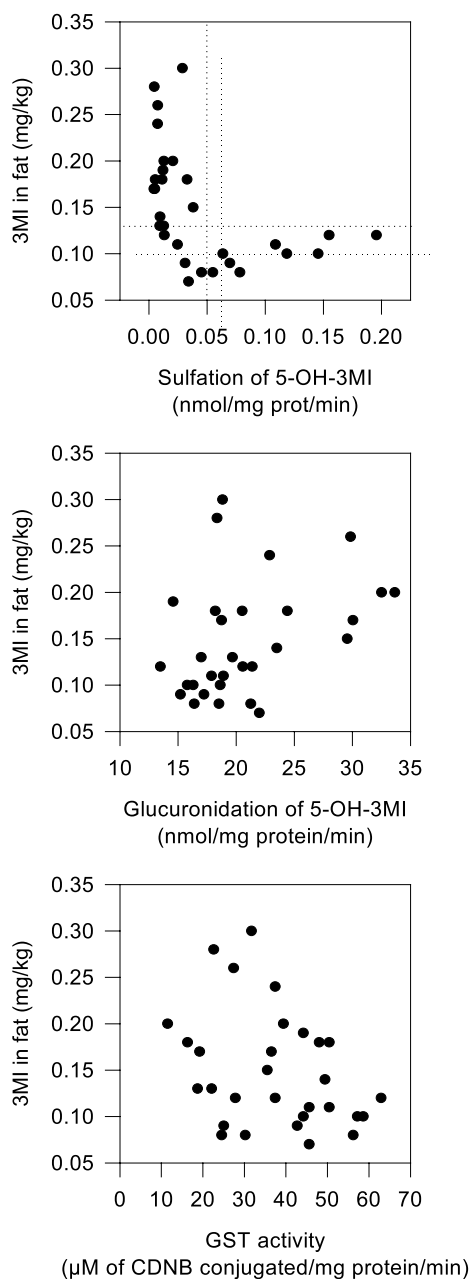


Figure 5. Relationship between *in vitro* hepatic 5-OH-3MI sulphation, 5-OH-3MI glucuronidation and GST activity, and 3MI accumulation in fat in pigs ($n = 30$).

fat but pigs with high 3MI levels in fat consistently showed low sulphation activity. Conversely, pigs with high sulphation activity had low levels of 3MI in fat. The glucuronidation of 5-OH-3MI and the GST activity, estimated as μmol CDNB conjugated with GSH $\text{min}^{-1} \text{mg}^{-1}$ cytosolic protein were not correlated with the 3MI content in fat and no particular pattern was observed in the plots for low and high 3MI accumulators.

The content of 3MI in fat in all samples ranged from 0.07 to 0.3 mg kg^{-1} and had a mean of 0.15 mg kg^{-1} . The 5-OH-3MI sulphation activity ranged from 0.004 to 0.196 nmol 5-sulphatoxyskatole mg^{-1} protein min^{-1} and had a mean of 0.045 nmol sulphatoxyskatole mg^{-1} protein min^{-1} . The 5-OH-3MI glucuronidation activity ranged from 13.5 to 33.6 nmol 5-OH-3MI glucuronide mg^{-1} protein min^{-1} with a mean of 20.9 nmol 5-OH-3MI glucuronide mg^{-1} protein min^{-1} . The GST activity ranged from 11.5 to 62.9 μmol CDNB conjugated mg^{-1} protein min^{-1} and had a mean of 37.3 μmol CDNB conjugated mg^{-1} protein min^{-1} . The results were grouped in three categories according to the degree of 3MI accumulation in fat for each pig as follows: large 3MI accumulators ($\geq 0.2 \text{ mg kg}^{-1}$ 3MI), moderate 3MI accumulators (0.11–0.19 mg kg^{-1} 3MI) and low accumulators ($\leq 0.1 \text{ mg kg}^{-1}$ 3MI). Lundström and Bonneau (1996) suggested that levels of 3MI of $\geq 0.2\text{--}0.25 \text{ mg kg}^{-1}$ cause unacceptable taint by sensory analysis. The means for 3MI content in fat, 5-OH-3MI sulphation activity, 5-OH-3MI glucuronidation activity and GST activity for these three categories of pigs are shown in table 1.

Discussion

Diaz *et al.* (1999) reported that porcine liver microsomes produce seven major metabolites from 3MI: 3-hydroxy-3-methylindolenine, 3MOI, HMOI, I3C, 2-aminoacetophenone, 5-OH-3MI and 6-hydroxy-3-methylindole (6-OH-3MI). Conjugation of 6-OH-3MI with sulphate to form 6-sulphatoxyskatole was demonstrated by Bæk *et al.* (1997) and Babol *et al.* (1998), and glucuronidation of 6-OH-3MI was reported by Bæk *et al.* (1995) and Babol *et al.* (1998). On the other hand, 3-hydroxy-3-methylindolenine was reported to be converted into HMOI by the action of the cytosolic enzyme aldehyde oxidase (Diaz *et al.* 2000b). Therefore, for the present study, it was decided to investigate the possible conjugation with

Table 1. Sulphation, glucuronidation and glutathione-sulphotransferase activities in pigs with different 3-methylindole content in fat.

	Degree of 3-methylindole accumulation		
	High (n = 6)	Moderate (n = 15)	Low (n = 9)
3-Methylindole content in fat (mg kg^{-1})	≥ 0.2	0.11–0.19	≤ 0.1
5-OH-3MI sulphation activity (nmol 5-sulphatoxyskatole mg^{-1} protein min^{-1})	0.014 ± 0.004^b	0.043 ± 0.060^{ab}	0.071 ± 0.038^a
5-OH-3MI glucuronidation activity (nmol 5-OH-3MI glucuronide mg^{-1} protein min^{-1})	26.0 ± 6.9^a	20.6 ± 4.7^a	17.9 ± 2.4^a
GST activity (μmol CDNB conjugated mg^{-1} protein min^{-1})	28.3 ± 10.3^a	37.6 ± 14.2^a	42.7 ± 13.5^a

Data are mean $\pm$ SD.
^{a-c}Means within a row without a common superscript are significantly different ($p < 0.05$).

sulphate and glucuronic acid of five 3MI metabolites: 3MOI, HMOI, I3C, 2-aminoacetophenone and 5-OH-3MI, using porcine cytosol as the source of PST and porcine microsomes as the source of UDP-GT. Additionally, the possible formation of GSH adducts in microsomal incubations was also investigated, either with or without the addition of cytosol as a source of GST activity.

A GSH adduct identified as 3MI-GSH by comparison with authentic standard was produced in microsomal incubations in the present study. The structure of this adduct was elucidated by Nocerini *et al.* (1985) who isolated it from goat lung microsomal incubations. The detection of the 3MI-GSH adduct in the supernatant of porcine microsomal incubations containing GSH confirm that the porcine species is capable of forming a reactive electrophile from 3MI that reacts with GSH. Formation of the 3MI-GSH adduct in the porcine species had not been reported previously but the mercapturate adduct expected as the processed 3MI-GSH adduct had been isolated from pig urine by Bæk *et al.* (1997). The conjugation of the putative 3MI electrophile and GSH occurred spontaneously and the reaction was not enhanced by the addition of cytosol with GST activity. The spontaneous reaction of GSH with an electrophile is common and depends on the nature of the electrophile (Ketterer 1986). Since the GST activity in the porcine cytosol samples could not be determined as the rate of formation of the 3MI-GSH adduct, it was decided to measure the GST activity using another substrate. CDNB was selected for this determination because it has been reported that swine conjugate CDNB as effectively as other mammalian species but have extremely low activity toward other prototype substrates such as ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and sulphobromophthalein (Watkins and Klaassen 1986). The average GST activity in pigs with low levels of 3MI in fat was numerically higher than in those with high levels (table 1); however, this difference failed to reach statistical significance. Further, the lack of correlation between 3MI fat levels and GST activity (figure 5) suggests that GST activity does not play an important role in the accumulation of 3MI in entire male pigs.

From the five metabolites studied, only 5-OH-3MI was found to conjugate with glucuronic acid. The glucurono-conjugation of 6-OH-3MI has been reported in a pig given a large oral dose of 3MI and which excreted the 6-OH-3MI glucuronide in the urine (Bæk *et al.* 1995). Evidence for glucuronidation of 6-OH-3MI was also provided by Babol *et al.* (1998) who reported that the amount of 6-OH-3MI produced in microsomal incubations decreased in a concentration-dependent manner with the addition of UDP-GA. Glucuronidation of 5-OH-3MI had not been reported previously in pigs, but given the structural similarity of 5-OH-3MI with the 6 isomer, it is not surprising that this conjugation reaction occurs. The rate of glucuronidation of 5-OH-3MI, however, failed to correlate with 3MI levels in fat (figure 5) and no statistical significance in the rate of glucuronidation of 5-OH-3MI was found between high and low accumulators of 3MI in fat (table 1). It is of interest to note that porcine microsomes did not glucuronidate HMOI. Goats excrete HMOI in the form of HMOI glucuronide but mice do not glucuronidate HMOI (Smith *et al.* 1996). Therefore, the glucuronidation of HMOI in pigs is similar to that of mice and different from that of goats.

As was the case for the glucuronidation assays, the only metabolite found to be conjugated with sulphate was 5-OH-3MI. This finding is in agreement with the current knowledge about Phase II biotransformation of 3MI metabolites in

different animal species since no reports of 3MOI, HMOI, I3C or 2-aminoacetophenone sulphation could be found in the literature. Sulphation of 5-OH-3MI is not surprising since it had already been shown that 6-OH-3MI is conjugated with sulphate (Bæk *et al.* 1997, Babol *et al.* 1998). Sulphation of ring-hydroxylated 3MI has been reported in humans and rats but not in goats. In humans, sulphated 5-, 6- and 7-hydroxy-3MI have been detected in the urine (Mahon and Mattok 1967), whereas rats given 3MI either orally or intraperitoneally excreted 6-sulphatoxy-skatoles in the urine (Nakao and Ball 1960). In contrast, goats given jugular infusions of 3MI did not excrete hydroxyskatoles or their sulphate esters in the urine, which suggests that aromatic hydroxylation and subsequent sulphation is not the major metabolic route in goats (Hammond *et al.* 1979) as it is in humans and rats.

The thermostable form of PST catalyses the *O*-sulphation of simple phenolic compounds (Weinshilboum and Ottorness 1994) and is selectively inhibited by DCNP and PCP (Koster *et al.* 1982, Rein *et al.* 1982, Meerman *et al.* 1983); in contrast, the thermolabile form of PST and the hydroxysteroid sulphotransferase are resistant to inhibition by these two compounds (Weinshilboum and Ottorness 1994). The model substrate for TS-PST is considered to be *p*-nitrophenol (Weinshilboum 1990). The strong concentration-response inhibition of the sulphoconjugation of 5-OH-3MI caused by the addition of DCNP and PCP to the cytosolic incubations (figure 2) strongly suggests that TS-PST is the enzyme responsible for this reaction. In addition, the high correlation coefficient ($r = 0.94$, $p < 0.001$) found between *p*-nitrophenol sulphation and 5-OH-3MI sulphation suggests that both reactions are catalysed by the same enzyme and further suggests that TS-PST is the sulphotransferase involved in the conjugation of 5-OH-3MI with sulphate.

In humans, comparison of platelet PST activities with those in liver of the same subjects showed that levels of platelet TS-PST activities were significantly correlated with levels of TS-PST activity in liver ($r = 0.79$, $n = 14$, $p < 0.001$); however, levels of platelet TL-PST activity were not significantly correlated with TL-PST activity in the same organ ($r = 0.44$, $n = 14$, $p > 0.10$) (Weinshilboum 1990). Since the platelet is an easily accessible tissue, this finding could be of practical significance for the genetic selection of pigs based on high platelet sulphation activity against 5-OH-3MI, which possibly would correlate with high hepatic TS-PST activity.

In humans, 10 sulphotransferase genes have been identified and it is now known that TS-PST actually reflects the activity of two sulphotransferases, namely ST1A2 and ST1A3 (Parkinson 2001). Genetic polymorphisms have been reported on human sulphotransferases ST1A2, ST1A3 and ST2A3 (Nagata and Yamazoe 2000). The same kind of polymorphism could possibly exist in the porcine species and might explain the large interindividual variation in *p*-nitrophenol and 5-OH-3MI sulphation activities observed in the present study.

In summary, the results of the present study show that the porcine liver glucuronidates and sulphates 5-OH-3MI and is also capable of forming an electrophile that reacts with GSH. Glucuronidation and GST activities are not correlated with 3MI levels in fat but pigs with high 3MI levels in fat consistently show low 5-OH-3MI sulphation activity. The results of the sulphation studies suggest that the sulphotransferase activity TS-PST is important in the Phase II metabolism of 3MI in the pig and that a low catalytic activity of TS-PST is

associated with a less than adequate 3MI clearance. The enzymatic activity of TS-PST might be used as a potential marker to identify pigs containing low levels of 3MI in fat, which might help in the genetic selection of pigs that are not prone to suffer from 'boar taint'. In humans, TS-PST activity in platelets is highly correlated with TS-PST activity in the liver. It is important to determine if these two activities are also correlated in the porcine species because it would allow for the selection of pigs with high sulphation activity by measuring TS-PST activity in platelets.

Acknowledgements

Thanks are due to Kerstin Lundström and Leif Andersson of the Swedish University of Agricultural Sciences for access to pig liver samples. Financial support for G. J. D. was provided by the University of Guelph, the Colombian Institute for the Development of Science and Technology (Colciencias) and the National University of Colombia. This work was supported by NSERC research grants and grants from Ontario Pork and the Ontario Ministry of Agriculture, Food and Rural Affairs. In addition, this project was supported by the Canada-Ontario Research and Development Program, administered by the Agricultural Adaptation Council. Funding was provided by Agriculture and Agri-Food Canada under the Canada-Ontario Agricultural Safety Net Management Agreement.

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Identification of novel metabolites of pioglitazone in rat and dog

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Received 31 October 2002

1. Four new metabolites of pioglitazone were identified by liquid chromatography-mass spectrometry (LC-MS/MS) as being formed by hydroxylation (M-VII and M-VIII), opening of the thiazolidinedione ring (M-X) and by desaturation of the terminal ethyl side chain or tether ethoxy moiety (M-IX), respectively. The structure of one of the hydroxylated metabolites (M-VII) was confirmed by chemical modification using the Jones reaction.

2. Oxidative cleavage of the thiazolidinedione ring is a novel pathway not previously reported for pioglitazone.

3. The hydroxylated M-VII was detected in incubations with rat, dog and human liver and kidney microsomes, and in plasma from rats and dogs dosed orally with [³H]pioglitazone.

4. The carboxylic acid derivative of M-VII (M-V) and its taurine conjugate were the major radioactive components in dog bile.

Introduction

Type 2 diabetes mellitus is a growing problem not only in the USA, but also across the world. Pioglitazone is a member of a recently developed class of glucose-lowering agents, the glitazones or thiazolidinediones, that are used in the treatment of type 2 diabetes mellitus (Chilcott *et al.* 2001, Mudaliar and Henry 2001). These compounds increase insulin sensitivity in target tissues by way of their interaction with the peroxisome proliferator-activated receptor gamma (PPAR γ) (Sood *et al.* 2000, Hanefeld 2001). Pioglitazone is metabolized in the human liver to several products, designated M-I to M-VI (figure 1) (Sohda *et al.* 1995, Tanis *et al.* 1996, Kiyota *et al.* 1997, Eckland and Danhof 2000). Three of the metabolites, M-III, M-IV and, to a lesser extent, M-II, have pharmacological activity and exert an anti-hyperglycemic potency about 40–60% of that of pioglitazone (Eckland and Danhof 2000). A number of reports describe the disposition of pioglitazone in species used in preclinical studies, such as rats, dogs and monkeys (Zhong and Lakings 1989, Krieter *et al.* 1994, Kiyota *et al.* 1997). All metabolites (M-I to M-VI inclusive) were found circulating in the plasma of rats, dogs and monkeys. Interestingly, the total plasma clearance for pioglitazone was fivefold higher in dogs than in rats and monkeys (Maeshiba *et al.* 1997). Furthermore, it was reported that pioglitazone was subject to renal metabolism (the Shinseishiryo Gaiyo 2000). In an effort to explain the wide range of clearances reported for the

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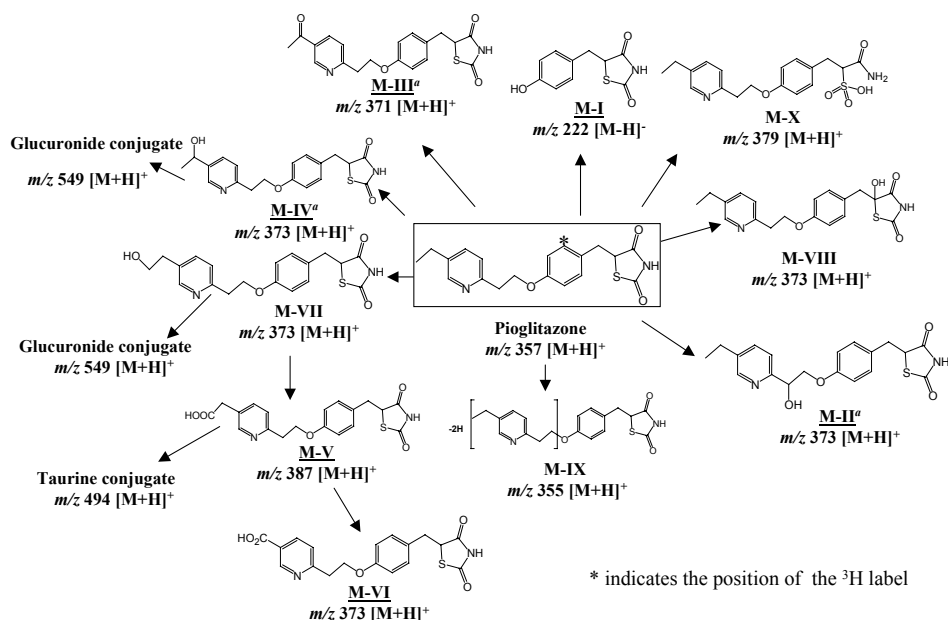


Figure 1. Proposed structures of pioglitazone metabolites in dog kidney and liver microsomes. Underlined M's indicate the literature reported metabolites; ^astandards were available.

various preclinical species, the *in vitro* metabolism of pioglitazone by liver and kidney microsomes from rats, dogs and humans was compared, and this resulted in the discovery of four novel metabolites, as described herein. In addition, in order to determine the importance of the newly identified metabolites in the overall elimination of this compound, metabolite profiles were obtained for rat plasma and for dog plasma, bile and urine following oral administration of [^3H]pioglitazone. Metabolite profiles in rat bile and urine have already been reported (Krieter *et al.* 1994).

Materials and methods

Chemicals

[Benzyl-3- ^3H]pioglitazone (figure 1) was synthesized by the Labeled Compound Synthesis Group, Department of Drug Metabolism (Merck Research Laboratories). The radiochemical purity was 98% by HPLC. Pioglitazone metabolites M-II to M-IV (figure 1) were synthesized by the Department of Medicinal Chemistry (Merck Research Laboratories). Their chemical purity was >90%, as determined by HPLC. Chromium oxide and concentrated sulfuric acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade water and all analytical organic solvents used were purchased from Sigma or Fisher Scientific (Fair Lawn, NJ, USA).

In vitro formation of metabolites

Rat, dog and human liver microsomes as well as rat and dog kidney microsomes were prepared according to standard literature procedures. Protein concentrations were determined using the bicinchoninic acid procedure (Smith *et al.* 1985). Human kidney microsomes were purchased from Tissue Transformation Technologies (Edison, NJ, USA). The *in vitro* metabolism of pioglitazone was determined in 0.2-ml incubations containing 50 μM [^3H]pioglitazone (7 $\mu\text{Ci mg}^{-1}$, acetonitrile solution), 1.5 mg ml^{-1} microsomal protein, 3.3 mM MgCl_2 , 1 mM ethylenediaminetetraacetic acid (EDTA), 1.3 mM nicotinamide adenine dinucleotide phosphate (NADP^+), 3.3 mM glucose 6-phosphate (G6P) and 0.4 units G6P dehydrogenase in Tris buffer (0.1 M, pH 7.4) at 37°C for 90 min. The final

concentration of acetonitrile was 1%. Incubations were terminated by the addition of 0.1 ml acetonitrile to precipitate microsomal proteins.

Animal studies and sample collection

All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Two bile duct-cannulated male Beagle dogs (about 10–15 kg) were fasted overnight and then given an oral dose of 6.25 mg kg⁻¹ [³H]pioglitazone (about 400 µCi per dog) by gastric intubation. The dogs were allowed free access to water and were fed following collection of the 4-h blood samples. The dosing solution was prepared by mixing [³H]pioglitazone in ethanol: 0.5% methylcellulose (20:80, v/v) and the dosing volume was 2.5 ml kg⁻¹. Blood was collected by jugular venipuncture at various times up to 24 h into heparin-coated tubes. The blood was centrifuged at 1850g for 15 min at 4°C and plasma was stored in a freezer at -80°C. Bile was collected at 0–2, 2–4, 4–8, 8–24 and 24–48 h, and urine at 0–24 and 24–48 h. Concentrations of radioactivity in plasma, urine and bile were determined by liquid scintillation counting.

Eight male Sprague-Dawley rats (400–500 g; Charles River, Worcester, MA, USA) acclimatized to the Merck Animal Facility and maintained on a 12 light/dark cycle were fasted overnight and dosed orally with 5 mg kg⁻¹ [³H]pioglitazone. The dosing solution was prepared by mixing [³H]pioglitazone in ethanol:0.5% methylcellulose (20:80, v/v), and the dosing volume was 2.0 ml kg⁻¹. The rats were allowed free access to water and were fed after 4 h post-dose. Blood samples were collected into heparinized tubes by cardiac puncture from two rats at 2, 4, 8 and 24 h post-dose, and pooled according to each time point. The pooled blood was centrifuged and the plasma was stored at -80°C before analysis.

HPLC-MS/MS analysis

HPLC analysis of samples from the *in vitro* incubations and rat and dog plasma was carried out on a Zorbax XD-C18 column (4.6 × 250 mm, 5 µm, MAC-MOD Analytical, Chadds Ford, PA, USA) using a Shimadzu LC-10ADvp binary pump system (Columbia, MD, USA). The pumps were controlled by a Shimadzu SCL-10Avp system controller. Samples were injected onto the column via a Shimadzu SIL-10ADvp autoinjector, maintained at 4°C. The mobile phase consisted of H₂O with 5 mM ammonium acetate (pH 4.7 adjusted with formic acid, mobile phase A) and acetonitrile with 5 mM ammonium acetate (mobile phase B). The flow rate was 1 ml min⁻¹. The initial mobile phase consisted of 20% B for 30 min followed by a linear gradient to 80% B over 40 min that was held at 80% B for 5 min. The column was equilibrated with initial mobile phase at 1 ml min⁻¹ for 15 min before sample injection. The LC system was connected to a β-Ram radioactivity monitor (IN/US system, Tampa, FL, USA) and a Finnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The analogue output from the ³H channel of β-Ram radiometric detector was coupled to the LCQ analogue input in order for radiochromatograms to be simultaneously recorded on both the LCQ and radiometric detector. For the LC-MS/MS analyses, a portion of the HPLC effluent was introduced into the LCQ ionization source at a rate of 200 µl min⁻¹. Mass spectral analysis was conducted using electrospray ionization (ESI) in both positive- and negative-ion modes. The electrospray ionizing voltage was 4 kV and the heated capillary temperature 200°C. The isolation width and collision energy were set at 3 and 35%, respectively, for MS² and MS³ experiments.

Dog bile and urine samples were analysed on a Zorbax SB-C8 column (4.6 × 250 mm, 5 µm) eluted at 1 ml min⁻¹ with a linear gradient from 80% A (5 mM ammonium acetate in water plus 0.05% acetic acid) to 45% B (5 mM ammonium acetate in methanol plus 0.05% acetic acid) in 20 min, and to 95% B in 17 min, followed by an isocratic elution at 95% B for 7 min. The LC-MS/MS system consisted of a Shimadzu SCL-10Avp system controller, two Shimadzu LC-10ADvp pumps, a Perkin-Elmer series 200 autosampler (Norwalk, CT, USA), a Packard flow scintillation analyser (Downers Grove, IL, USA) and a ThermoFinnigan Deca XP ion-trap mass spectrometer. The column eluent was split between the mass spectrometer (one-fifth) and the flow scintillation analyser, where it was mixed with 3 vols Packard Ultima Flo-M scintillation fluid. The LCQ was operated in the electrospray positive-ion mode. The source voltage was maintained at 5 kV. The capillary temperature was set to 350°C and the sheath gas to 40. The MS² and MS³ data were obtained using a relative collision energy of 35%. Data acquisition and reduction were carried out using Xcalibur software (v.1.2). Detection of the metabolites was achieved based on radioactivity monitoring as well as MS² and MS³ spectral data.

Oxidation of M-IV and M-VII by the Jones reagent

The Jones reagent was used for the oxidation of primary and secondary alcohols (Fieser *et al.* 1967, Epifanio *et al.* 1988, Evans *et al.* 1996). It was prepared as follows: 6.67 g chromium oxide (Sigma) were dissolved in water, followed by the addition of 5.3 ml concentrated sulfuric acid and water to a volume of 25 ml. Synthetic M-IV (hydroxylated metabolite; 100 µl 1 mg ml⁻¹ in ethanol) and an extract of a kidney microsome incubation containing M-IV and M-VII were dried under a stream of nitrogen and reconstituted in 100 µl acetone. A total of 2 µl Jones reagent were added into the acetone solution, which was mixed well for about 1 h at room temperature. An aliquot of isopropanol was added to react with the

excess Jones reagent before drying under nitrogen and reconstituting with 200 µl starting mobile phase for LC-MS/MS analysis.

Results and discussion

Identification of new metabolites of pioglitazone in incubations with rat, dog and human liver and kidney microsomes

A summary of the metabolites detected in incubations with rat, dog and human liver and kidney microsomes is shown in table 1. Figure 2 shows representative radioactivity chromatograms of [^3H]pioglitazone incubations with dog kidney and liver microsomes for 90 min. Six metabolites (M-I to M-VI; figure 1) have been reported in literature (Sohda *et al.* 1995, Tanis *et al.* 1996, Kiyota *et al.* 1997, Eckland and Danhof 2000). In the present study, authentic standards of M-II to M-IV were used to search for metabolites or for structure confirmation. In addition to M-II and M-IV, four new metabolites (M-VII to M-X) were identified. However, M-I, M-III, M-V and M-VI were not detected in the incubation samples in the present experiment. Low amounts of M-IV, M-VII, M-IX were found in [^3H]pioglitazone incubations with dog kidney microsomes, whereas M-II, M-IV and M-VII to M-X were found in incubations with dog liver microsomes. In the dog liver microsomal incubations, M-IV, M-VII, M-VIII, and M-X were associated with detectable radioactivity, whereas M-II and M-IX could only be detected by mass spectrometry (figure 2). Similar metabolites were also found in incubations of [^3H]pioglitazone with rat and human liver and kidney microsomes (table 1), although the turnover in these species was lower than in dog. In human liver microsomes, M-IV was the only major metabolite that could be detected by radioactivity, whereas the amount of M-VII was at the limit of detection of radioactivity. Trace amounts of M-II, M-III, M-VIII and M-IX were detected by mass spectrometry. In rat liver microsomes, there were trace levels of M-II, M-IV and M-VII to M-IX, whereas in rat and human kidney microsomes, only M-IV and M-VII could be detected. The lack of detection of previously reported metabolites M-I, M-V and M-VI maybe due to the sensitivity of the instrument or they might not be generated in the systems described here.

Table 1. Occurrence of pioglitazone metabolites in incubations with liver and kidney microsomes and in plasma following oral administration of [^3H]pioglitazone.

Compound	Retention time (min)	Liver microsomes			Kidney microsomes			Plasma	
		Rat	Dog	Human	Rat	Dog	Human	Rat	Dog
M-II	46.3	trace	trace	trace	ND	ND	ND	trace	trace
M-III	45.6	ND	ND	trace	ND	ND	ND	✓	✓
M-IV	40.2	trace	✓	✓	trace	✓	trace	✓	✓
M-VII	38.3	trace	✓	✓	trace	✓	trace	trace	✓
M-VIII	48.3	trace	✓	trace	ND	ND	ND	trace	trace
M-IX	51.5	trace	trace	trace	ND	trace	ND	trace	trace
M-X	10.0	ND	✓	ND	ND	ND	ND	ND	ND

ND, not detected.

The retention time of pioglitazone under these HPLC conditions, as described in the Materials and methods, was 51.9 min.

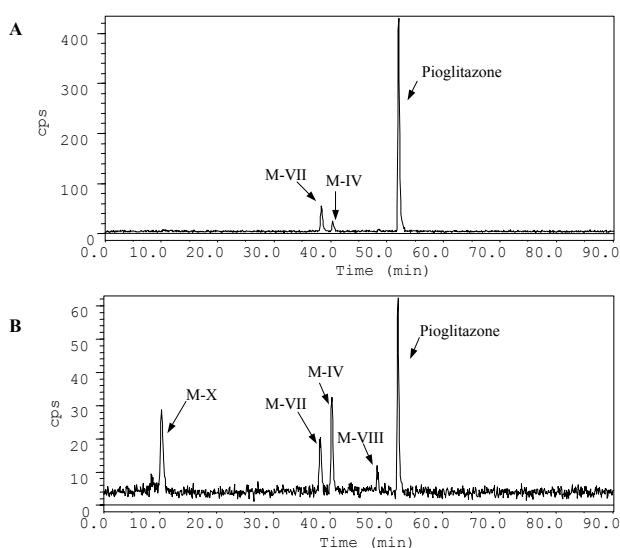
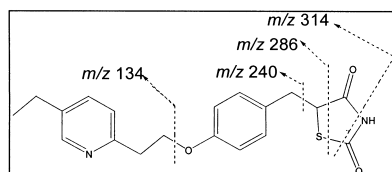


Figure 2. Radioactivity chromatograms of samples obtained following incubation of [^3H]pioglitazone with dog kidney (A) and liver (B) microsomes.

Identification of M-VII

The proposed fragmentation patterns of pioglitazone and its newly identified metabolites are shown in table 2. M-VII gave a protonated molecule $[\text{M} + \text{H}]^+$ at m/z 373, which was 16 amu higher than that of pioglitazone (m/z 357). The major product ions had the following m/z values (relative abundance): 355 (8%), 150 (100%) and 132 (18%) (table 2). Further fragmentation of the most intense precursor ion at m/z 150 resulted in one major fragment ion at m/z 132. The fragment ion at m/z 150 indicated the introduction of a hydroxyl group on the 2-(5-ethyl-2-pyridyl)ethyl moiety. The major product ions of the authentic standard M-IV, another hydroxylated pioglitazone, were also at m/z 355 (100%),

Table 2. Product ion mass spectral data of pioglitazone and its novel metabolites.



Pioglitazone, m/z 357

Metabolites	$[\text{M} + \text{H}]^+$	MS^2 (m/z)	MS^3 (m/z)
M-VII	373	355, 150 ^a , 132	132
M-VIII	373	313 ^a	268, 134
M-IX	355	238, 132	not available
M-X	379	362, 299, 282 ^a	264, 240, 134
M-IV glucuronide conjugate	549	373, 355 ^a	132
M-VII glucuronide conjugate	549	373 ^a	150

^aPrecursor ions for MS^3 .

150 (3%) and 132 (4%); however, their relative abundance was different from those of M-VII. Further fragmentation of the most intense ion at m/z 355 gave the fragment ion at m/z 132. Clearly, M-VII can be distinguished from M-IV based on their characteristic fragmentation patterns. Furthermore, the HPLC retention time of M-VII was different from that of authentic standards of the other hydroxylated metabolites, M-IV and M-II. Hence, M-VII represented a new hydroxylated metabolite. The loss of the element of water from m/z 373 to 355 and the fragmentation patterns led us to speculate that the hydroxylation had occurred on the terminal carbon of the alkyl side chain. This was confirmed by chemical modification, namely oxidation by the Jones reagent, as described below.

As a control experiment, the authentic standard of M-IV was allowed to react with the Jones reagent to form, as expected, M-III the keto form of M-IV (data not shown). The retention time and MS² spectrum of the keto-product were identical with those of the authentic standard of M-III. The dog kidney microsome incubation sample, which contained mainly M-IV and M-VII, was allowed to react with the Jones reagent. As expected, M-III was detected due to the oxidation of the M-IV (data not shown). In addition, a newly generated peak gave a protonated molecule $[M + H]^+$ at m/z 387 that was 14 amu higher than that of M-VII (figure 3). The MS² spectrum of m/z 387 gave the characteristic fragment ion at m/z 164 that was 14 amu higher than the corresponding fragment of M-VII (figure 3). These results suggested that a carboxylic acid group was formed on the 2-(5-ethyl-2-pyridyl)ethyl moiety. These data unequivocally indicated the oxidation of a primary alcohol, confirming the structure of M-VII (figure 1). M-VII is most probably the precursor of M-V, a previously reported carboxylic acid metabolite (Krieter *et al.* 1994, Sohda *et al.* 1995, Tanis *et al.* 1996, Kiyota *et al.* 1997, Eckland and Danhof 2000).

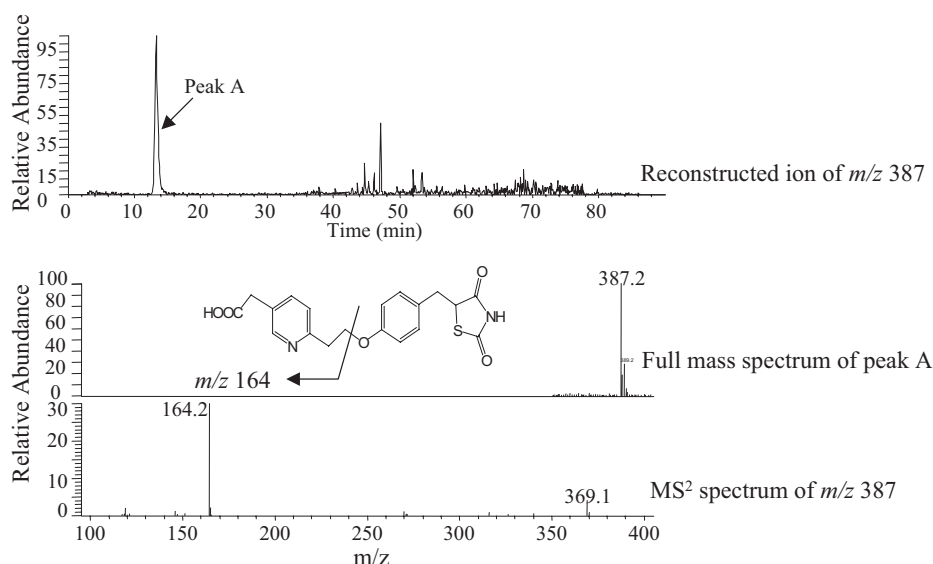


Figure 3. Reconstructed ion chromatogram of m/z 387 illustrating peak A, which was formed on the treatment of M-VII with the Jones reagent (upper); MS spectrum of peak A and MS/MS spectrum of m/z 387 (lower).

Identification of M-VIII

The full-scan mass spectrum of M-VIII revealed a protonated molecule $[M+H]^+$ at m/z 373 that was 16 amu greater than that of pioglitazone. However, its fragmentation pattern was quite different from that of pioglitazone and from the other hydroxylated metabolites M-II, M-IV and M-VII. A unique product ion was observed at m/z 313 that resulted from loss of 60 amu from the precursor ion of m/z 373, which was formed, presumably, by the elimination of -SCO (table 2). In addition, further fragmentation of m/z 313 produced an ion at m/z 134, indicating that the 2-(5-ethyl-2-pyridyl)ethyl moiety was intact. This suggested the introduction of an -OH group on the tertiary carbon of the 2,4-thiazolidinedione (TZD) ring, which made the TZD ring more susceptible to the elimination of -SCO. The proposed structure of M-VIII is shown in figure 1.

Partial characterization of M-IX

A minor metabolite M-IX, eluting closely to pioglitazone, had a molecular ion $[M+H]^+$ at m/z 355, 2 amu less than that of pioglitazone (m/z 357). The MS² spectrum of the precursor ion at m/z 355 showed fragment ions at m/z 238 and 132 (table 2). This suggested that the 5-(4-hydroxybenzyl)-2,4-thiazolidinedione moiety had not been modified. Thus, formation of M-IX may have involved the desaturation of the terminal ethyl or the tether ethoxy (figure 1). The structure of M-IX was not studied further since it was present only at trace levels. Other researchers (Baillie 1988, Baillie and Sheffels 1995) have reported the bioactivation reactions as well as the nature of the enzymes involved in side-chain terminal desaturation.

Identification of M-X

The early eluting metabolite peak M-X exhibited a protonated molecule $[M+H]^+$ at m/z 379 (figure 1). MS² spectrum of the peak showed fragment ions at m/z 362 $[M+H-NH_3]^+$, 299 $[M+H-SO_3]^+$ and 282 $[M+H-SO_3-NH_3]^+$, which indicated opening of the TZD ring (table 2). Further fragmentation of precursor ion m/z 282 resulted in two major fragment ions at m/z 240 and 134, indicating that 2-(5-ethyl-2-pyridyl)ethyl moiety and 4-hydroxybenzyl moiety were intact. Thus, a sulfonic acid amide structure was proposed for M-X (figure 1). The mechanism of the thiazolidinedione ring scission may involve a novel biotransformation pathway (Kassahun *et al.* 2001), although the precise details of the underlying mechanism remain to be established. Metabolic cleavage of the TZD ring of pioglitazone has not been reported previously.

Pioglitazone metabolites in dog and rat plasma

It was of interest to determine whether these newly found metabolites were also formed *in vivo* and if they were species specific. Figures 4 and 5 show representative radioactivity chromatograms of dog and rat plasma, respectively, following oral administration of [³H]pioglitazone. The major metabolites circulating in dog plasma were M-IV and M-VII. Other metabolites, including M-II, M-III, M-VIII and M-IX, were present only at trace levels and could only be detected by mass spectrometry. The parent compound was the major radioactive

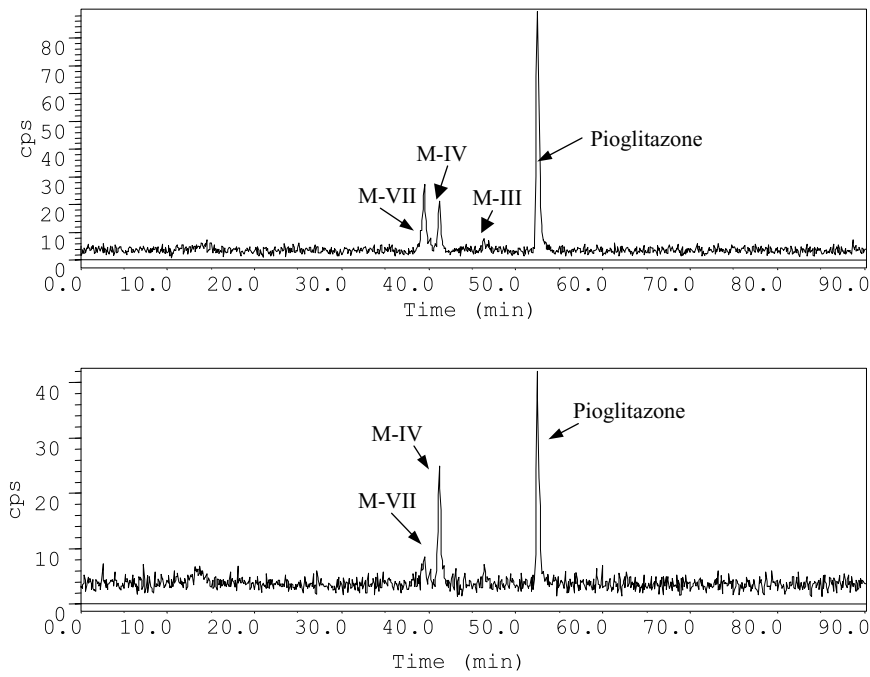


Figure 4. Representative radioactivity chromatograms of dog plasma at 2 (upper) and 4 h (lower) following oral administration of [^3H]pioglitazone at 6.25 mg kg^{-1} .

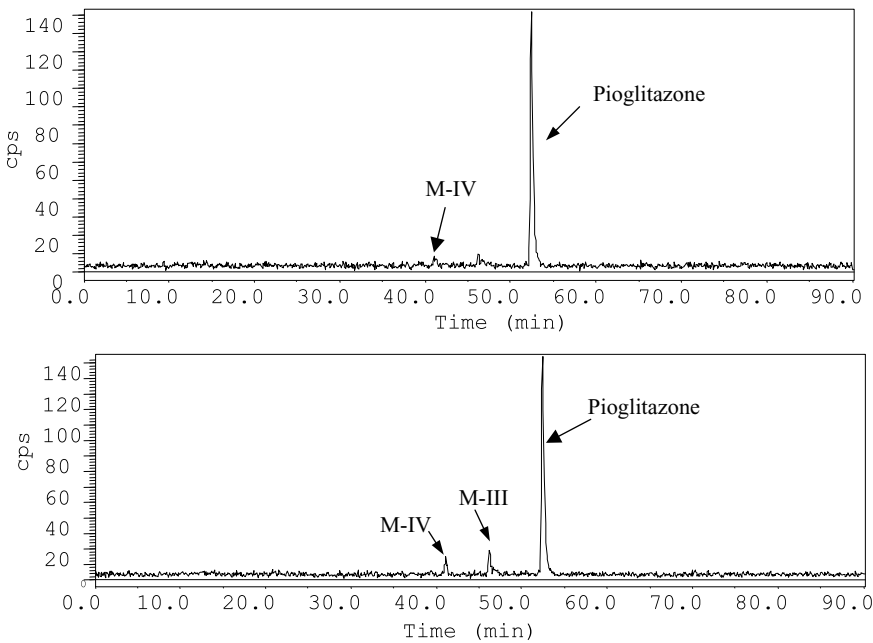


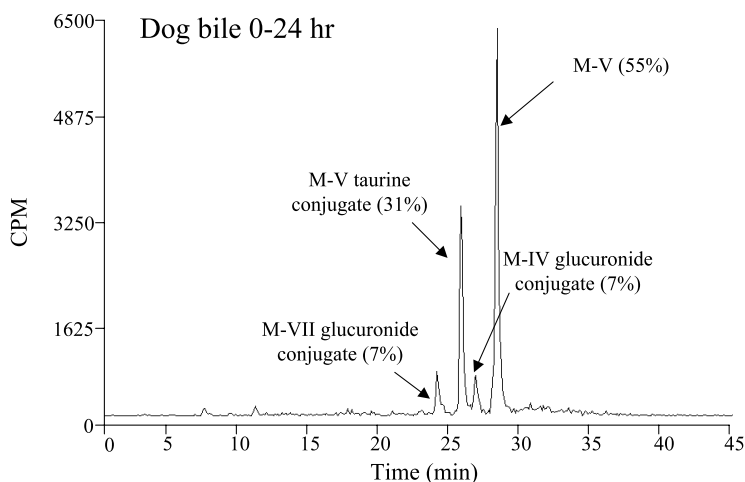
Figure 5. Representative radioactivity chromatograms of rat plasma at 2 (upper) and 4 h (lower) following oral administration of [^3H]pioglitazone at 5 mg kg^{-1} .

component in dog plasma at 30 min to 4 h. The level of M-VII was higher than that of M-IV when samples were collected at 30 min and 2 h after dosing. However, M-IV became the predominant metabolite at 4 h. Eight hours after dosing, pioglitazone levels started to diminish, and M-IV became the major drug-related component in the plasma. After 24 h, all the metabolites and parent compound were eliminated and only trace amount of M-IV and M-VII could be detected by mass spectrometry.

Rat plasma contained lower levels of metabolites than dog plasma. M-III and M-IV were the only major metabolites, which could be detected by radiometric detection (figure 5). Trace amounts of M-II and M-VII to M-IX were detected only by mass spectrometry (table 1). Pioglitazone was circulating in the plasma for up to 8 h after dosing, but levels diminished, along with those metabolites, at 24 h after dosing.

Pioglitazone metabolites in dog bile and urine

The excretion of radioactivity into bile and urine was determined following oral dosing of dogs with [^3H]pioglitazone. Approximately 34 and 12% of the radioactive dose were recovered in bile and urine, respectively, within 48 h. LC-MS/MS analysis of pooled bile revealed the presence of M-V, which is the carboxylic acid product of M-VII, and its taurine conjugate. These metabolites, which were identified previously by others (Krieter *et al.* 1995, Maeshiba *et al.* 1997), represented about 55 and 30% of the radioactivity in dog bile (corresponding to about 40 and 22% of absorbed dose), respectively (figure 6). M-IV was also detected, but at trace levels, and it was not resolved from M-V under the HPLC conditions used to analyse the bile. Additionally, the glucuronide conjugates of M-IV and M-VII were detected, each contributing about 7% of the radioactivity excreted into bile, which have not been previously reported. The



A trace amount of M-IV was detected under the M-V peak

Figure 6. Representative radioactivity chromatogram of pooled dog bile collected at 0–24 h following oral administration of [^3H]pioglitazone to dogs at 6.25 mg kg^{-1} .

identification of these glucuronides was based on mass spectral data. The full-scan mass spectral data revealed protonated molecules $[M + H]^+$ at m/z 549 for both compounds, which were 176 amu greater than that of hydroxylated-pioglitazone at m/z 373. MS² spectrum of parent ion at m/z 549 gave fragment ions at m/z 373, which further fragmented into m/z 150 for M-VII glucuronide conjugate. However, for the M-IV glucuronide conjugate, the MS² spectrum of parent ion at m/z 549 gave fragment ions at m/z 373 and 355; the latter was the most intense ion and could be further fragmented into m/z 132 (table 2). Thus, the spectra of these glucuronides were consistent with the fragmentation patterns of their corresponding aglycones, M-IV and M-VII. This allowed us to assign the structures for M-VII glucuronide and M-IV glucuronide accordingly. The amount of radioactivity recovered in urine was not enough to allow the construction of a radiochromatogram. However, M-V and its taurine conjugate were also detected by LC-MS/MS in dog urine, along with low amounts of pioglitazone and several other unidentified metabolites (data not shown).

In conclusion, we confirmed that pioglitazone is subject to oxidative metabolism in dog kidney microsomes. Additionally, four new metabolites (M-VII to M-X) were identified by mass spectrometry, and the structure of M-VII was confirmed using the Jones reaction, showing that it could be converted to the carboxylic acid, M-V. The newly identified hydroxylated M-VII was one of the metabolites circulating in dog plasma, in addition to M-IV, especially at the early time points. Although the pharmacological activity of M-VII is unknown, it is not expected to contribute significantly to the pharmacological activity of pioglitazone since it is only a minor component both in dog and rat plasma at most time points and in *in vitro* incubations with human liver microsomes. Nevertheless, formation of M-VII appears to be a major elimination pathway for pioglitazone in dogs, since the majority of the absorbed dose was excreted as M-V, the carboxylic acid product of M-VII, and its taurine conjugate.

Acknowledgements

The authors thank Mr Chris Kochansky for preparation of the rat and dog kidney microsomes, Dr Peter Meinke for guidance in the synthesis of metabolites M-II to M-IV, and Mr Paul Cunningham, Ms Allison Parlapiano and Ms Gina Seeburger for assistance in the dog study. The authors also thank Drs Tom Baillie and Reza Anari for helpful discussions.

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Studies on the induction of rat hepatic CYP1A, CYP2B, CYP3A and CYP4A subfamily form mRNAs *in vivo* and *in vitro* using precision-cut rat liver slices

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Received 14 October 2002

1. Real-time quantitative reverse transcription-polymerase chain reaction methodology (TaqMan[®]) was used to examine the induction of some selected rat hepatic cytochrome P450 (CYP) forms *in vivo* and *in vitro* using cultured precision-cut liver slices.

2. TaqMan primers and probe sets were developed for rat CYP1A1, CYP1A2, CYP2B1, CYP2B1/2, CYP3A1, CYP3A2 and CYP4A1 mRNAs.

3. To characterize the responsiveness of the rat CYP mRNA TaqMan primers and probe sets, rats were treated *in vivo* with a single intraperitoneal dose of 500 mg kg⁻¹ Aroclor 1254 (ARO) and with four daily oral doses of either 50 mg kg⁻¹ day⁻¹ dexamethasone (DEX) or 75 mg kg⁻¹ day⁻¹ methylclofenapate (MCP). Treatment with ARO produced 22 600-, 5480-, 648-, 52-, 47- and 9-fold increases in levels of CYP1A1, CYP2B1, CYP2B1/2, CYP1A2, CYP3A1 and CYP3A2 mRNA, respectively. DEX treatment produced 97-, 24-, 8- and 4-fold increases, respectively, in CYP3A1, CYP2B1, CYP2B1/2 and CYP3A2 mRNA levels, and MCP produced 339-, 126- and 25-fold increases, respectively, in CYP4A1, CYP2B1 and CYP2B1/2 mRNA levels. All three CYP inducers also increased microsomal CYP content and produced corresponding increases in CYP1A, CYP2B, CYP3A and CYP4A form marker enzyme activities.

4. Rat liver slices were cultured for 6 and 24 h in medium containing 0.1 µM insulin and 0.1 µM DEX, and also for 24 h in medium containing only 0.1 µM insulin (DEX-free medium). Liver slices were cultured in control medium or in medium containing either 10 µM β-naphthoflavone (BNF), 10 µg ml⁻¹ ARO, 500 µM sodium phenobarbitone (NaPB), 20 µM pregnenolone-16α-carbonitrile (PCN), 50 µM Wy-14,643 (WY) or 50 µM MCP.

5. With the exception of the effect of BNF on CYP1A1 mRNA levels, the induction of all the CYP mRNAs studied was greater after 24- than after 6-h treatment. Generally, the magnitude of induction of CYP mRNA levels was greater after 24 h in liver slices cultured in DEX-free than in DEX-supplemented medium.

6. Treatment of liver slices with BNF and ARO for 24 h in DEX-free medium produced 21- and 35-fold increases, respectively, and 38- and 37-fold increases, respectively, in CYP1A1 and CYP1A2 mRNA levels. NaPB, PCN, WY and MCP did not increase either CYP1A1 or CYP1A2 mRNA levels.

7. After 24 h, levels of CYP2B1/2 mRNA were increased 18-, 20-, 9-, 16- and 13-fold by treatment with ARO, NaPB, PCN, WY and MCP, respectively. PCN also produced 56- and 4-fold increases, respectively, in CYP3A1 and CYP3A2 mRNA levels.

8. Treatment with WY and MCP for 24 h produced 437- and 186-fold increases, respectively, in levels of CYP4A1 mRNA. None of the other CYP inducers studied had any effect on CYP4A1 mRNA levels.

9. The results demonstrate the utility of cultured precision-cut liver slices as an *in vitro* model system to evaluate the effects of xenobiotics on rat CYP1A, CYP2B, CYP3A and CYP4A form mRNA levels.

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Introduction

Mammalian hepatic cytochrome P450 (CYP) forms have a major role in the oxidative metabolism of drugs and other xenobiotics (Conney 1986, Nelson *et al.* 1996, Parkinson 1996). CYP form-dependent enzyme activities can be either induced or inhibited by a wide range of chemicals including therapeutic agents, pesticides, industrial chemicals, food chemicals and natural products (Conney 1986, Okey 1990, Parkinson 1996, Pelkonen *et al.* 1998). In the development of new therapeutic agents, it is important to ascertain whether the compound will be either an inducer or an inhibitor of CYP forms in order to minimize potential drug interactions (Lin and Lu 1998). Moreover, CYP induction may also be important in preclinical studies as several non-genotoxic CYP inducers have been shown to produce liver tumours in rodents (Grasso and Hinton 1991, Lake and Lewis 1996, Nims and Lubet 1996).

The effects of xenobiotic on hepatic CYP forms may be evaluated by both *in vivo* and *in vitro* techniques. CYP induction can be determined by measurement of enzyme activities, Western immunoblotting for CYP apoproteins and by measurement of CYP mRNA levels. Many studies have employed cultured rat hepatocytes as a model system to evaluate the induction of CYP forms (LeCluyse *et al.* 1996, Coecke *et al.* 1999, Davila and Morris 1999, Burczynski *et al.* 2001). The *in vitro* induction of CYP forms has also been evaluated in more limited studies performed in cultured precision-cut liver slices (reviewed in Lake *et al.* 1998a, Lerche-Langrand and Toutain 2000). In a recent study (Pan *et al.* 2002), we reported that the induction of some rat CYP form mRNAs could be demonstrated in cultured precision-cut rat liver slices employing real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) methodology (TaqMan[®]).

The objective of the present study was to investigate the induction of a wider range of CYP form mRNAs in liver slices treated with known CYP inducers. In order to undertake these studies, TaqMan primers and probe sets were developed for rat hepatic CYP1A1, CYP1A2, CYP2B1, CYP2B1/2 (i.e. to CYP2B1 and CYP2B2), CYP3A1, CYP3A2 and CYP4A1 forms. To characterize the responsiveness of the rat CYP mRNA TaqMan primers and probe sets, rats were treated *in vivo* with Aroclor 1254 (ARO), dexamethasone (DEX) and methylclofenapate (MCP). Previous studies have shown that the polychlorinated biphenyl mixture ARO induces several CYP subfamilies including CYP1A, CYP2B and CYP3A forms; DEX induces CYP3A and to a lesser extent CYP2B forms; whereas peroxisome proliferators including MCP induce CYP4A and to a lesser extent other CYP forms (Okey 1990, Lake and Lewis 1996, Maurel 1996, Lake *et al.* 1998b).

Studies were then performed in cultured rat liver slices treated with known CYP inducers for either 6 or 24 h. These time points were selected on the basis of previous studies on the induction of CYP mRNA levels in cultured rat liver slices (Drahushuk *et al.* 1996, Müller *et al.* 1998, 2000, Pan *et al.* 2002). The CYP inducers studied comprised β -naphthoflavone (BNF), ARO, sodium phenobarbital (NaPB), pregnenolone-16 α -carbonitrile (PCN), Wy-14,643 (WY) and MCP. Previous studies have demonstrated that BNF induces CYP1A forms; NaPB induces CYP2B and other CYP forms; PCN induces CYP3A and to a lesser extent CYP2B forms; and the peroxisome proliferator WY induces CYP4A and to a lesser extent other CYP forms (Conney 1986, Okey 1990, Debri *et al.* 1995, Lake

and Lewis 1996, Maurel 1996, Nims and Lubet 1996, Parkinson 1996, Pelkonen *et al.* 1998). Finally, the effect of DEX supplementation of the tissue culture medium was also investigated as previous studies have demonstrated that CYP induction in cultured rat hepatocytes can be modulated by the presence of glucocorticoids in the medium (Sinclair *et al.* 1990, Waxman *et al.* 1990, Sidhu *et al.* 1993, Burczynski *et al.* 2001).

Materials and methods

Materials

Tissue culture materials were obtained from Life Technologies Ltd (Paisley, UK) and β -naphthoflavone (BNF), pregnenolone-16 α -carbonitrile (PCN), dexamethasone (DEX), palmitoyl-CoA, 7-ethoxyresorufin, 7-pentoxoresorufin, testosterone, resorufin, 6 β -hydroxytestosterone, Tris and enzyme cofactors were from Sigma-Aldrich Co. Ltd (Poole, UK). Sodium phenobarbitone (NaPB) was purchased from BDH Chemicals (Poole, UK), methylofenapate (MCP) from Lancaster Synthesis (Morecambe, UK), Wy-14,643 (WY) from Chemsyn Science Laboratories (Lenexa, KS, USA) and [4-¹⁴C]testosterone (specific activity 56 mCi mmol⁻¹) from Amersham Pharmacia Biotech UK Ltd (Little Chalfont, UK). Aroclor 1254 (ARO) was the generous gift of the Monsanto Chemical Co. (St Louis, MO, USA). QIAGEN RNeasy[®], QIAGEN RNeasy[®] mini kits and QIAGEN Omniscript[®] Reverse transcriptase kits were obtained from QIAGEN Ltd (Crawley, UK) and TaqMan Universal Master Mix Reagents, RNase inhibitor and random hexamer primers from Applied Biosystems (Warrington, UK). The oligonucleotide primers and probes for rat CYP1A1, CYP1A2, CYP2B1, CYP2B1/2, CYP3A1, CYP3A2, CYP4A1 and albumin were synthesized by Applied Biosystems.

Animals

Male Sprague-Dawley rats were obtained from Harlan Olac (Bicester, UK) and were allowed free access to R&M No. 1 laboratory animal diet (Special Diets Services, Witham, UK) and water. The animals were housed in polypropylene cages with stainless-steel grid tops and floors in rooms maintained at 22 $\pm$ 3°C with a relative humidity of 40–70% and were allowed to acclimatize to these conditions for at least 14 days before use. Rats (200–220 g) were given a single i.p. dose of 500 mg kg⁻¹ ARO or four daily oral doses of either 50 mg kg⁻¹ day⁻¹ DEX or 75 mg kg⁻¹ day⁻¹ MCP. The vehicle (5 ml kg⁻¹ body weight) was corn oil for ARO and MCP and 0.56% (w/v) gum tragacanth for DEX. Control animals received no treatment.

Biochemical investigations

Rats were killed (24 h after the last doses of DEX and MCP and 96 h after the single dose of ARO) by exposure to carbon dioxide gas followed by cervical dislocation and the livers immediately excised. Small samples of liver from the left lobe were snap-frozen in liquid nitrogen and stored at -80°C before extraction of total RNA. Whole liver homogenates (0.25 g fresh tissue ml⁻¹) were prepared in ice-cold 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, using a Potter-type, Teflon-glass, motor-driven homogenizer (A. H. Thomas, Philadelphia, PA, USA). Liver-washed microsomal fractions were prepared by differential centrifugation and assayed for total CYP content (Lake 1987). Whole homogenate and microsomal protein content was determined by the method of Lowry *et al.* (1951) employing bovine serum albumin as standard. Aliquots of whole homogenate and washed microsomes were stored at -80°C. Liver whole homogenate palmitoyl-CoA oxidation was determined as described by Gray *et al.* (1983), but with the addition of 10 μ M FAD to each cuvette. Liver microsomes were assayed for 7-ethoxyresorufin O-deethylase, 7-pentoxoresorufin O-depentyase and testosterone 6 β -hydroxylase activities employing substrate concentrations of 2, 20 and 250 μ M, respectively, as described (Lake 1987, Japenga *et al.* 1993, Lake *et al.* 1998b).

Preparation of liver slices

Untreated rats were killed by carbon dioxide asphyxiation followed by cervical dislocation. The livers were perfused *in situ* before excision with 20 ml ice-cold oxygenated (95% O₂/5% CO₂) Earle's balanced salt solution (EBSS) containing 25 mM D-glucose, 50 μ g ml⁻¹ gentamicin and 2.5 μ g ml⁻¹ fungizone (Price *et al.* 1998). Tissue cylinders from liver samples were prepared with a 10-mm diameter motor-driven tissue coring tool. From the cylinders, liver slices of around 250 μ m thickness were prepared in cold, oxygenated EBSS containing the above additions using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA). Liver slice thickness was determined as described (Price *et al.* 1998).

Culture of liver slices

Liver slices were floated onto Vitron (Vitron, Inc., Tucson, AZ, USA) type C titanium roller inserts (two slices per insert) and cultured in glass vials containing 1.7 ml culture medium at 37°C employing a Vitron dynamic organ culture incubator. The culture medium consisted of Williams' medium E containing 2 mM L-glutamine, 0.1 µM insulin, 0.1 µM dexamethasone (DEX), 50 µg ml⁻¹ gentamicin and 2.5 µg ml⁻¹ fungizone under an atmosphere of 95% O₂/5% CO₂. In addition to the above medium (DEX-supplemented medium), liver slices were also cultured in the same medium, but without the addition of 0.1 µM DEX (DEX-free medium). After 1 h, treatment was commenced by replacing the culture medium with medium containing the required concentrations of the test compounds. The test compounds were dissolved in dimethyl sulphoxide (DMSO) and added to the culture medium so that the final DMSO concentration was 0.4% (v/v) in all vials including the control cultures.

RNA extraction from liver slices and liver samples

After culture, liver slices were washed in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, and individual slices stored in QIAGEN RNeasy[®] RNA stabilization reagent. Total RNA was extracted from cultured rat liver slices and liver samples using QIAGEN RNeasy[®] mini kits and eluted in 30 µl RNase-free water.

Reverse transcription

The reaction mixture (final volume 20 µl) was prepared with final concentrations as follows: 4 units Omniscript Reverse Transcriptase, 1 × RT buffer, 10 units RNase inhibitor, 1 µM random hexamer primers, 0.5 mM each of dATP, dGTP, dCTP and dTTP and 8 µl total RNA extract. The RT reaction was performed for 60 min at 42°C and then 5 min at 93°C to stop the reaction.

Design of TaqMan primers and probes

Rat CYP and albumin primers and probes (table 1) were designed using Primer Express[®] v.1.5 software (Applied Biosystems). The RNA sequences of CYP1A1, CYP1A2, CYP2B1, CYP2B1/2 (i.e. for CYP2B1 and CYP2B2), CYP3A1, CYP3A2, CYP4A1 and albumin were obtained from GenBank with accession numbers X00469, NM012541, J00719, M37134, D13912, U09742, M14972 and V01222. Nucleotide primers and probe sequences were checked against the NCBI BLAST database to ensure specificity for the selected gene. This analysis confirmed that the selected primers and probe set for

Table 1. TaqMan[®] primers and probe sequences for rat CYP forms and albumin.

Gene	Primers/probes	Location	5' → 3' sequence
CYP1A1	forward	750–772	TGAGTTTGGGGAGGTTACTGGTT
	reverse	838–818	TGAAGGCATCCAGGGAAGAGT
	probe	777–808	ATACCCAGCTGACTTCATTCCCTATCCTCCGTT
CYP1A2	forward	937–959	TTTGGAGCTGGATTTGAAACAGT
	reverse	1026–1006	TCATGAATCTTCCCTCTGCACCTT
	probe	961–989	ACAACAGCCATCTTCTGGAGCATTTTGCT
CYP2B1	forward	989–1008	ACCGGCTACCAACCCTTGAT
	reverse	1093–1070	TGTGTGGTACTCCAATAGGGACAA
	probe	1011–1039	CCGCAGTAAATGCCATACACTGATGCAG
CYP2B1/2	forward	434–453	CCCAATGTTTGGTGGAGGAA
	reverse	511–490	CTGTGATGCACTGGAAGAGGAA
	probe	461–482	AATCCCAGGGAGCCCCACTGA
CYP3A1	forward	1001–1022	CAGCAGCACACTTTCCTTTTGTC
	reverse	1073–1049	CTCCTCCTGCAGTTTCTTCTGTGTA
	probe	1024–1044	TGCATTCCCTGGCCACTACC
CYP3A2	forward	77–99	GCTTTCAGCTCTCACACTGGAAA
	reverse	155–133	TCTATGGGTTCCAAGTCGGTAGA
	probe	106–129	TCCTCCTGGCAGTCATCCTGGTGC
CYP4A1	forward	576–602	CTCCTTAATGACCCTAGACACTGTCAT
	reverse	655–631	TC TTGTAATTTCCATCCACCTGAAC
	probe	629–605	CTGCCATTGTGGCTGAAGGCACACT
Albumin	forward	1076–1096	CAAGAAGGCACCCCGATTACT
	reverse	1165–1147	CGCCTTCAGCACAGCACTT
	probe	1146–1116	CTCCAGTGTGGCTTCATATTCTTAGCAAGTCTCA

CYP2B1/2 would detect both CYP2B1 and CYP2B2. While a TaqMan primers and probe set could be designed for CYP2B1, attempts to design a primers and probe set for CYP2B2 proved unsuccessful.

Real-time quantitative PCR

Real-time quantitative PCR for rat CYP and albumin mRNA levels was performed employing a TaqMan 7700 sequence detector system (Applied Biosystems). The reaction mixture (final volume 25 µl) for PCR was prepared with final concentrations as follows: 1× Universal Master Mix Reagents (Applied Biosystems; containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dATP, dGTP, dCTP, dUTP, passive reference and optimized buffer components), 1.25 µl of the products of the RT reaction in RNase-free water and 50–300 nM of each primer and probe for each rat CYP and albumin mRNA assay. The PCR reaction was performed for 40 cycles: denaturation at 95°C for 15 s and anneal/extension at 60°C for 1 min. The levels of the CYP mRNAs determined were expressed as a ratio to albumin (co-amplified in a duplex reaction), which was employed to normalize RNA loading.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance. Comparisons between means were made using the least significant difference test.

Results

In vivo studies

TaqMan primers and probe sets were developed for rat CYP1A1, CYP1A2, CYP2B1, CYP2B1/2 (i.e. for CYP2B1 and CYP2B2), CYP3A1, CYP3A2 and CYP4A1. To characterize the responsiveness of the TaqMan primers and probe sets (table 1) for induction of CYP form mRNAs, rats were treated with a single i.p. dose of 500 mg kg⁻¹ ARO and four daily oral doses of either 50 mg kg⁻¹ day⁻¹ DEX or 75 mg kg⁻¹ day⁻¹ MCP. Treatment with ARO, DEX and MCP significantly increased the relative liver weight and microsomal CYP content (table 2). ARO, but not DEX and MCP, also significantly increased microsomal protein content (table 2). Liver microsomes were assayed for CYP1A-, CYP2B- and CYP3A-dependent enzyme activities and liver whole homogenates for cyanide-insensitive palmitoyl-CoA oxidation. Previous studies have demonstrated a good correlation between the induction of peroxisomal (palmitoyl-CoA oxidation) and

Table 2. Effect of treatment of male Sprague–Dawley rats with Aroclor 1254 (ARO), dexamethasone (DEX) and methylclofenapate (MCP) on relative liver weight and some hepatic parameters.

Treatment ^{a,b}	Relative liver weight ^c	Microsomal protein ^d	Microsomal CYP ^e	Palmitoyl-CoA oxidation ^f
Control	4.35 ± 0.09	21.4 ± 2.3	0.97 ± 0.02	4.13 ± 0.10
ARO (500 mg kg ⁻¹)	7.38 ± 0.21*** (1.7)	30.9 ± 1.9** (1.4)	3.75 ± 0.13*** (3.9)	3.26 ± 0.06 (0.8)
DEX (50 mg kg ⁻¹ day ⁻¹)	7.29 ± 0.18*** (1.7)	23.9 ± 1.0 (1.1)	1.58 ± 0.09*** (1.6)	2.77 ± 0.09 (0.7)
MCP (75 mg kg ⁻¹ day ⁻¹)	6.64 ± 0.13*** (1.5)	21.7 ± 1.9 (1.0)	1.37 ± 0.02** (1.4)	53.46 ± 2.01*** (12.9)

^aAroclor 1254 was given as a single i.p. dose, whereas dexamethasone and methylclofenapate were given by gavage for 4 days.

^bResults are the mean ± SEM for groups of five rats. Figures in parentheses are fold changes compared with control levels.

^cUnits: g liver/100 g body weight.

^dUnits: mg protein g⁻¹ liver.

^eUnits: nmol mg⁻¹ microsomal protein.

^fUnits: nmol min⁻¹ mg⁻¹ whole homogenate protein.

Values significantly different from control: ***p* < 0.01; ****p* < 0.001.

Table 3. Effect of treatment of male Sprague–Dawley rats with Aroclor 1254 (ARO), dexamethasone (DEX) and methylclofenapate (MCP) on some hepatic microsomal enzyme activities.

Treatment ^a	Enzyme activity ^b		
	7-Ethoxyresorufin <i>O</i> -deethylase ^c	7-Pentoxoresorufin <i>O</i> -deethylase ^c	Testosterone 6 β -hydroxylase ^d
Control	80 $\pm$ 7	32 $\pm$ 3	1.73 $\pm$ 0.05
ARO (500 mg kg ⁻¹)	10150 $\pm$ 448 ^{***} (127)	1114 $\pm$ 49 ^{***} (34.8)	2.85 $\pm$ 0.14 [*] (1.7)
DEX (50 mg kg ⁻¹ day ⁻¹)	209 $\pm$ 59 ^{***} (2.6)	221 $\pm$ 25 ^{***} (6.9)	11.03 $\pm$ 0.52 ^{***} (6.4)
MCP (75 mg kg ⁻¹ day ⁻¹)	55 $\pm$ 9 [*] (0.7)	111 $\pm$ 8 ^{***} (3.5)	2.07 $\pm$ 0.25 (1.2)

^a Aroclor 1254 was given as a single i.p. dose, whereas dexamethasone and methylclofenapate were given by gavage for 4 days.

^b Results are the mean $\pm$ SEM for groups of five rats. Figures in parentheses are fold changes compared with control levels.

^c Units: pmol min⁻¹ mg⁻¹ protein.

^d Units: nmol min⁻¹ mg⁻¹ protein.

Values significantly different from control: * p < 0.05; ** p < 0.01; *** p < 0.001.

microsomal (CYP4A-dependent) fatty acid oxidizing enzyme activities (Lake and Lewis 1996).

Treatment with ARO resulted in a 127-fold induction of CYP1A-dependent 7-ethoxyresorufin *O*-deethylase activity and a 34.8-fold induction of CYP2B-dependent 7-pentoxoresorufin *O*-deethylase activity (table 3). In contrast, treatment with DEX resulted in only a 2.6-fold increase in 7-ethoxyresorufin *O*-deethylase activity, with MCP treatment producing a small decrease in enzyme activity. Treatment with DEX and MCP produced 6.9- and 3.5-fold increases, respectively, in 7-pentoxoresorufin *O*-deethylase activity (table 3). DEX treatment also resulted in a 6.4-fold increase in CYP3A-dependent testosterone 6 β -hydroxylase activity, with ARO producing a small 1.7-fold increase and MCP having no significant effect (table 3). Treatment with MCP, but not ARO and DEX, resulted in a 12.9-fold induction of whole homogenate palmitoyl-CoA oxidation activity (table 2).

Total RNA was extracted from liver samples from control and CYP inducer treated rats and cDNAs prepared by reverse transcription. Levels of CYP mRNAs were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, the levels of all CYP mRNAs were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction.

Treatment with ARO resulted in a marked 22 600-fold induction (compared with control rats) in CYP1A1 mRNA levels (figure 1A). Levels of CYP1A2 mRNA were significantly increased by 52-fold after treatment with ARO, whereas both DEX and MCP produced significant decreases in CYP1A2 mRNA levels (figure 1B).

Treatment with ARO, MCP and DEX produced significant 5480-, 126- and 24-fold, respectively, increases in CYP2B1 mRNA levels (figure 1C). In addition, treatment with the CYP inducers also produced significant 648-, 25- and 8-fold increases, respectively, in CYP2B1/2 mRNA levels (figure 1D).

Treatment with ARO and DEX produced significant 47- and 97-fold increases in CYP3A1 mRNA levels, whereas MCP produced only a 2-fold increase

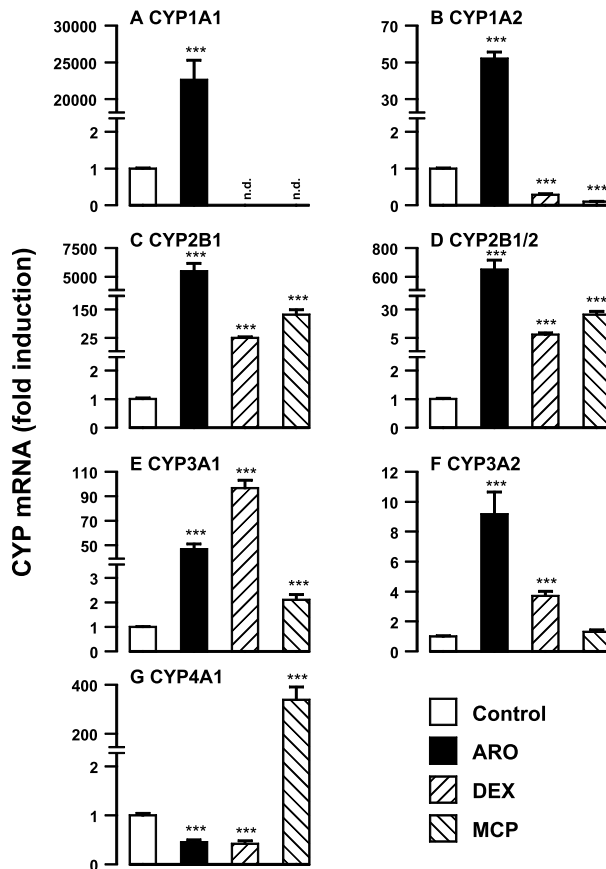


Figure 1. Effect of treatment of male Sprague–Dawley rats with Aroclor 1254 (ARO), dexamethasone (DEX) and methylclofenapate (MCP) on the levels of hepatic CYP1A1 (A), CYP1A2 (B), CYP2B1 (C), CYP2B1/2 (D), CYP3A1 (E), CYP3A2 (F) and CYP4A1 (G) mRNA. Rats received either no treatment (controls) or a single i.p. dose of 500 mg kg^{-1} ARO or four daily oral doses of either $50 \text{ mg kg}^{-1} \text{ day}^{-1}$ DEX or $75 \text{ mg kg}^{-1} \text{ day}^{-1}$ MCP. Total RNA was extracted from liver samples and levels of CYP form mRNAs determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of each CYP form mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. The effect of DEX and MCP on CYP1A1 mRNA was not determined (n.d.). Results are the mean $\pm$ SEM for groups of five rats. Values significantly different from control: *** $p < 0.001$.

(figure 1E). Levels of CYP3A2 mRNA were less markedly stimulated, with ARO and DEX producing 9- and 4-fold increases, respectively (figure 1F). Treatment with MCP resulted in a significant 339-fold increase in CYP4A1 mRNA levels (figure 1G). In contrast, levels of CYP4A1 mRNA were significantly reduced by treatment with ARO and DEX.

Induction of CYP mRNAs in cultured rat liver slices

Precision-cut rat liver slices were cultured in Williams' medium E supplemented with $0.1 \mu\text{M}$ insulin and $0.1 \mu\text{M}$ DEX for 6 and 24 h. In addition, liver slices were also cultured for 24 h in medium containing $0.1 \mu\text{M}$ insulin but no

DEX. Rat liver slices were cultured in medium containing no CYP inducers (control, with 0.4% v/v DMSO) and in media containing either 10 μ M BNF, 10 μ g ml⁻¹ ARO, 500 μ M NaPB, 20 μ M PCN, 50 μ M WY or 50 μ M MCP. The concentrations of the CYP inducers were selected from the results of previous studies on the induction of CYP forms and peroxisomal enzyme activities in cultured precision-cut rat liver slices (Beaman *et al.* 1993, Lake *et al.* 1993, 1996, unpublished observations). At the end of the culture periods, liver slice total RNA was extracted and processed for determination of CYP1A1, CYP1A2, CYP2B1/2, CYP3A1, CYP3A2 and CYP4A1 mRNA levels. No *in vitro* studies were performed with the TaqMan primers and probe set for CYP2B1 mRNA.

The treatment of rat liver slices with BNF produced a marked 135-fold induction (compared with liver slices cultured in control medium) of CYP1A1 mRNA after 6 h, with a much smaller increase being produced by ARO (figure 2A). After 24 h, CYP1A1 mRNA levels were increased 21- and 35-fold by treatment with BNF and ARO, respectively, in DEX-free medium (figure 2C), whereas CYP1A1 mRNA levels were not significantly increased after 24-h treatment with BNF and ARO in DEX-supplemented medium (figure 2B). None of the other CYP inducers significantly increased CYP1A1 mRNA levels, with in some

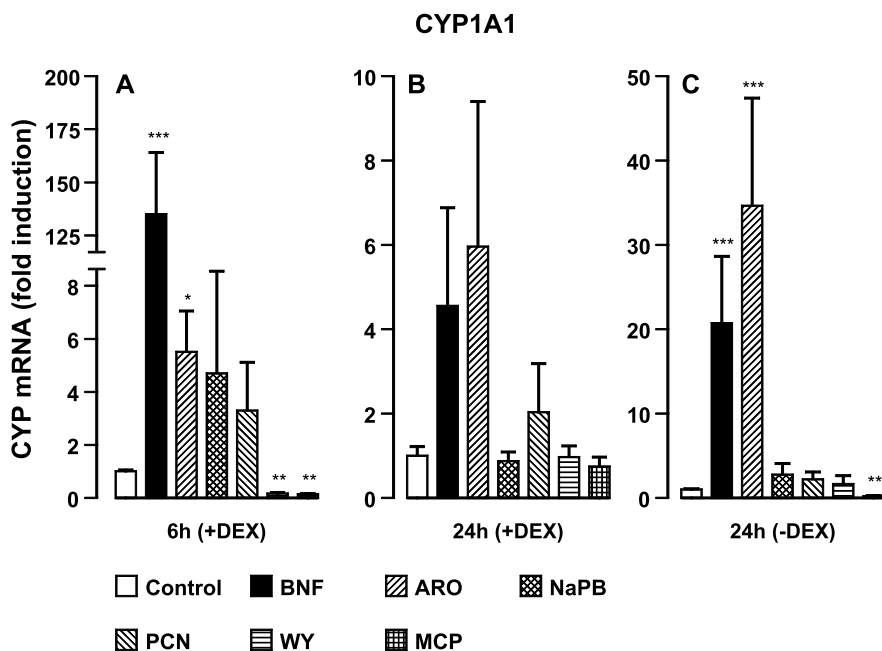


Figure 2. Effect of treatment of rat liver slices with 10 μ M β -naphthoflavone (BNF), 10 μ g ml⁻¹ Aroclor 1254 (ARO), 500 μ M sodium phenobarbitone (NaPB), 20 μ M pregnenolone-16 α -carbonitrile (PCN), 50 μ M Wy-14,643 (WY) and 50 μ M methylclofenapate (MCP) on levels of CYP1A1 mRNA. Precision-cut rat liver slices were cultured in DEX-supplemented medium for 6 (A) and 24 h (B) and in DEX-free medium for 24 h (C). Total RNA was extracted from whole liver slices and levels of CYP1A1 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of CYP1A1 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are mean $\pm$ SEM of three separate experiments. In each experiment, four control (DMSO only treated) and two liver slices for each CYP inducer studied were analysed. Values significantly different from control: * p < 0.05; ** p < 0.01; *** p < 0.001.

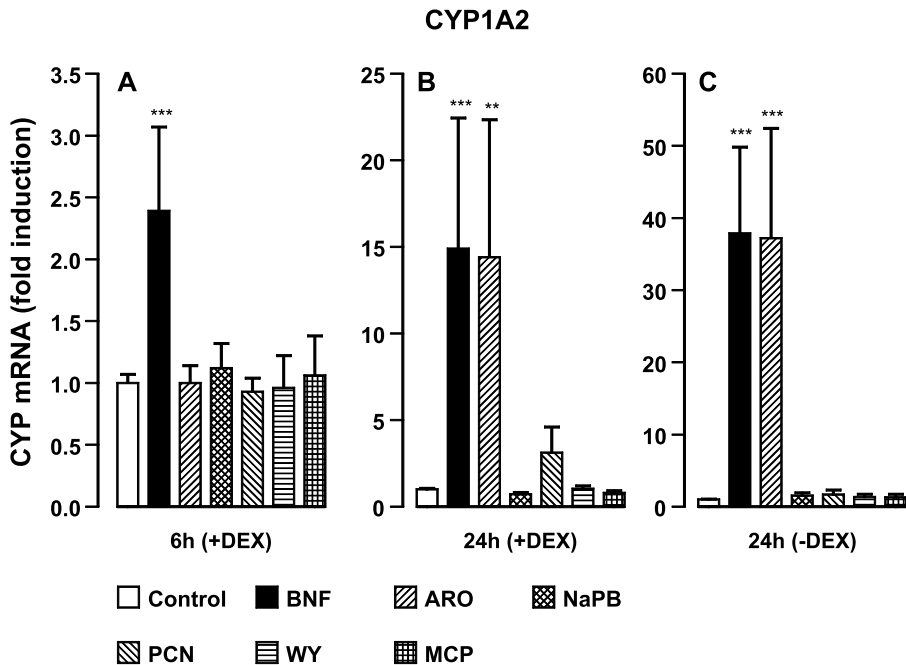


Figure 3. Effect of treatment of rat liver slices with 10 μ M β -naphthoflavone (BNF), 10 μ g ml⁻¹ Aroclor 1254 (ARO), 500 μ M sodium phenobarbitone (NaPB), 20 μ M pregnenolone-16 α -carbonitrile (PCN), 50 μ M Wy-14,643 (WY) and 50 μ M methylclofenapate (MCP) on levels of CYP1A2 mRNA. Precision-cut rat liver slices were cultured in DEX-supplemented medium for 6 (A) and 24 h (B) and in DEX-free medium for 24 h (C). Total RNA was extracted from whole liver slices and levels of CYP1A2 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of CYP1A2 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are mean $\pm$ SEM of three experiments. In each experiment, four control (DMSO only treated) and two liver slices for each CYP inducer studied were analysed. Values significantly different from control: ** p < 0.01; *** p < 0.001.

instances WY and MCP producing significant decreases in CYP1A1 mRNA levels (figure 2).

Treatment with BNF for 6 h produced a small increase in CYP1A2 mRNA levels, whereas both BNF and ARO significantly increased CYP1A2 levels after 24 h (figure 3). After 24 h, levels of CYP1A2 mRNA were increased around 38-fold in DEX-free medium (figure 3C), but only around 15-fold in DEX-supplemented medium (figure 3B). None of the other CYP inducers had any significant effect on CYP1A2 mRNA levels.

Treatment with ARO and NaPB for 6 and 24 h produced significant increases in CYP2B1/2 mRNA levels (figure 4). Whereas the magnitude of CYP2B1/2 mRNA induction by ARO and NaPB after 24 h was greater in DEX-supplemented (figure 4B), than in DEX-free medium (figure 4C), a marked variability was observed in the experiments conducted in the DEX-supplemented medium. In addition, significant 9–16-fold increases in CYP2B1/2 mRNA levels were only observed after treatment with PCN, WY and MCP for 24 h using the DEX-free medium (figure 4C).

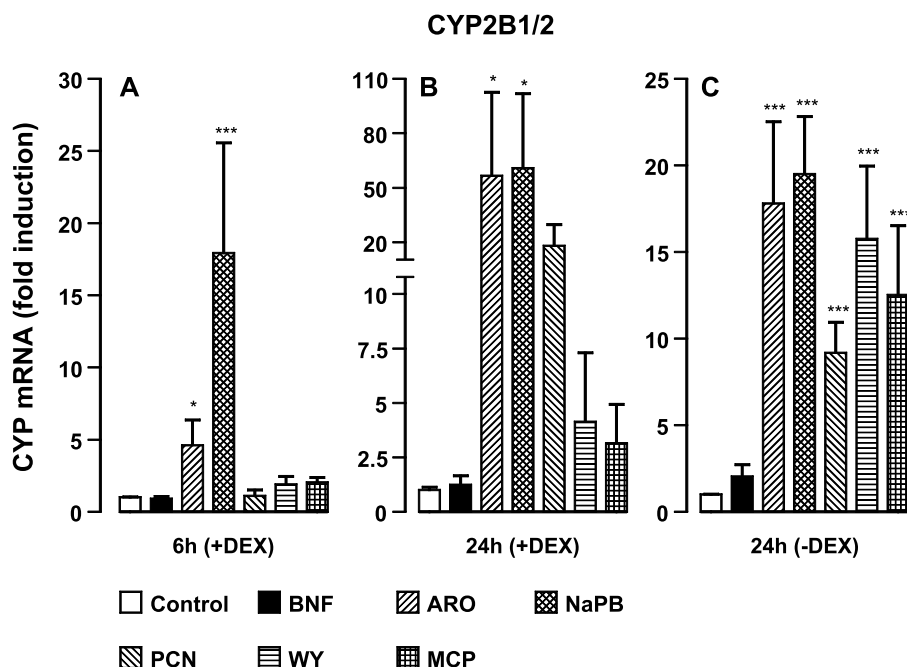


Figure 4. Effect of treatment of rat liver slices with 10 μ M β -naphthoflavone (BNF), 10 μ g ml⁻¹ Aroclor 1254 (ARO), 500 μ M sodium phenobarbitone (NaPB), 20 μ M pregnenolone-16 α -carbonitrile (PCN), 50 μ M Wy-14,643 (WY) and 50 μ M methylclofenapate (MCP) on levels of CYP2B1/2 mRNA. Precision-cut rat liver slices were cultured in DEX-supplemented medium for 6 (A) and 24 h (B) and in DEX-free medium for 24 h (C). Total RNA was extracted from whole liver slices and levels of CYP2B1/2 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of CYP2B1/2 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are mean $\pm$ SEM of three experiments. In each experiment, four control (DMSO only treated) and two liver slices for each CYP inducer studied were analysed. Values significantly different from control: * p < 0.05; *** p < 0.001.

The treatment of rat liver slices with PCN resulted in significant increases in CYP3A1 mRNA levels (figure 5). After 24 h, a 56-fold increase was observed in DEX-free medium (figure 5C), whereas CYP3A1 mRNA levels were only increased 7-fold in DEX-supplemented medium (figure 5B). None of the other CYP inducers significantly increased CYP3A1 mRNA levels and in some instances significant decreases were observed (figure 5).

The treatment of rat liver slices with PCN for 24 h resulted in significant increases in levels of CYP3A2 mRNA (figure 6). Levels of CYP3A2 mRNA were increased around 4-fold when liver slices were cultured in either DEX-supplemented or DEX-free medium. None of the other CYP inducers examined had any significant effect on levels of CYP3A2 mRNA in cultured rat liver slices (figure 6).

The treatment of rat liver slices with WY and MCP for 6 and 24 h resulted in significant increases in CYP4A1 mRNA levels (figure 7). In DEX-supplemented medium, CYP4A1 mRNA levels were increased around 6- and 10–15-fold, respectively (figure 7A, B). However, in DEX-free medium, treatment with WY and MCP for 24 h produced 437- and 186-fold, respectively, increases in CYP4A1 mRNA levels (figure 7C).

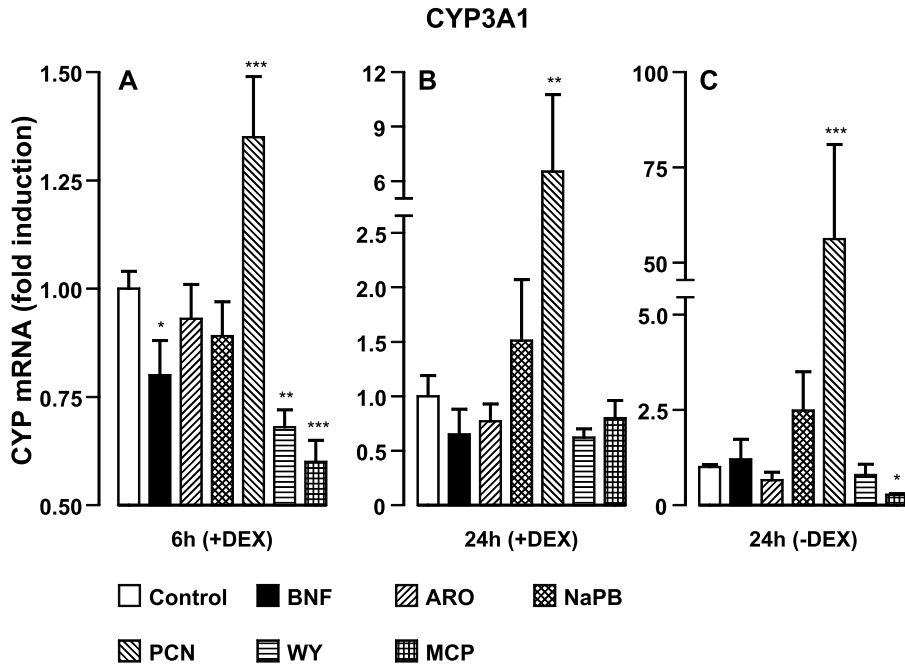


Figure 5. Effect of treatment of rat liver slices with 10 μ M β -naphthoflavone (BNF), 10 μ g ml⁻¹ Aroclor 1254 (ARO), 500 μ M sodium phenobarbitone (NaPB), 20 μ M pregnenolone-16 α -carbonitrile (PCN), 50 μ M Wy-14,643 (WY) and 50 μ M methylclofenapate (MCP) on levels of CYP3A1 mRNA. Precision-cut rat liver slices were cultured in DEX-supplemented medium for 6 (A) and 24 h (B) and in DEX-free medium for 24 h (C). Total RNA was extracted from whole liver slices and levels of CYP3A1 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of CYP3A1 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are mean $\pm$ SEM of three experiments. In each experiment, four control (DMSO only treated) and two liver slices for each CYP inducer studied were analysed. Values significantly different from control: * p < 0.05; ** p < 0.01; *** p < 0.001.

Discussion

The first objective of these studies was to develop TaqMan primers and probe sets for a number of rat hepatic CYP1A, CYP2B, CYP3A and CYP4A subfamily forms. To characterize the responsiveness of the TaqMan primers and probe sets for the selected CYP forms, rats were treated with ARO, DEX and MCP. Liver-washed microsomes were assayed for CYP1A-, CYP2B- and CYP3A-dependent enzyme activities and liver whole homogenates for palmitoyl-CoA oxidation as a surrogate marker of CYP4A form induction (Lake and Lewis 1996). As expected from previous studies, ARO induced CYP1A-, CYP2B- and to a lesser extent CYP3A-dependent enzyme activities. In addition, treatment with DEX resulted in an induction of CYP3A and CYP2B enzyme activities, whereas MCP induced the CYP4A marker and to a lesser extent the CYP2B marker.

Treatment with ARO, a known CYP1A, CYP2B and CYP3A inducer (Okey 1990), resulted in marked increases in CYP1A1, CYP1A2, CYP2B1, CYP2B1/2 and CYP3A1 mRNA levels. In addition, a small increase in CYP3A2 mRNA levels was also observed, whereas ARO treatment significantly reduced CYP4A1 mRNA levels. DEX, which is known to induce CYP3A and also CYP2B forms in

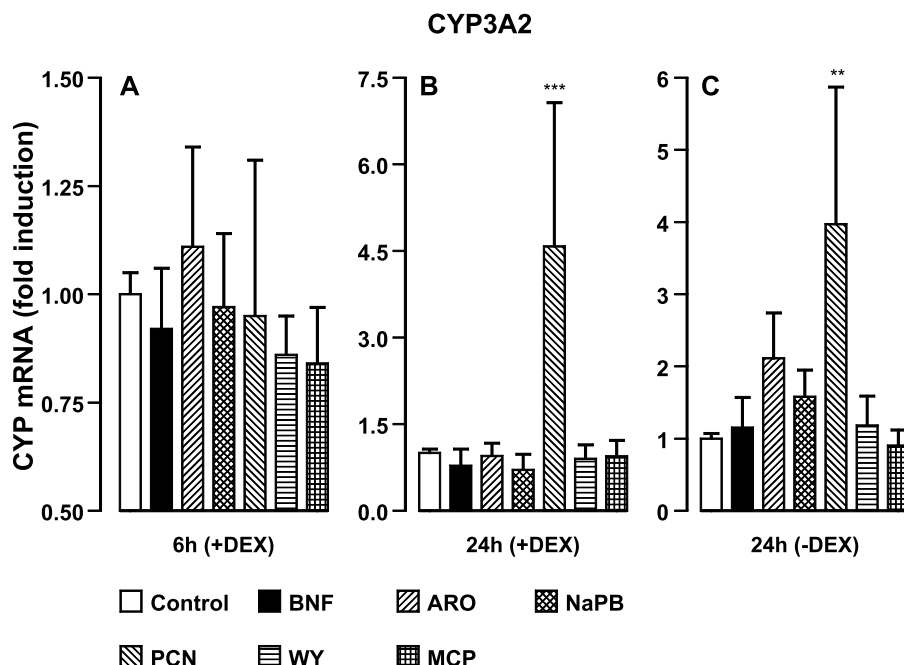


Figure 6. Effect of treatment of rat liver slices with 10 μ M β -naphthoflavone (BNF), 10 μ g ml⁻¹ Aroclor 1254 (ARO), 500 μ M sodium phenobarbitone (NaPB), 20 μ M pregnenolone-16 α -carbonitrile (PCN), 50 μ M Wy-14,643 (WY) and 50 μ M methylclofenapate (MCP) on levels of CYP3A2 mRNA. Precision-cut rat liver slices were cultured in DEX-supplemented medium for 6 (A) and 24 h (B) and in DEX-free medium for 24 h (C). Total RNA was extracted from whole liver slices and levels of CYP3A2 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of CYP3A2 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are mean $\pm$ SEM of three experiments. In each experiment, four control (DMSO only treated) and two liver slices for each CYP inducer studied were analysed. Values significantly different from control: ** p < 0.01; *** p < 0.001.

rat liver (Maurel 1996, Lake *et al.* 1998b), significantly increased CYP2B1, CYP2B1/2 and CYP3A1 mRNA levels. In addition, DEX also produced a small increase in CYP3A2 mRNA levels, but did not increase levels of either CYP1A2 or CYP4A1 mRNA. Finally, MCP markedly induced levels of CYP4A1 mRNA and also produced smaller increases in levels of CYP2B1, CYP2B1/2 and CYP3A1 mRNA. These results are in agreement with other studies, where peroxisome proliferators have been shown to markedly induce CYP4A forms and also to induce other CYP forms in rat hepatocytes (Bars *et al.* 1989, 1993, Lake and Lewis 1996).

Generally, there was a good concordance between the effects of ARO, DEX and MCP on the CYP enzyme activities and the CYP mRNA levels determined (table 3 and figure 1). Thus, the marked induction of 7-ethoxyresorufin *O*-deethylase activity by ARO was paralleled by large increases in CYP1A1 and CYP1A2 mRNA levels. The comparatively small effect of DEX on 7-ethoxyresorufin *O*-deethylase activity may be attributable to the induction of other CYP forms (e.g. CYP2C forms). ARO produced a marked induction of 7-pentoxoresorufin *O*-deethylase activity, which was paralleled by large increases in

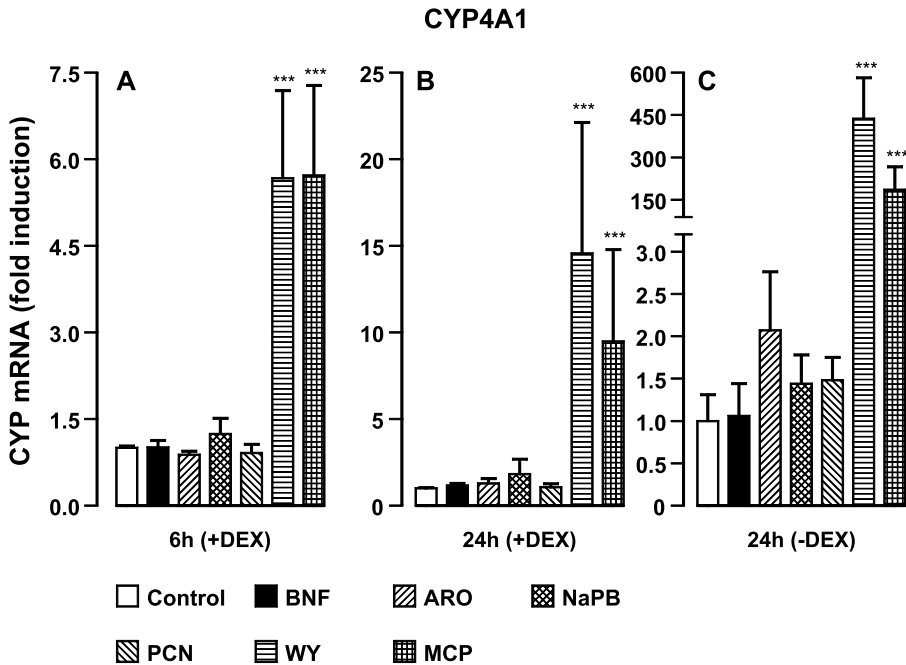


Figure 7. Effect of treatment of rat liver slices with 10 μ M β -naphthoflavone (BNF), 10 μ g ml⁻¹ Aroclor 1254 (ARO), 500 μ M sodium phenobarbitone (NaPB), 20 μ M pregnenolone-16 α -carbonitrile (PCN), 50 μ M Wy-14,643 (WY) and 50 μ M methylclofenapate (MCP) on levels of CYP4A1 mRNA. Precision-cut rat liver slices were cultured in DEX-supplemented medium for 6 (A) and 24 h (B) and in DEX-free medium for 24 h (C). Total RNA was extracted from whole slices and levels of CYP4A1 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan). To normalize for RNA loading, levels of CYP4A1 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are mean $\pm$ SEM of three experiments. In each experiment, four control (DMSO only treated) and liver slices for each CYP inducer studied were analysed. Values significantly different from control: *** p < 0.001.

CYP2B1 and CYP2B1/2 mRNA levels. Both DEX and MCP also induced CYP2B enzyme activity and mRNA levels. However, DEX produced a greater effect on 7-pentoxyresorufin *O*-depentylase activity, whereas MCP produced a greater effect on CYP2B1 and CYP2B1/2 mRNA levels. Treatment with DEX produced the greatest increases in testosterone 6 β -hydroxylase activity and CYP3A1 mRNA levels. Smaller increases in testosterone 6 β -hydroxylase activity and CYP3A1 mRNA levels were produced by ARO, whereas MCP had little effect on CYP3A enzyme activity and mRNA levels. Although no CYP4A-dependent enzyme activity was determined, the large increases in CYP4A1 mRNA levels observed after MCP treatment were paralleled by an induction of peroxisomal fatty acid β -oxidation activity.

Overall, the results of the *in vivo* studies demonstrate a good concordance between the induction of CYP form mRNA levels and the enzyme activities measured. The three CYP inducers examined produced the expected effects on CYP form mRNA levels, confirming the usefulness of the developed TaqMan primers and probe sets for investigating CYP form mRNA induction in rat liver.

Precision-cut rat liver slices were cultured for 6 and 24 h in DEX-supplemented medium and for 24 h in DEX-free medium. Generally, the CYP inducers examined produced the expected CYP mRNA induction profiles. Thus, levels of CYP1A1 and CYP1A2 mRNA were induced by BNF and ARO, but not by the CYP2B/CYP3A inducers NaPB and PCN and the peroxisome proliferators WY and MCP. With the exception of BNF, levels of CYP2B1/2 mRNA were induced by treatment with all the CYP inducers examined. Only PCN induced CYP3A1 and CYP3A2 mRNA levels and only WY and MCP induced CYP4A1 mRNA levels.

Overall, the results obtained for the *in vitro* induction of CYP1A1, CYP1A2, CYP2B1/2 and CYP4A1 mRNA levels are in agreement with the known *in vivo* properties of BNF, ARO, NaPB, PCN, WY and MCP (Conney 1986, Okey 1990, Lake and Lewis 1996, Maurel 1996, Nims and Lubet 1996, Parkinson 1996, Lake *et al.* 1998b, Pelkonen *et al.* 1998). However, whereas the CYP3A inducer PCN (Debri *et al.* 1995, Maurel 1996) increased CYP3A1 and CYP3A2 mRNA levels, no effect was observed with ARO and NaPB, which are also known to induce CYP3A forms in rat liver (Okey 1990, Maurel 1996, Nims and Lubet 1996, Lake *et al.* 1998b). The reason for the lack of effect of ARO and NaPB on CYP3A form mRNA levels in this study is unclear. Possibly, this may be related to the sensitivity of the response, as in the *in vivo* study ARO produced a 648-fold induction of CYP2B1/2 mRNA levels but only a 47- and 9-fold induction of CYP3A1 and CYP3A2 mRNA levels, respectively (figure 1). In addition, only single concentrations of each CYP inducer were examined, with possibly higher concentrations of ARO and NaPB being required to induce CYP3A as well as CYP2B mRNA levels in cultured rat liver slices. Apart from CYP3A1 and CYP3A2, other CYP3A subfamily members present in rat liver include CYP3A9, CYP3A18 and CYP3A23 (Komori and Oda 1994, Debri *et al.* 1995, Nelson *et al.* 1996, Mahnke *et al.* 1997). CYP3A23 has been shown to be markedly inducible in rat liver and hence it would be of interest to design a TaqMan primers and probe set for CYP3A23 for *in vitro* induction studies in cultured rat liver slices.

Examination of the data for all six CYP form mRNAs studied in cultured liver slices (figures 2–7) revealed both time-dependent and medium composition differences in mRNA levels. Apart from the effects of BNF on CYP1A1 levels, CYP mRNA induction was always greater after 24 than after 6 h of treatment. These results are in agreement with a previous study using different TaqMan primers and probe sets where induction of CYP1A2, CYP2B1 and CYP4A1 mRNA levels was generally greater after 24 than after 3 or 6 h (Pan *et al.* 2002). Hence, in terms of selecting one time point for rat liver slice CYP1A, CYP2B, CYP3A and CYP4A form mRNA induction studies, a 24-h inducer treatment period appears preferable, even though this time point may be past the peak of CYP1A1 mRNA induction by some compounds.

As with cultured rat hepatocytes, the literature contains many variations in culture conditions for precision-cut liver slices. For example, in some studies, liver slices have been cultured in media supplemented with foetal calf serum, insulin and a glucocorticoid (DEX or hydrocortisone), whereas in other studies liver slices have been cultured in media containing either some or none of these additions (Lake *et al.* 1993, 1996, Drahushuk *et al.* 1996, Müller *et al.* 1998, 2000, Toutain *et al.* 1998). Although a given set of culture condition may be optimal for one

particular application, such conditions may not be optimal for other applications. In the present study, the effect of DEX supplementation was investigated as other studies have demonstrated that CYP induction in cultured rat hepatocytes can be modulated by the presence of glucocorticoids in the medium. For example, the presence of DEX has been shown to enhance the induction of CYP2B forms by NaPB (Sinclair *et al.* 1990, Waxman *et al.* 1990, Sidhu *et al.* 1993, Burczynski *et al.* 2001). DEX is also known to potentiate the induction of CYP2B, CYP2C and CYP3A forms in cultured human hepatocytes (Gerbal-Chaloin *et al.* 2001).

In the current study, marked differences were observed in the magnitude of CYP form mRNA induction in liver slices cultured for 24 h in DEX-free as against DEX-supplemented medium. The magnitude of induction of levels of CYP1A1, CYP1A2, CYP3A1 and CYP4A1 mRNA were all greater in DEX-free than in DEX-supplemented medium. Indeed, in some instances CYP mRNA induction was only significant when the liver slices were cultured in DEX-free medium. As might be anticipated from studies in cultured rat hepatocytes (see above), the magnitude of CYP2B1/2 mRNA induction by ARO and NaPB after 24-h treatment was greater in DEX-supplemented than in DEX-free medium. However, a marked variability was observed in the experiments conducted in DEX-supplemented medium and the induction of CYP2B1/2 mRNA levels by PCN, WY and MCP was only significant when the liver slices were cultured in DEX-free medium. Overall, for the majority of the CYP forms studied, a DEX-free medium appears preferable for CYP mRNA induction studies. The only exception was CYP3A2, where PCN produced a similar increase in mRNA levels after 24 h in both media examined.

In summary, the present results demonstrate the utility of cultured precision-cut liver slices as an *in vitro* model system to study the induction of rat hepatic CYP forms. For the concurrent analysis of the CYP1A, CYP2B, CYP3A and CYP4A forms examined, liver slices are best cultured for 24 h with the test compounds in medium supplemented with insulin but without DEX.

Acknowledgements

The authors are grateful to the UK Food Standards Agency (Contract Number T01011) and to the European Union (EU BIOTECH Contract Number BI04-CT97-2145) for the financial support of these studies.

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More potent inhibition of human CYP2A6 than mouse CYP2A5 enzyme activities by derivatives of phenylethylamine and benzaldehyde

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Received 10 October 2002

1. A rapid 96-well plate assay method was developed and validated to measure liver microsomal coumarin 7-hydroxylation *in vitro*.

2. The method was used to test inhibition of human and mouse CYP2A enzymes by three phenylethylamine derivatives 2-(*p*-tolyl)-ethylamine, amphetamine, 2-phenylethylamine and benzaldehyde, and two of its derivatives, 4-methylbenzaldehyde and 4-methoxybenzaldehyde.

3. The benzaldehyde derivatives were more potent inhibitors of CYP2A5 than the phenylethylamines. The K_{ic} value of 4-methylbenzaldehyde was 3.4 μ M and for 4-methoxybenzaldehyde it was 0.86 μ M for CYP2A5.

4. Amphetamine is a weak inhibitor of CYP2A6, whereas benzaldehyde is a suicide inhibitor with $K_{inact} = 0.16 \text{ min}^{-1}$ and $K_I = 18 \mu\text{M}$. The K_{ic} values of 2-phenylethylamine, 2-(*p*-tolyl)-ethylamine, 4-methylbenzaldehyde and 4-methoxybenzaldehyde were 1.13, 0.23, 0.36 and 0.73 μ M for CYP2A6, respectively.

5. Novel potent inhibitors were found for CYP2A6 and, except for 4-methoxybenzaldehyde, all the compounds inhibited CYP2A5 and CYP2A6 enzymes differentially. These data add to the refinement of CYP2A enzyme active sites and provide chemical leads for developing novel chemical inhibitors of the CYP2A6 enzyme.

Introduction

Human CYP2A6 and mouse CYP2A5 are biotransformation enzymes that metabolize a number of drugs and toxic compounds, including methoxyflurane, tegafur, SM12502, aflatoxin B1, 1,3-butadiene and several nitrosamines (Raunio *et al.* 2001). CYP2A6 and CYP2A5 enzymes show 82% amino acid homology and are expressed predominantly in the liver in humans and mice (Miles *et al.* 1990, Yamano *et al.* 1990, Koskela *et al.* 1999). The CYP2A6 enzyme has been the focus of much attention recently because it is a major and high-affinity metabolizer of nicotine and many tobacco-specific nitrosamines (Oscarson 2001, Tyndale and Sellers 2001). The *CYP2A6* gene is polymorphic with several inactive alleles having been characterized to date (www.imm.ki.se/CYPalleles). Genetic differences in the *CYP2A6* gene have been shown to affect nicotine kinetics markedly (Kitagawa *et al.* 1999, Inoue *et al.* 2000, Nakajima *et al.* 2000), smoking behaviour (Tyndale and Sellers 2002) and the risk of lung cancer due to cigarette smoking (Miyamoto *et al.* 1999). Pilot studies have suggested that deliberate inhibition of

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CYP2A6 activity may favourably modulate smoking habits (Sellers *et al.* 2000, Tyndale and Sellers 2002).

Coumarin 7-hydroxylation is a specific and well-characterized marker activity for both of these CYP2A forms (Chang and Waxman 1996, Pelkonen *et al.* 2000). The most convenient assay to quantitate this reaction is to measure the fluorescence of 7-hydroxycoumarin formed from coumarin under alkaline conditions. Several methods based on manual spectrophotofluorometry are available for this purpose, including the popular Greenlee–Poland (Greenlee and Poland 1978) and Aitio (1978) methods. In addition, more complicated and time-consuming HPLC, thin-layer chromatography and capillary electrophoresis techniques have been developed for detection of 7-hydroxycoumarin (Lamiable *et al.* 1993, van Iersel *et al.* 1994, Bogan *et al.* 1995, Hiller and Cole 1995). Clearly, a higher throughput assay method is needed for screening purposes.

The inhibitory properties of some compounds for CYP2A6 and CYP2A5 have been studied. Metirapone is a potent inhibitor of mouse CYP2A5 (Wood 1979), but curiously, it is a weak inhibitor of human CYP2A6 (Mäenpää *et al.* 1993). Similarly, many other compounds differ in their inhibition potency against these enzymes indicating that there are crucial differences in their active sites. The active site of CYP2A5 seems to be sterically larger than that of CYP2A6, and the CYP2A6 enzyme poorly tolerates negative charges near the lactone moiety of coumarin (Poso *et al.* 2001). The most potent inhibitors of CYP2A6 found to date are methoxsalen, tranilcypromine, miconazole, clotrimazole, 4,4-dipyridyl sulfide and 4,4-dipyridyl disulfide which all have $K_i < 1 \mu\text{M}$ (Mäenpää *et al.* 1993, Draper *et al.* 1997, Fujita and Kamataki 2001, Taavitsainen *et al.* 2001, Zhang *et al.* 2001). Methoxsalen, tranilcypromine, miconazole and clotrimazole also potently inhibit CYP2A5, as do 7-methylcoumarin, butylcyclohexane and several δ - and γ -lactone derivatives (Mäenpää *et al.* 1993, Juvonen *et al.* 2000).

The study of CYP2A6 and CYP2A5 enzyme inhibitors has not been systematic. In general, previously known CYP inhibitors and several coumarin derivatives have been characterized to date. It is therefore important to test new types of compounds to expand the library of chemicals known to inhibit CYP2A enzymes and to obtain more detailed structure-activity relationships. Therefore, the aims of this study were: (1) to develop a higher throughput assay method for the assay of coumarin 7-hydroxylation, (2) to determine inhibition potencies and mechanisms of previously uncharacterized aromatic ethylamine as well as benzaldehyde derivatives (figure 1), (3) to determine which structural features are important for inhibition potency in these compounds and (4) to compare the relative inhibition potencies for the CYP2A6 and CYP2A5 enzymes. The ultimate goal is to develop potent, specific and safe chemical inhibitors of CYP2A6 for clinical use.

Materials and methods

Chemicals

Coumarin, 7-hydroxycoumarin, 2-phenylethylamine, 2-(*p*-tolyl)ethylamine, amphetamine, benzaldehyde, 4-methylbenzaldehyde and 4-methoxybenzaldehyde were obtained from Sigma Chemical Co. (St Louis, MO, USA) and NADPH from Merck (Darmstadt, Germany). The chemicals were at least 97% pure.

Biological material. Male and female (7–12 weeks old, 15–25 g body weight) DBA/2N/Kuo mice were given pyrazole in saline (150 mg kg⁻¹, i.p.) on three consecutive mornings and killed 24 h after the last

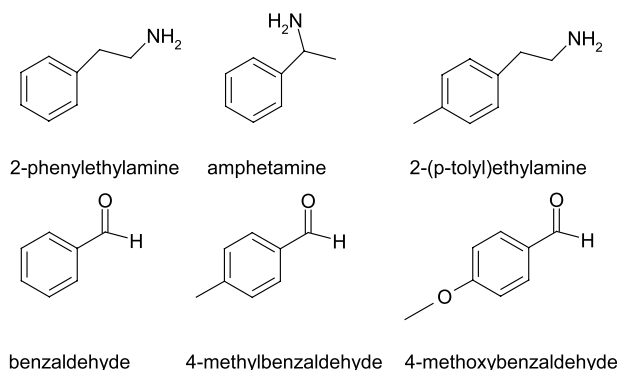


Figure 1. Structures of the inhibitors.

injection. Pyrazole was administered to these mice to maximize their CYP2A5 oxidizing capacity (Juvonen *et al.* 1985). The livers were removed into ice-cold saline and pooled microsomes prepared as described (Lang and Nebert 1981). The Ethics Committee for Animal Experiments, University of Kuopio, approved the experiments. Two sets of human liver samples were used. First, pilot studies were carried out with samples from the liver bank in Kuopio (Kimonen *et al.* 1995, Taavitsainen *et al.* 2001). The actual experiments were performed with microsomes obtained from the liver of a 40-year-old woman who died of intracranial haemorrhage. This microsome preparation was selected because previous screening showed that it had a high CYP2A6 content. The use of surplus human tissue was approved by the Ethics Committees of the University of Kuopio and the University of Oulu. Microsomes were prepared from liver tissue stored in liquid nitrogen essentially as described above. The use of mouse and human liver microsomes was justified by the fact that the coumarin 7-hydroxylation reaction is known to be mediated exclusively by the CYP2A5 and CYP2A6 enzymes in pyrazole-treated mouse and human liver, respectively (Chang and Waxman 1996, Draper *et al.* 1997, Pelkonen *et al.* 1997, 2000, Juvonen *et al.* 2000).

Biochemical assays

Microsomal protein was determined by the method of Smith *et al.* (1985). The coumarin 7-hydroxylation activity assay is based on detection of fluorescence emitted by 7-hydroxycoumarin in alkaline conditions by manual spectrophotofluorometry as described by Aitio (1978). In the original method, the incubation mixtures contained 50–200 µg microsomal protein, 0.2–100 µM coumarin, 0–0.1 M inhibitor, 2% ethanol (as a solvent for coumarin and inhibitor), 5 mM MgCl₂, 0.5 mM NADPH in 50 mM Tris-HCl buffer, pH 7.4, in a total volume of 0.5 ml. The mixture was incubated at 37 °C for 10 min in the IC₅₀ determination experiments, with the reaction terminated by addition of 0.5 ml 6% trichloroacetic acid, centrifuged for 10 min at 3000g, and 0.5 ml of supernatant was mixed with 2 ml 1.6 M glycine buffer (pH 10.4) just before transfer into a cuvette. Fluorescence was measured promptly using 390 nm excitation and 440 nm emission wavelengths. Two blank reactions were included in the inhibition experiments, one containing inhibitor but not coumarin, the other being the mixture in which the reaction was stopped before addition of NADPH. This 7-hydroxycoumarin assay can detect as low as 10 pmol 7-hydroxycoumarin and its reproducibility on different working days is better than 15%.

The above method was adapted to a 96-well plate format to facilitate higher throughput inhibition analysis. In each well, a 100-µl incubation volume contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 10 µM coumarin, about 20 µg microsomal protein, 0.3 mM NADPH and 0–10 mM inhibitor. The reaction was terminated by adding 60 µl 10% TCA, and just before the measurement 140 µl 1.6 M glycine-NaOH buffer, pH 7.4, was added. Fluorescence was measured with Victor2TM plate counter (Perkin Elmer Life Sciences, Wallac, Turku, Finland) in each well for 1 s at 355 nm excitation and 460 nm emission. Under these conditions, the reaction proceeded linearly for 10 min at 37 °C. The detection limit was 10 pmol 7-hydroxycoumarin. The reproducibility of this method on different working days was better than 10%. Microsomes or coumarin were omitted from the blank wells.

Both the type and irreversibility of inhibition were determined for selected compounds. When the type of inhibition was determined, the concentration of coumarin varied between 0.4 and 100 µM at four fixed inhibitor concentrations around the determined *K_i* values. Studies to determine irreversible inhibition were undertaken for 2-phenylethylamine, 2-(p-tolyl)-ethylamine, benzaldehyde, 4-methylbenzaldehyde and 4-methoxybenzaldehyde. Various concentrations of inhibitors were pre-incubated up to 20 min 37 °C at 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 200 µg microsomal protein and 0.3 mM NADPH. This was diluted 100 times with 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂,

10 μ M coumarin, 0.3 mM NADPH and continued the incubation for 10 min 37 °C. The reaction was terminated and measured as described above.

Data analysis

Ordinary least products (OLP) regression analysis was used to compare the two coumarin 7-hydroxylase determination methods (Ludbrook 1997). The LOSS function used was:

$$(y - (a + bx))^2 / I b I,$$

where b is the regression coefficient and $I b I$ its absolute (positive) value. The IC_{50} values were calculated using non-linear regression analysis with Prism 3.0TM software (San Diego, CA, USA), which was used also in the analysis of other data. Inhibition types were analysed and inhibition constants were calculated by first doing Hanes plot and then plotting $K_m^{apparent}/V_{max}^{apparent}$ and $1/V_{max}^{apparent}$ against the inhibitor concentration (Cornish-Bowden 1995). If the plot of the natural logarithm of the fraction of remaining activity as a function of pre-incubation time linearly decreased, then decreasing the inhibitor was irreversible. The half maximal inactivation K_I value and the maximal rate of inactivation K_{inact} value were calculated when the inverse individual inactivation rate was plotted against inverse of benzaldehyde concentration ($t_{1/2} = 0.693 (1 + K_I/I)/K_{inact}$) (Kitz and Wilson 1962, Koenigs *et al.* 1997, Raner *et al.* 1997, Wen *et al.* 2002).

Results

Comparison of coumarin 7-hydroxylation assay methods

The original Aitio method and the newly developed 96-well plate method were compared in two ways (figure 2). First, liver microsomal samples with different levels of coumarin 7-hydroxylation activities were measured using both methods. Second, the IC_{50} values of eight known CYP2A inhibitors were determined by the 96-well plate method in mouse and human liver microsomes and these values were compared with published values (Juvonen *et al.* 2000). OLP regression analysis in both cases indicated that the results obtained with these methods were statistically similar with each other because both regression lines pass through the origin within the 95% confidence limits and the regression coefficients of both lines were practically 1.00 (figure 2). Thus, the newly developed 96-well plate method produces the same results as the original Aitio method.

Inhibition of mouse CYP2A5

The basic inhibition properties of three aromatic ethylamine derivatives (2-(*p*-tolyl)-ethylamine, amphetamine, 2-phenylethylamine) and three benzaldehyde derivatives (benzaldehyde, 4-methylbenzaldehyde, 4-methoxybenzaldehyde) were determined. The structures of these compounds are shown in figure 1. Based on the determined IC_{50} values, the benzaldehyde derivatives were more potent inhibitors of CYP2A5 than the phenylethylamines (table 1). 4-Methoxybenzaldehyde was the most potent inhibitor with an $IC_{50} = 5.8 \mu$ M. 4-Methylbenzaldehyde was approximately three times less potent while all of the other compounds were at least one order of magnitude less potent inhibitors of mouse CYP2A5.

Since 4-methoxybenzaldehyde and 4-methylbenzaldehyde were more potent inhibitors of CYP2A5 than the other compounds, their inhibition kinetics were studied in more detail. 4-Methoxybenzaldehyde and 4-methylbenzaldehyde were not irreversible inhibitors for CYP2A5, but both were competitive inhibitors with $K_{ic} = 0.86$ and 3.4μ M, respectively (table 2).

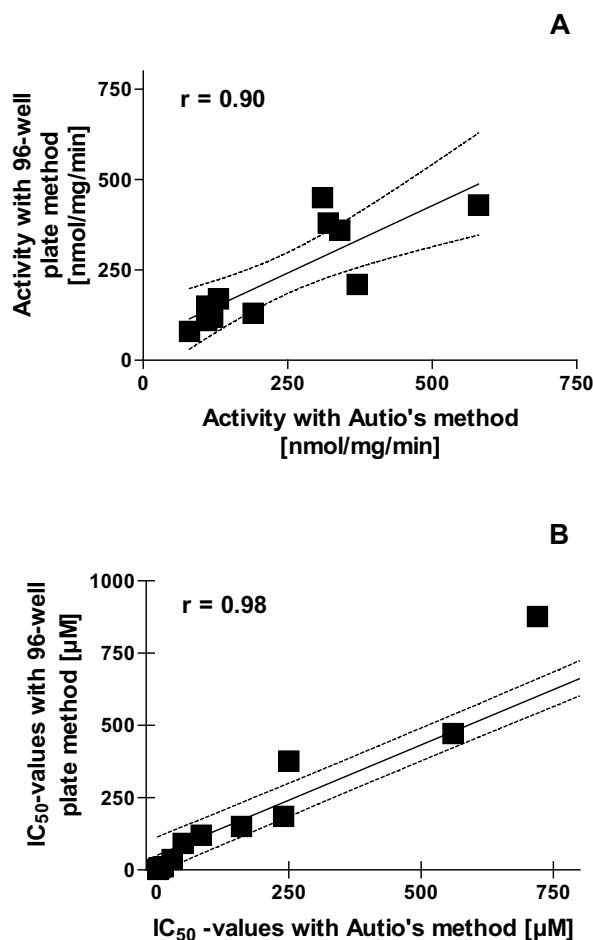


Figure 2. Comparison of the original Aitio method and the 96-well plate method using OLP regression analysis. (A) Coumarin 7-hydroxylation activity in various samples was measured using both methods. (B) Eight IC₅₀ values of CYP2A5 and CYP2A6 inhibitors were determined with the 96-well plate method and compared with published values obtained with the original Aitio method. Ordinary least product (OLP) analysis and the 95% confidence limits are shown.

Inhibition of human CYP2A6

The IC₅₀ values of 2-(*p*-tolyl)-ethylamine and 4-methoxybenzaldehyde were less than 10 μM for the CYP2A6 enzyme and the IC₅₀ value of the weakest inhibitor, amphetamine, was 320 μM (table 1). The type of inhibition caused by these amine and aldehyde derivatives was also studied. Only benzaldehyde appeared to be an irreversible inhibitor of CYP2A6 since the inhibition was dependent on both concentration and incubation time (figure 3). The K_{inact} value, i.e. the maximal rate of enzyme inactivation at saturated benzaldehyde concentration, was 0.16 min⁻¹. The K_I value, i.e. the concentration required for half-maximal enzyme inactivation, was 18 μM.

The inhibition kinetics of 2-(*p*-tolyl)-ethylamine, 2-phenylethylamine, 4-methoxybenzaldehyde and 4-methylbenzaldehyde were examined in greater detail. They all appeared to be competitive inhibitors. The lowest K_{ic} value was for 2-(*p*-tolyl)-ethylamine 0.23 μM and the highest 1.1 μM for 2-phenylethylamine (table 2).

Table 1. IC₅₀ values of inhibitors against CYP2A5 and CYP2A6. Human and mouse liver microsomal coumarin 7-hydroxylation using 10 µM coumarin was inhibited by increasing concentrations of indicated inhibitors. A CYP2A6/CYP2A5 inhibition ratio for each inhibitor was calculated by dividing the IC₅₀ for human coumarin 7-hydroxylation by the IC₅₀ for mouse coumarin 7-hydroxylation.

Inhibitor	IC ₅₀ (µM) (95% confidence limit)		CYP2A6/CYP2A5 inhibition ratio
	CYP2A5	CYP2A6	
2-Phenylethylamine	> 1000	39 (29–48)	< 0.039
Amphetamine	1400	320 (240–390)	0.22
2-(<i>p</i> -Tolyl)-ethylamine	100 (67–140)	4.9 (3.2–6.7)	0.048
Benzaldehyde	520 (380–690)	120 (83–160)	0.24
4-Methylbenzaldehyde	21 (11–30)	13 (6.3–19)	0.61
4-Methoxybenzaldehyde	5.8 (3.9–7.6)	7.1 (6.0–8.2)	1.22

Table 2. Competitive inhibition constant K_{ic} values of 2-phenylethylamine, 2-(*p*-tolyl)-ethylamine, 4-methylbenzaldehyde and 4-methoxybenzaldehyde. Coumarin 7-hydroxylation activity was measured at different coumarin and inhibitor concentrations and the results analysed by Hanes plots to obtain apparent (app) kinetic constants. In a secondary plot, K_m^{app}/V^{app} values were plotted against inhibitor concentration so that the intercepts of straight lines on the *x*-axis gave competitive inhibitor K_{ic} values.

Inhibitor	K_{ic} (µM)	
	CYP2A5	CYP2A6
2-Phenylethylamine	ND	1.1
2-(<i>p</i> -Tolyl)-ethylamine	ND	0.23
4-Methylbenzaldehyde	3.4	0.36
4-Methoxybenzaldehyde	0.86	0.73

ND, not determined.
Data are the results of two independent experiments.

Comparison of inhibition of mouse and human coumarin 7-hydroxylation

Only 4-methoxybenzaldehyde inhibited equally potently mouse CYP2A5 and human CYP2A6-mediated coumarin 7-hydroxylation (table 1). It was a competitive inhibitor for both enzymes. All the other compounds tested differed in their inhibition potency of CYP2A5 and CYP2A6 enzymes as they were more potent inhibitors of human CYP2A6 than mouse CYP2A5 (CYP2A6/CYP2A5 inhibition ratio < 1). The compounds showing the largest interspecies differences were 2-(*p*-tolyl)-ethylamine and 2-phenylethylamine.

Discussion

This study describes a rapid method to assay microsomal coumarin 7-hydroxylation activity in a 96-well plate format and an application of this method for

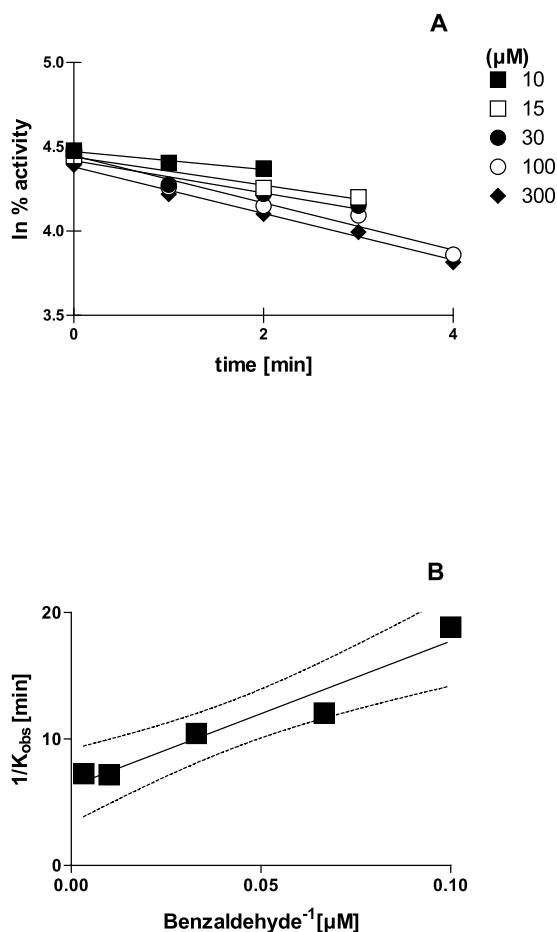


Figure 3. Mechanism-based inhibition of human CYP2A6 by benzaldehyde. (A) Time- and concentration-dependent inactivation of the enzyme activity. (B) Double-reciprocal plot of the relationship between inactivation rate and benzaldehyde concentration. $K_{\text{inact}} = 0.16 \text{ min}^{-1}$; $K_I = 18 \mu\text{M}$. Data are the results of two independent experiments.

screening and characterizing CYP2A inhibitors. Validation of the 96-well plate method showed that it yields identical results with the traditional and well-characterized methods based on manual fluorometry. The new method facilitates higher throughput analysis of chemicals interacting with CYP2A enzymes.

Mouse CYP2A5 and human CYP2A6 mediated coumarin 7-hydroxylation reactions were inhibited by three amine and three aldehyde compounds. The most important finding of this study was that 2-phenylethylamine, amphetamine, 2-(*p*-tolyl)-ethylamine, benzaldehyde and 4-methylbenzaldehyde were all more potent inhibitors of the human CYP2A6 than the mouse CYP2A5 enzyme. 2-(*p*-Tolyl)-ethylamine and 4-methylbenzaldehyde were the most potent CYP2A6 inhibitors. Interestingly, benzaldehyde was a mechanism-based inhibitor of CYP2A6.

This study confirms earlier studies showing that CYP2A5 and CYP2A6 enzymes exhibit distinct substrate and inhibitor specificities (Mäenpää *et al.* 1993, Juvonen *et al.* 2000, Poso *et al.* 2001). Amino acid homology between

CYP2A5 and CYP2A6 was 82% (Miles *et al.* 1990, Yamano *et al.* 1990). Site-directed mutational studies of CYP2A4 and CYP2A5 revealed that very few amino acid changes can account for the variations in substrate specificities among CYP2A enzymes (Lindberg and Negishi 1989, Lewis and Lake 2002). 2-(*p*-Tolyl)-ethylamine and 2-phenylethylamine showed the highest difference in the CYP2A6:CYP2A5 IC₅₀ ratio, which was 30 times or more. Most previously studied compounds, e.g. metyrapone, furanocoumarins, miconazole and lactone derivatives, inhibited CYP2A5 more potently than CYP2A6 (Wood 1979, Mäenpää *et al.* 1993, Juvonen *et al.* 2000). These studies showed that larger compounds inhibited CYP2A5 more potently than CYP2A6, suggesting that the active site of CYP2A6 is smaller (Poso *et al.* 2001). However, 2-phenylethylamine, amphetamine, 2-(*p*-tolyl)-ethylamine, benzaldehyde and 4-methylbenzaldehyde are not large compounds. Therefore, their size cannot be the most critical parameter explaining their difference in CYP2A5 and CYP2A6 inhibition potency. Rather, it is likely to be due to their chemical character and the different interactions at the active sites of these enzymes.

The three most potent CYP2A6 inhibitors, 2-(*p*-tolyl)-ethylamine, 4-methylbenzaldehyde and 4-methoxybenzaldehyde, in this study are not as potent as the most potent known inhibitors of CYP2A6. Methoxsalen, tranilcypromine, 4,4'-dipyridyl sulfide and 4,4'-dipyridyl disulfide are known to be 10 times more potent than these aldehydes (Draper *et al.* 1997, Fujita and Kamataki 2001, Taavitsainen *et al.* 2001). Tranilcypromine is relatively specific for CYP2A6 such that 10 times higher concentrations are required to inhibit the next most sensitive form, CYP2E1 (Taavitsainen *et al.* 2001). The specificities of the inhibitors in the current study need to be studied in more detail. Nevertheless, the current inhibitors provide leads for increasing inhibition potency by structural modification.

One important finding was that benzaldehyde was an irreversible inhibitor of CYP2A6. This contrasts with the more potent compounds 4-methylbenzaldehyde and 4-methoxybenzaldehyde, which were reversible inhibitors. This is probably due to the small structural differences between these compounds. The presence of a methyl or methoxy substitution at the 4-position in benzaldehyde changes this inhibition pattern. Although these compounds are inhibitors, they can potentially also be substrates of CYP2A6 as 4-methyl- and 4-methoxybenzaldehyde are competitive inhibitors and benzaldehyde is activated to a suicide inhibitor. Benzaldehyde and some other aldehydes are known to be irreversible inhibitors of CYP2B4 (Raner *et al.* 1997). CYP enzymes oxidize aldehydes to carboxylic acids via oxenoid-iron species or to $n - 1$ olefins and to alcohols via peroxo-iron species (Vaz *et al.* 1996, Raner *et al.* 1997, Coon *et al.* 1998). The pathway via the peroxo-iron species may also lead to inactivation of the CYP enzyme, but the pathway via oxenoid-iron does not inactivate the enzyme (Raner *et al.* 1997). Therefore, a 4-position substitution may lead to different interaction and the oxidizing pathways of 4-methyl- and 4-methoxybenzaldehyde may differ from benzaldehyde at the active site of CYP2A6. Benzaldehyde is smaller in size and its IC₅₀ value is greater than the IC₅₀ values of 4-methyl- and 4-methoxybenzaldehyde. Therefore, it is concluded that this difference in inhibition pattern is due to higher mobility of benzaldehyde compared with 4-methyl or 4-methoxybenzaldehyde at the active site and differences of oxygenation.

The findings of this study will help in identifying some of the important structural requirements to allow a compound to interact with CYP2A5 and CYP2A6 enzymes. First, the results indicate that the aldehyde group of benzaldehyde is oriented towards the haem iron of CYP2A6 since it is an irreversible inhibitor. 4-Methyl- and 4-methoxybenzaldehyde may also orientate similarly with their aldehyde groups facing the haem iron. Analogously, the amino groups of the ethylamine compounds in this study are probably also oriented towards haem iron. Further, as CYP2A5 and CYP2A6 are orthologous enzymes, a similar orientation may occur also in CYP2A5. Second, methyl or methoxy substitution in the *para*-position of benzaldehyde or 2-phenylethylamine increases the interaction since the IC₅₀ values are lower with these derivatives for both enzymes. Third, an amino group in position 1- of 2-phenylethylamine compared with position 2 strengthens the interaction with CYP2A6 as the IC₅₀ value of amphetamine is one order of magnitude higher than that of 2-phenylethylamine. However, this effect is not seen with CYP2A5 because the IC₅₀ values of amphetamine and 2-phenylethylamine are similar. Fourth, in the case of CYP2A6, the IC₅₀ value of 2-phenylethylamine is lower and in the case of CYP2A5 is higher than the corresponding IC₅₀ value of butylbenzene (Juvonen *et al.* 2000). This means that an ethylamine substitution in the benzene ring has an opposite effect on CYP2A5 and CYP2A6. In summary, it is concluded that amine groups in inhibitors have different effects on CYP2A5 and CYP2A6, and that a small 4-alkyl substitution of benzaldehyde or 2-phenylethylamine increases the potency of these compounds for both enzymes.

Tyndale and co-workers have reported that individuals carrying inactive CYP2A6 alleles are less likely to become smokers and if they do so, they smoke fewer cigarettes per day. Kinetic studies indicate that mimicking this genetic defect by inhibiting the CYP2A6 enzyme *in vivo* results in decreased smoking and makes nicotine orally bioavailable (Sellers *et al.* 2000, Tyndale and Sellers 2002). Thus, chemical inhibitors of CYP2A6 could be used as adjunct drugs in smoking cessation therapy. A major obstacle to this long-term goal is the lack of chemical inhibitors that are safe, potent and still selective enough to be used clinically.

In conclusion, novel chemical structures were identified showing preferential inhibition of the human CYP2A6 enzyme yielding initial structure-activity data. The most potent of these compounds could be used as lead molecules for developing potent, selective and safe CYP2A6 inhibitors for clinical use.

Acknowledgements

The authors thank Ms Virpi Koponen for excellent technical help and Dr Ewen MacDonald for comments on the manuscript. Some liver samples were provided by Dr Päivi Taavitsainen (Department of Pharmacology and Toxicology, University of Oulu, Oulu, Finland). The study was partially financed by Perkin Elmer Life Sciences.

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Pharmacokinetics and disposition of a novel NMDA glycine site antagonist (UK-240,455) in rats, dogs and man

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Received December 2002

1. UK-240,455 ((+) 6,7-dichloro-5-{*N*-(2-hydroxyethyl)methanesulphonamido}-2,3 (1*H*,4*H*)-quinoxalinedione) is a potent, selective *N*-methyl D-aspartate (NMDA) glycine site antagonist that is being evaluated for the potential treatment of stroke.

2. UK-240,455 is predominately excreted unchanged in urine (58–68%) in rats, dogs and man following intravenous administration. The remainder of the dose is excreted unchanged in the faeces. It is considered that UK-240,455 is predominantly cleared by active renal tubular secretion and active hepatobiliary transport. In man, there is evidence that UK-240,455 undergoes glucuronidation. However, there is no evidence for this in rats and dogs.

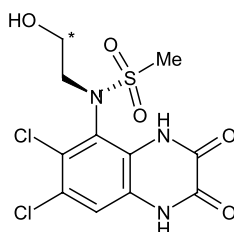
3. UK-240,455 has a short elimination half-life in rats, dogs and man (0.4–1.4 h) and clearances of 12, 13 and 6 ml min⁻¹ kg⁻¹, respectively. The compound shows limited tissue distribution with volumes of distribution of 0.4–0.8 l kg⁻¹ in rats, dogs and man. The species' variation in pharmacokinetic parameters was related allometrically when plasma protein binding was taken in to account. Hence, active hepatic or renal clearance processes for this compound were conserved across species.

4. Cerebrospinal fluid and brain concentrations of UK-240,455 were determined in rats. The cerebrospinal fluid/plasma concentration ratio of UK-240,455 was 4.3%, which was similar to the plasma-free fraction of this compound (3%), indicating good blood–brain barrier permeability. Brain tissue concentrations were low (0.7% of the total plasma concentrations).

Introduction

UK-240,455 ((+) 6,7-dichloro-5-{*N*-(2-hydroxyethyl)methanesulphonamido}-2,3 (1*H*,4*H*)-quinoxalinedione) (figure 1) is a representative of a series of novel potent, selective, competitive and reversible *N*-methyl D-aspartate (NMDA) glycine site antagonists. NMDA glycine site antagonists have been proposed as a potential therapy for stroke and head trauma. In stroke, NMDA glycine site antagonists act in the penumbra of the infarct to limit excitotoxicity (Bordi *et al.* 2000). An NMDA glycine site antagonist would be required to cross the blood–brain barrier (BBB) efficiently in order to penetrate to the site of action rapidly, the central nervous system (CNS). Rapid BBB penetration would allow efficacious concentrations to be readily achieved in the extracellular fluid (ECF) of the brain, the biophase from which this class of compounds is believed to act. Efficient BBB penetration is also important as the time window for clinical intervention with NMDA glycine site antagonists may be narrow. Animal data

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* position of ^{14}C radiolabel.

Figure 1. Structure of UK-240,455. *Position of ^{14}C radiolabel.

in models of focal or excitotoxic lesions have suggested that the maximum time window for effective intervention may be as low as 1–2 h (Schulz *et al.* 1995). However, others have indicated that the therapeutic time window may be up to 12 h (Cao and Phillis 1994). Slow BBB penetration of a compound would reduce the likelihood of achieving efficacious ECF concentrations if the time window for therapeutic intervention were narrow.

There are also concerns with respect to the potential therapeutic window of a potent NMDA glycine site antagonist in man as the NMDA receptor has been strongly implicated in cognitive processes (Smith *et al.* 1994). It was therefore decided to target an NMDA glycine site antagonist with a short half-life to allow for rapid removal of drug from the systemic circulation and rapid remediation of potential side-effects. Such a compound would have to be administered as a constant rate intravenous (i.v.) infusion to maintain the plasma concentration at efficacious levels for the treatment period.

This paper describes the pharmacokinetics, distribution, metabolism and excretion of UK-240,455 following single i.v., oral and infusion administration to rats and dogs. It also describes the i.v. pharmacokinetics in man and the allometric relationship between the animal and human pharmacokinetics.

Materials and methods

Preparation of radioactive UK-240,455

[Ethanol-1- ^{14}C]-UK-240,455 was prepared by Amersham International (Amersham, UK) with a nominal specific activity of $43 \mu\text{Ci mg}^{-1}$. The location of the ^{14}C radiolabel is shown in figure 1. The radiolabel preparation had a radiochemical purity of $>99\%$.

Log $D_{7.4}$ octanol determination

The log $D_{7.4}$ octanol value of UK-240,455 was determined using the methods developed by Stopher and McClean (1990). UK-240,455 (1 mg) was dissolved in 5 ml of 0.1 M NaH_2PO_4 buffer (pH 7.4). An equal volume of octanol (previously saturated with buffer) was added to this solution. The phases were then mixed for 1 h, separated by centrifugation and a sample of each phase analysed by direct injection onto the following hplc system. UK-240,455 was chromatographed using a BDS C18 hplc column (4.6×250 mm, Fischer Scientific, UK) and a mobile phase consisting of 0.1 M ammonium acetate, pH 4.0 (60%)/methanol (40%). The flow rate was 1.5 ml min^{-1} and UK-240,455 was detected by its ultraviolet (UV) adsorption (wavelength = 330 nm). UK-240,455 had a retention time of approximately 5 min.

pK_a determination

The ionization constant of UK-240,455 was determined by potentiometric titration in water/methanol with the GlpKa apparatus of Sirius Analytical Instruments (Forrest Row, UK). All titrations were conducted under argon to exclude atmospheric CO₂ and at 25 °C. Six separate semi-aqueous solutions in 19–36% (w/w) methanol of 0.5–0.75 mM UK-240,455 and 0.15% KCl were acidified with 0.1 M HCl to pH 2. The solutions were then titrated with standardized KOH to pH 12. Bjerrum difference plots were deduced from each titration curve and used to calculate approximate values of the apparent ionization constant in the mixed solvent. These approximate values were used in a weighted non-linear least-squares procedure to refine the *pK_a* values by including previously determined values of citric acid as unrefined constituents. The refined values were extrapolated to zero co-solvent by the Yasuda-Shedlovsky procedure (Gorby *et al.* 2000).

Determination of plasma protein binding in rat, dog and man

UK-240,455 plasma protein binding values were determined in pooled male rat (250–300 g Sprague-Dawley; Charles River, UK), male beagle dog plasma (10–15 kg, *n* = 3 different animals) and human plasma (adult, 50–100 kg, *n* = 3 donors) by equilibrium dialysis using a semi-micro Teflon dialysis cell (Spectrum Medical Industries, Inc., USA). Cellulose dialysis membranes with a molecular weight cut-off of 4000–6000 kD (Spectrum Laboratories, Inc., USA) were used.

UK-240,455 was added to plasma at nominal concentrations of 2 µM. This plasma (1 ml/cell) was added to one side of the dialysis apparatus and dialysed against Krebs buffer (pH 7.4, 1 ml). The dialysis cells were slowly rotated (20 rpm) at room temperature for at least 12 h. After this period of dialysis, plasma and buffer samples (0.5 ml) were removed from the apparatus and diluted with the opposite phase (0.5 ml of either plasma or buffer). These samples were analysed using the appropriate assay methodology detailed for plasma. Buffer containing 2 µM UK-240,455 was also dialysed against unspiked buffer to ensure that equilibrium had occurred during the course of the experiment. Concentrations in both buffer samples following dialysis had to be essentially equal for the dialysis experiment to be considered valid. Plasma protein binding values were determined using the following equation:

$$\text{Plasma protein binding (\%)} = 100 - \left(\frac{\text{buffer concentration}}{\text{plasma concentration}} \times 100 \right) \quad (1)$$

Caco-2 studies

Caco-2 cells (American Type Culture Collection, USA) were seeded in 24-well Falcon Multiwell[®] plates at 4.0×10^4 cells/well. The cells were grown in culture medium consisting of minimum essential medium (Gibco 21090-022, obtained from Invitrogen Life Technologies, UK) supplemented with 20% foetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine and 2 mM sodium pyruvate. The culture medium was replaced three times every week and the cells were maintained at 37 °C, with 5% CO₂ and at 90% relative humidity. Permeability studies were conducted when the monolayers were between 15 and 18 days old. Cells were used between passages 23 and 40. UK-240,455 was prepared as a 25 µM solutions in transport buffer (Hank's balanced salt solution, HBSS) at pH 7.4. Before commencing the study, each monolayer was washed three times with HBSS. Control transport buffer was placed in each acceptor well, 250 µl on the apical side and 1 ml into the basolateral well. The study was commenced by adding UK-240,455 solution to each donor well, 250 µl to the apical wells and 1 ml to the basolateral well. A Tecan Genesis robot was used for all pipetting steps. Following a 2-h incubation at 37 °C, samples were removed from all wells and mixed with acetonitrile (final concentration 20% v/v) before analysis. Membrane integrity was determined following the 2-h incubation using Lucifer yellow added to the apical wells at a concentration of 100 µM (Irvine *et al.* 1999). The concentration of UK-240,455 in each sample was analysed by direct injection of HBSS onto a desalting column switching chromatography system with a mass spectrometry (MS/MS) end-point. The chromatography column used was Hypersil HS C18 (50 × 4.6 mm Thermo Hypersil Runcorn, UK). The HBSS was directly injected onto the column and the salts were then washed to waste with an aqueous mobile phase consisting of 90:10 water:methanol with added 0.027% formic acid and 2 mM ammonium acetate. The mobile phase was then switched to 90:10 methanol:water, again with added 0.027% formic acid and 2 mM ammonium acetate. Flow rate was constant at 2 ml min⁻¹. UK-240,455 was detected by a Sciex API 2000 mass spectrometer with an ionspray interface using specific MS/MS transitions. The amount measured in each sample was determined from a calibration line constructed from known standards. Apparent permeabilities ($P_{\text{app}} \times 10^{-6}$ cm⁻¹) were calculated using equation (2):

$$P_{\text{app}} = (\delta Q / \delta t) \times (1 / C_o) \times (1 / A) \quad (2)$$

where δQ is the amount of compound (nmol) in the acceptor well at the end of the experiment, δt is the duration (s) of the experiment, C_o is the initial concentration (nmol ml⁻¹) and A is the surface area (cm²) of each well.

Animal experimentation

All studies involving animals were carried out in accordance with the requirements of UK national legislation and conducted under necessary authorities. At all stages, consideration was given to experiment refinement, reduction in animal numbers and replacement with *in vitro* techniques.

Intravenous and oral pharmacokinetics in the rat

UK-240,455 was administered both i.v. and orally at a dose of 2 mg kg^{-1} to male rats (Sprague-Dawley). Before and during the i.v. and oral studies, animals had free access to food (rat diet pellets) and water. For i.v. studies, UK-240,455 was dissolved at a concentration of 2 mg ml^{-1} in sterile saline and administered to rats (250–300 g) via the tail vein. At the following time points ($n=3/\text{time point}$) blood samples were taken under terminal anaesthesia: predose, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 4.0 and 7.0 h post-dose. Blood samples were collected into heparinized tubes and centrifuged to separate plasma. The plasma was removed and stored frozen.

Three further rats were dosed with UK-240,455 as above and placed in metabolism cages to collect urine. After 24 h, these animals were killed and the urine collected and stored frozen.

For oral studies, UK-240,455 was dissolved in water (2 mg ml^{-1}) and administered by gavage ($n=3$ rats/time point). Blood samples were collected at the following time points and the plasma stored as above: predose, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0 and 11 h.

Evaluation of CNS and cerebrospinal fluid (CSF) concentrations in the rat following infusion administration

Male Sprague-Dawley rats (about 300 g, Charles River) were prepared with jugular vein catheters and UK-240,455 was dissolved in sterile saline at a concentration of 2.25 mg ml^{-1} . The loading bolus was administered over 5 min by infusion via the jugular vein catheter at a rate sufficient to achieve a dose of 2.5 mg kg^{-1} in the individual animal. Maintenance infusions were carried out again by tailoring the infusion rate for the individual animal. The target maintenance infusion rate was $4.5 \text{ mg kg}^{-1} \text{ h}^{-1}$.

At the following time points post-dose, blood samples were collected under terminal anaesthesia and plasma stored frozen at 0.25, 0.5, 1, 3, 6, 12, 24, 48, 96 and 168 h (all time points were $n=1$, except for 168 h when $n=3$ rats were evaluated). CSF samples were collected by cisterna magna puncture and the brains removed. Brains were washed with saline to remove excess blood and homogenized in water (33% homogenate). Brain homogenate and CSF samples were stored frozen before analysis.

Biliary excretion in the rat

Three male Sprague-Dawley rats (300–350 g, Charles River) were anaesthetized, the bile duct located and cannulated. Anaesthesia was maintained for the duration of the experiment and animals were placed on a temperature-controlled blanket to maintain body temperature at 37°C . A total UK-240,455 dose of 1 mg (0.5 ml of a 2 mg ml^{-1} solution in saline) was administered via the tail vein. Bile was collected for 2 h following administration of the dose. At the end of this period, the animals were killed. Bile samples were stored frozen.

Whole-body autoradiography (WBA) in the rat (including analytical methodologies)

Four male Lister Hooded rats (about 200 g, Charles River) were weighed and dosed with a 10 mg ml^{-1} solution (in saline) of [^{14}C]-UK-240,455 via the tail vein (1 ml kg^{-1} , about $100 \mu\text{Ci}/\text{animal}$). The animals were then housed in plastic metabolism cages with free access to food (rat diet pellets) and water. At 0.1, 1, 24 and 48 h post-dose, the rats were killed and frozen rapidly by immersion in a bath of hexane and solid carbon dioxide. The carcasses were stored frozen at -20°C before analysis. For analysis, the frozen carcasses were mounted on the stage of a cryomicrotome (PMV, Sweden) maintained at -25°C in a cell of carboxymethyl cellulose. Sagittal sections $25 \mu\text{m}$ thick were cut and lifted onto pressure-sensitive tape (Taab, UK). The sections were freeze-dried at -25°C in the cryomicrotome cabinet for 24 h. The samples were then sprayed lightly with PTFE and apposed to a photostimulable phosphor plate for 8 days. The stored image of radioactivity in the tissues was converted to a computer image using a phosphorimager (Molecular Dynamics, CA, USA). The concentration of drug-related material in various tissues was determined by reference to calibrated radioactivity polymer standards (A. R. C., Inc., USA), which were also apposed to each phosphor plate at the same time as the tissue section.

Disposition studies in the rat (including analytical methodologies)

[^{14}C]-UK-240,255 and unlabelled UK-240,455 were dissolved in saline to achieve a nominal concentration of 2 mg ml^{-1} . This dose was administered to three male and three female Sprague-Dawley rats (250–300 g, Charles River) via the tail vein at dose volumes equivalent to 1 ml kg^{-1} . Each animal received a nominal radioactive dose of $20 \mu\text{Ci}$. Following dosing, the rats were housed individually in plastic metabolism cages. The total output of urine and faeces was collected and

weighed daily for 6 days post-dose. Urine was stored frozen at -20°C before analysis. Faeces was macerated with an equal weight of water and stored frozen (-20°C) before analysis. Six days following dosing, the rats were killed and the carcasses stored frozen. Before analysis, the carcasses were dissolved in 500 ml of a mixture of 3 M aqueous sodium hydroxide, methanol and Triton X-100 (20:10:3 v/v) overnight at 60°C .

The radioactivity in urine (1 g) was measured by liquid scintillation counting of duplicate weighed samples in 4 ml of Starscint (Packard, USA). The radioactivity in faecal homogenates (0.5 g) was measured by combustion of triplicate weighed samples in an Oximate Sample Oxidizer (Packard, USA) and liquid scintillation counting of the evolved $^{14}\text{CO}_2$ in 10 ml permafluor E+ (Packard, USA). The radioactivity in the digested carcass was measured by liquid scintillation counting of triplicate weighed samples (1 g) in Hionic-fluor (Packard).

Excretion of UK-240,455 and metabolites was evaluated by radio hplc of urine and extracts of faeces. Urine samples (0–24 h pooled from all animals) were cleared of suspended solids by centrifugation (5 min at 2000 rpm). Samples (200 μl) were injected onto the hplc. Samples (1 g) of faeces (0–72 h pooled from all animals) were mixed with 2 ml methanol and 0.25 ml 0.5 M aqueous sodium carbonate, then centrifuged for 10 min at 2000 rpm. The supernatant was removed and evaporated under nitrogen at 37°C , reconstituted in 1 ml mobile phase and 250- μl samples were injected onto the hplc. For purposes of comparison, hplc was also performed on samples of control urine and extracts of control faeces to which [^{14}C]-UK-240,455 had been added.

The hplc consisted of a Spherisorb 5 μ ODS-2 column (250 $\times$ 4.5 mm i.d.; Hichrom Ltd, UK) and an isocratic mobile phase consisting of water (85%)/acetonitrile (15%) containing 0.1% trifluoroacetic acid. The mobile phase flow rate was 1.5 ml min $^{-1}$. On-line radiodetection was used to evaluate the levels of radioactivity in the eluent (RAMONA, Isomess GMBH, with data analysis on LAURA software, Lablogic, UK).

Chromatographic eluate corresponding to the radioactive components were collected manually and evaporated under nitrogen at 37°C . The residues were reconstituted in mobile phase and diluted 1:1 with methanol/water containing ammonium acetate (2 mM) for mass spectrometric analysis. Samples were infused directly into the mass spectrometer (Sciex API III plus, using positive IonSpray), single stage mass analysis (Q1), followed by product, and precursor ion scans were performed.

Intravenous and oral pharmacokinetics in dog

UK-240,455 for i.v. administration was formulated in sterile saline. Two male and two female beagles dogs were selected from the Pfizer colony (weight 12–16 kg). Before the study, the animal were given food (dog diet pellets) and water *ad libitum*. Just before i.v. administration of UK-240,455, the animals were restrained in slings. The left and right saphenous veins were cannulated. The right saphenous vein was used for administration of the dose, whilst the left was used for blood sampling. Intravenous administration of UK-240,455 was carried out over 15 min at 30 ml h $^{-1}$. Each dose was tailored so that a 1 mg kg $^{-1}$ dose was administered in this 15-min infusion period. Blood samples (3 ml) were taken from the left saphenous vein, using the indwelling catheter, for the first 5 h; later samples were taken by vena puncture (time points predose, end of infusion, 0.1, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5 and 7 h after the end of the infusion). Blood samples were collected in heparinized tubes and plasma was prepared by centrifugation. Plasma was stored frozen before analysis. After the 5-h time point, the dosing and sampling cannula were removed and the dogs either returned to pens (male) or placed in metabolism cages for urine collection (females). Urine was collected for 24 h post-dose and stored frozen.

Oral doses of UK-240,455 were administered to the same dogs 2 weeks later by gavage (1 mg kg $^{-1}$ solution in water). The animals were placed in slings for the first 5 h of the study and blood samples taken at the following time points post-dose: predose, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 7 and 11 h post-dose. Plasma samples were prepared and stored as above. After 5 h, the dogs were returned to their pens.

When not in slings, the dogs had free access to food (at regular feeding times) and water throughout the study.

Disposition studies in dog (including analytical methodologies)

Two adult dogs (beagle strain from the Pfizer colony; one male and one female; 10–15 kg) were weighed then acclimatized (before dosing) for 1 day in individual metabolism cages. For dosing and to allow blood collection, the dogs were kept in slings for the first 6 h. Water and food (dog diet pellets, 400 g daily) were restored when the dogs were placed in metabolism cages.

Individual doses were infused into the saphenous vein at a rate (about 30 ml h $^{-1}$) that delivered the correct dose per body weight in 15 min. For i.v. administration, [^{14}C]-UK-240,455 and unlabelled UK-240,455 were dissolved in 25 ml sterile saline to give a concentration of 4 mg ml $^{-1}$. Dose volumes, equivalent to 0.5 ml kg $^{-1}$ bodyweight, were administered from weighed syringes to dogs. A total dose of 2 mg kg $^{-1}$ was administered to the dogs containing approximately 85 μCi of radioactivity.

The total output of urine and faeces was weighed daily for 6 days after dosing. Urine was stored frozen before analysis. Daily faecal output was macerated with water in a ratio of 4:1 with 1 g chlorhexidine to suppress bacterial activity, and stored frozen before analysis.

Blood samples (about 4 ml) were taken into heparinized tubes for preparation of plasma at specified times after dosing. Whole blood (1.5 ml) was retained and stored at about 4°C. The remaining samples were centrifuged, and plasma stored frozen until analysed.

The radioactivity in urine (about 1 g), plasma (about 0.2 g) and faecal homogenate (about 0.5 g) was measured by liquid scintillation as previously described for the rat. The radioactivity in whole blood (about 0.25 g) was measured by combustion of triplicate weighed samples in an Oximate Sample Oxidizer and liquid scintillation counting of the evolved $^{14}\text{CO}_2$ in 10 ml Permafluor E+. Excretions of drug and metabolites were evaluated using the same methodology previously described for the rat.

Administration to human volunteers

To determine the pharmacokinetics of UK-240,455 in humans, eight male volunteers (age range 18–45 years, weight 60–100 kg) participated in a randomized cross-over study conducted by Pfizer Central Research. The trial was reviewed and approved by the local ethics committee. The volunteers provided written informed consent and showed no evidence of any clinically significant diseases or abnormality following review of their laboratory data and full physical examination. UK-240,455 was administered i.v. (15 min infusion) at a dose of 2 mg kg⁻¹ to all volunteers and plasma samples prepared from blood collected at the following time points from the forearm: predose, 0.083, 0.17, 0.25, 0.42, 0.58, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 6, 8, 10 and 12 h. Urine samples were also collected up to 24 h post-dose.

Analysis of plasma, CNS homogenates, CSF, bile and urine samples from rats and dogs

UK-240,455 concentrations were determined in plasma, CNS homogenates, CSF, bile and urine using the following procedures.

UK-240,455 was separated from endogenous material using reverse-phase chromatography. It was chromatographed on a BDS C18 5 µ hplc column (250 × 4.6 mm, Fischer Scientific, UK) using a mobile phase consisting of 70% 0.1 M ammonium acetate, pH 4.0, and 30% methanol (flow rate 1.5 ml min⁻¹). UK-240,455 was detected by UV absorbance at 330 nm. It had a retention time of approximately 10 min. UK-224,085, a di bromo analogue of UK-240,455, was used as an internal standard in all assays and had a retention time of approximately 15 min.

UK-240,455 was extracted from 1 ml of rat and dog plasma using 3 ml 0.1 M disodium hydrogen phosphate buffer (pH 6.0) and ethyl acetate (5 ml). The samples were mixed by inversion for 5 min, centrifuged and the ethyl acetate removed. The ethyl acetate was evaporated to dryness under a stream of nitrogen, resuspended in 170 µl of mobile phase and 150 µl injected on to the hplc system. The assay had an analytical range of 20–1000 ng ml⁻¹. UK-240,455 was extracted from 1 ml of rat CNS homogenate and analysed using the same methodology as used for plasma. The assay had an analytical range of 40–2000 ng g⁻¹ of brain. Rat bile (50 µl) was analysed using the same methodology as used for plasma. The assay had a range of 2–40 µg ml⁻¹. UK-240,455 was extracted from 100 µl of rat and dog urine and analysed using the same methodology as used for plasma. The assay had a range of 1–20 µg ml⁻¹.

The CSF concentrations of UK-240,455 were determined by direct injection of 100 µl of rat CSF samples onto the above hplc system. The assay had an analytical range of 20–1000 ng ml⁻¹.

All assays were validated before use by analysis of replicate sample ($n = 5$) from the bottom, middle and top of the analytical range. An assay was considered to be valid if the cumulative variation of the assay at the bottom was <20% and at the middle and top was <15%. Quality control samples were analysed with all samples and the values had to be within 20, 15 and 15% of the expected values at the bottom, middle and top of the analytical range for the analysis to be deemed valid.

Analysis of human plasma samples

UK-240,455 concentrations were determined in plasma and urine using the following procedures. UK-240,455 was separated from endogenous material using reverse-phase chromatography. UK-240,455 was chromatographed on a Purospher 5 µ hplc column (125 × 3 mm, Merck BDH, UK) using a mobile phase consisting of 65% water, 35% acetonitrile and 0.1% trifluoroacetic acid (flow rate 0.5 ml min⁻¹). UK-240,455 was detected using fluorescence with excitation and emission wavelengths of 324 and 404 nm, respectively. UK-240,455 had a retention time of approximately 6 min.

UK-240,455 was extracted from 0.2 ml plasma by precipitation with 0.2 ml acetonitrile. Following precipitation, 0.4 ml water were added to the sample and the resultant mixture centrifuged to remove suspended precipitate. A 50-µl volume of the supernatant was injected on to the hplc system. The assay had an analytical range of 1–1000 ng ml⁻¹. A similar assay procedure was used for urine; however, the assay range was 0.2–20 µg ml⁻¹.

All assays were validated before use by analysis of replicate sample ($n = 7$) from the bottom, middle and top of the analytical range. An assay was considered to be valid if the cumulative variation of the

assay at the bottom was <20% and at the middle and top was <15%. Quality control samples were analysed with all samples and the values had to be within 20, 15 and 15% of the expected values at the bottom, middle and top of the analytical range.

Deconjugation of rat, dog and human plasma samples

Plasma (1 ml) from rat, dog and human pharmacokinetic studies was added to a vial containing 1000 units β -glucuronidase (Sigma G-5897-sulfatase free). This vial was incubated at 37 °C for 1 h. After this time, the mixture was extracted and quantified as described above.

Determination of the pharmacokinetic parameters

Standard algorithms were used to perform the pharmacokinetic analysis of the data. $C_{\max}$ and $T_{\max}$ values were obtained directly from the recorded data. Terminal elimination half-lives were calculated by log linear regression analysis. AUC_t values were calculated between time zero and the final time point at which measurable drug concentrations were observed using the linear trapezoidal rule. AUC_t values were extrapolated to infinity based on the terminal elimination rate constant. Values for the volume of distribution at steady-state were calculated using statistical moment analysis. Systemic clearances were calculated from AUC_{∞} values using the equation:

$$\text{Clearance} = \text{Dose}/AUC.$$

Relative bioavailabilities were calculated based on AUC values obtained by oral and i.v. routes.

Results

Physicochemical properties, plasma protein binding and membrane permeability

UK-240,455 is a weakly acidic (pK_a 7.9) and relatively polar molecule ($\log D_{7.4 \text{ octanol}} = 0.1$). Plasma protein binding values for UK-240,455 were determined in rat, dog and man by equilibrium dialysis and were 97, 85 and 87%, respectively. UK-240,455 shows poor flux across a Caco-2 monolayer with $P_{\text{app}} = 2 \times 10^{-6} \text{ cm}^{-1}$ in the apical to basolateral direction. In the basolateral to apical direction, $P_{\text{app}} = 3 \times 10^{-6} \text{ cm}^{-1}$, indicating that the compound may undergo active efflux in the Caco-2 permeability assay.

Pharmacokinetics of UK-240,455 in rats, dogs and man

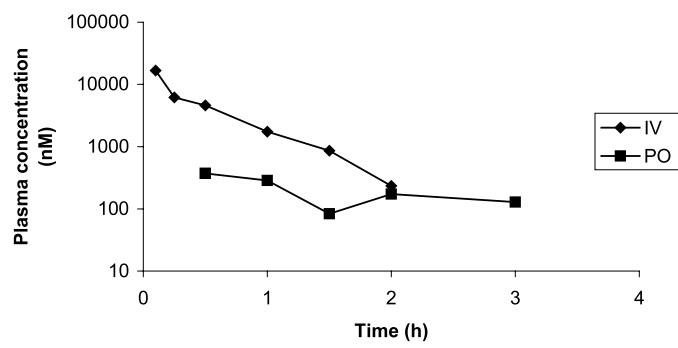
The mean plasma concentrations of UK-240,455 following single i.v. and oral administration to rats (male), dogs (male and female) and man are shown in figure 2. Table 1 summarizes the apparent pharmacokinetic parameters estimated for UK-240,455 in rats, dogs and man.

Following i.v. administration of UK-240,455 to male rats, the compound had a clearance of $12 \text{ ml min}^{-1} \text{ kg}^{-1}$ and a volume of distribution of 0.4 l kg^{-1} . The plasma concentration of UK-240,455 decreased with an apparent half-life of 0.4 h. Analysis of urine (0–24 h) for unchanged drug indicates that 57% of the dose administered is excreted unchanged in the urine. The urinary clearance of UK-240,455 in the rat is therefore $7 \text{ ml min}^{-1} \text{ kg}^{-1}$.

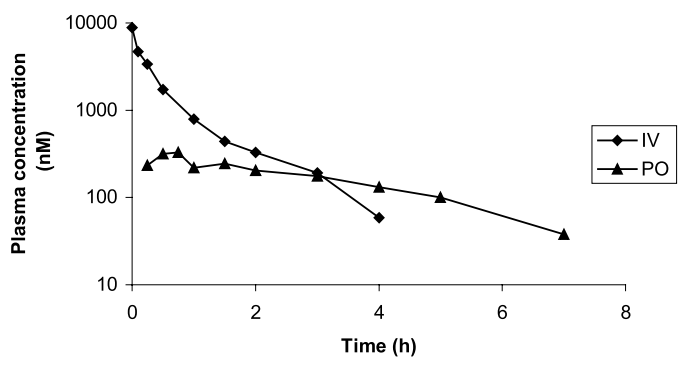
Studies carried out by i.v. infusion in both male and female rats (table 2) indicate that there is a sex difference in the clearance of UK-240,455 in the rat. Overall AUC_{1-24h} in female rats were approximately 55–70% of those seen for males.

Following oral administration of UK-240,255 to male rats (table 1), the apparent elimination half-life of UK-240,455 from plasma following oral administration was 1.6 h. This is four times greater than that seen following i.v. administration. The oral bioavailability in the male rat was 7%.

RAT



DOG



MAN

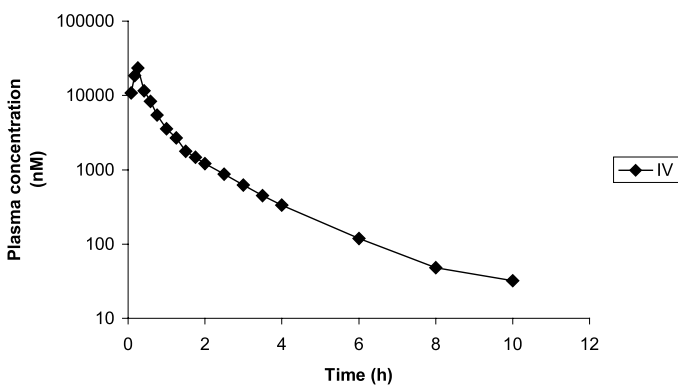


Figure 2. Mean plasma concentration profiles of UK-240,455 following i.v. and oral administration to rat (male, 2 mg kg^{-1} , $n = 3/\text{time point}$), dog (male/female $n = 2/\text{sex}$, 1 mg kg^{-1}) and man (i.v. only; $n = 8$ males; 2 mg kg^{-1}).

Table 1. Pharmacokinetics of UK-240,455 in rats (male), dogs (combined male and female) and man (males).

		Male rat (<i>n</i> = 3 rats/ time point)	Dog (male <i>n</i> = 2; female <i>n</i> = 2) Mean $\pm$ SD	Man (males <i>n</i> = 8) mean $\pm$ SD
Intravenous/oral dose (mg kg ⁻¹)		2.0	1.0	2.0
Intravenous administration	Elimination half-life (h)	0.4	1.1 $\pm$ 0.4	1.4 $\pm$ 0.2
	Volume of distribution (l kg ⁻¹)	0.4	0.8 $\pm$ 0.3	0.8 $\pm$ 0.1
	Systemic clearance (ml min kg ⁻¹)	12	13 $\pm$ 3	6 $\pm$ 1
	Percentage of dose excreted unchanged in urine*	57	41	61 $\pm$ 27
	Urinary clearance of unchanged drug (ml min ⁻¹ kg ⁻¹)	7	5	3–4
Oral administration	<i>C</i> _{max} (nM)	130–489	370 $\pm$ 131	–
	<i>T</i> _{max} (h)	0.5–1	0.5–2.0	–
	Apparent half-life (h)	1.6	5.3 $\pm$ 3.5	–
	Oral bioavailability (%)	7	31 $\pm$ 16	–

* Calculated using hplc analysis of urine.

Table 2. Plasma AUC_{1–24 h} (mean $\pm$ SD) of UK-240,455 in male and female rats following intravenous infusion administration for 24 h (*n* = 5 rats/sex/dose).

Infusion dose (mg kg ⁻¹ h ⁻¹)	Sex	AUC _{1–24 h} (μg h ⁻¹ ml ⁻¹) mean $\pm$ SD	Estimated clearance* (ml min ⁻¹ kg ⁻¹) mean $\pm$ SD
3	male	111 $\pm$ 19	10.8
	female	80 $\pm$ 25	15.0
9	male	343 $\pm$ 79	10.5
	female	243 $\pm$ 36	14.8
25	male	1087 $\pm$ 488	9.2
	female	611 $\pm$ 73	16.4

Clearance = dose/AUC_{1–24 h}.

In male and female beagle dogs there was no evidence of any sex differences in pharmacokinetics and therefore the data were combined (table 1 and figure 2). Following i.v. administration of UK-240,455 to dogs, the compound has mean clearance and volume of distribution of 13 $\pm$ 3 ml min⁻¹ kg⁻¹ (mean $\pm$ SD) and 0.8 $\pm$ 0.3 l kg⁻¹. In plasma, the concentration of UK-240,455 decreased with an apparent half-life of 1.1 $\pm$ 0.4 h. Analysis of urine (0–24 h) obtained from female beagle dogs for unchanged drug indicated that 41% of the dose administered was excreted unchanged by this route. These data indicate that the urinary clearance of unchanged UK-240,455 in dog is approximately 5 ml min⁻¹ kg⁻¹.

Following oral administration of UK-240,455 to dog, the mean bioavailability was 31 $\pm$ 16% and the apparent elimination half-life was 5.3 $\pm$ 3.5 h.

Following i.v. administration of UK-240,455 to man, the compound had a clearance and volume of distribution of 6 $\pm$ 1 ml min⁻¹ kg⁻¹ (mean $\pm$ SD) and 0.8 $\pm$ 0.1 l kg⁻¹. In plasma, the concentration of UK-240,455 decreased with a

Table 3. CNS/plasma and CSF/plasma ratios for UK-240,455 following continuous intravenous infusion to rats (loading bolus = 2.5 mg kg⁻¹, maintenance infusion 4.5 mg kg⁻¹ h⁻¹).

Time (h)	Plasma concentration (μM)	CNS/plasma ratio (%)	CSF/plasma ratio (%)
0.25 (n = 1)	34.0	0.5	8.5
0.5 (n = 1)	25.2	0.9	2.2
1 (n = 1)	15.9	0.8	2.6
3 (n = 1)	19.7	0.6	3.5
6 (n = 1)	22.5	0.5	4.7
12 (n = 1)	28.6	0.5	2.0
24 (n = 1)	34.2	0.7	3.2
48 (n = 1)	19.8	0.6	3.8
96 (n = 1)	10.9	0.9	7.7
168 (n = 3)	19.7 ± 2.7	0.7 ± 0.1	4.6 ± 0.28
Mean* (n = 12)	22.5 ± 7.0	0.7 ± 0.15	4.3 ± 2.1

* Mean (± SD) of all time points.

mean apparent half-life of 1.4 ± 0.2 h. Analysis of urine (0–24 h) obtained from man for unchanged drug indicates that $61 \pm 27\%$ of the dose administered was excreted unchanged by this route. These data indicate that the urinary clearance of unchanged UK-240,455 in man is approximately $3\text{--}4 \text{ ml min}^{-1} \text{ kg}^{-1}$.

Distribution of UK-240,455 into CNS and CSF in rats

The ability of UK-240,455 to cross the BBB and penetrate the CNS has been evaluated in the rat following infusion administration. The data obtained are summarized in table 3. UK-240,455 rapidly penetrates the BBB with concentrations in the CNS and CSF apparently in equilibrium at the first measured time point post-dose (0.25 h post-dose, respectively). The CNS:plasma concentration ratio (%) for UK-240,455 is low ($0.7 \pm 0.15\%$) and the CSF:plasma concentration ratio (%) for this compound is 4.3 ± 2.1 in the rat.

Circulating UK-240,455 glucuronide in rat, dog and human plasma samples

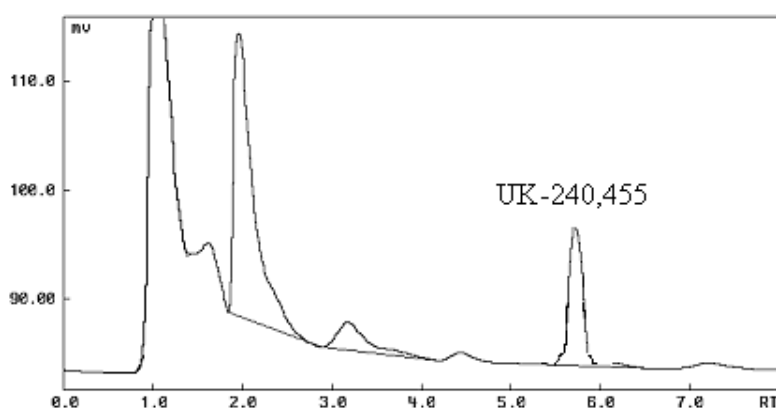
During the analysis of clinical samples from the human study, an unknown peak was detected in plasma (figure 3). A series of experiments were carried out using β -glucuronidase and using mass spectroscopic techniques that demonstrated that this metabolite was the glucuronide conjugate of UK-240,455. Neither MS-MS nor nuclear magnetic resonance analysis of the UK-240,455 glucuronide conjugate could define the site at which glucuronidation had occurred. Analysis of rat and dog plasma samples on the same hplc system as used for the human plasma samples showed that this metabolite did not circulate in either species.

Rat WBA

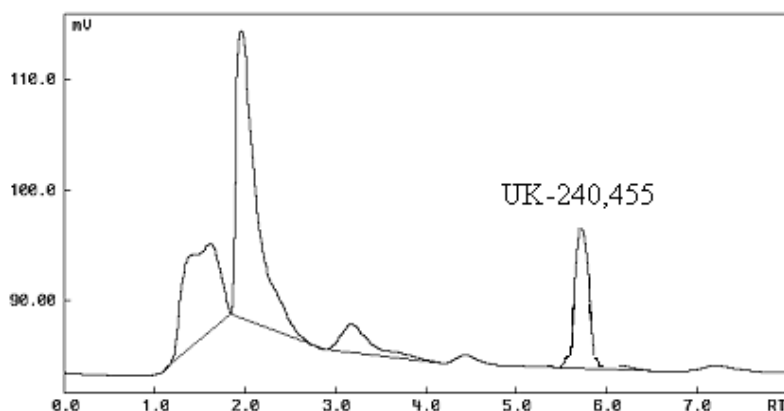
The tissue radioactivity levels of [¹⁴C]-UK-240,455 following i.v. administration (10 mg kg^{-1}) in pigment male rats are shown in table 4.

Shortly after dosing (0.1 h), radioactivity was present in most tissues. However, these tissue concentrations were approximately half those in blood ($21 \mu\text{g eq. g}^{-1}$). The lowest concentrations of UK-240,455 were in brain ($0.4 \mu\text{g eq. g}^{-1}$, about 2%

RAT



DOG



MAN

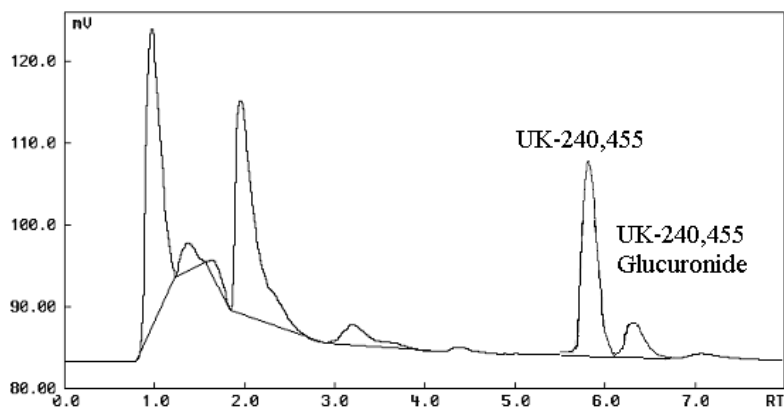


Figure 3. Example chromatograms of rat, dog and human plasma detailing the presence/absence of the glucuronide conjugate of UK-240,455.

of those in blood) and testis ($0.9 \mu\text{g eq. g}^{-1}$, about 4% of those in blood). High concentrations of radioactivity were seen in the contents of the small intestine, bile collecting ducts, kidney cortex, kidney medulla and urine. By 1 h post-dose, the concentrations of radioactivity in all tissues had fallen markedly. However, high

Table 4. Tissue concentration of [^{14}C] UK-240,455 in male pigmented rats following 10 mg kg $^{-1}$ intravenous administration.

Time after dose (h)	μg equivalents of UK-240,455 g $^{-1}$ tissue			
	0.1	1.0	24	48
Adipose tissue	3.1	0.28	blq	blq
Adrenal gland	12	1.3	blq	blq
Bile	147	22	not seen	not seen
Bone marrow	4.7	0.54	0.15	blq
Blood	21	2.8	blq	blq
Brain	0.4	0.11	blq	blq
Brown fat	9.4	1.0	blq	blq
Cardiac muscle	8.1	0.93	blq	blq
Retina	8.1	0.96	blq	blq
Aqueous humour	0.23	0.073	blq	blq
Vitreous humour	0.25	0.073	blq	blq
Testis	0.88	0.56	blq	not seen
Small intestine	172	88	0.14	blq
Large intestine	0.11	5.9	2.1	0.30
Harderian gland	6.7	0.51	blq	blq
Kidney cortex	72	6.8	blq	blq
Kidney medulla	58	6.6	blq	blq
Liver	45	13	0.14	blq
Lung	18	13	blq	blq
Naso-optic sinus	4.2	0.41	blq	blq
Pancreas	7.8	1.0	blq	blq
Preputial gland	8.0	1.2	blq	blq
Pineal gland	6.7	0.64	blq	blq
Pituitary gland	4.8	0.75	blq	blq
Prostate gland	3.5	0.63	blq	blq
Salivary gland	9.4	0.84	blq	blq
Seminal vesicle	0.87	0.14	blq	blq
Skeletal muscle	2.6	0.37	blq	blq
Skin epidermis	1.9	0.39	blq	blq
Skin dermis	7.5	1.3	blq	blq
Sebaceous gland	7.4	0.78	0.17	blq
Spleen	not seen	0.66	not seen	blq
Stomach content	0.25	0.18	blq	blq
Thymus gland	5.1	0.35	blq	blq
Thyroid gland	8.9	1.32	blq	blq
Urine	173	24	not seen	blq
Pancreatic ducts	148	11	not seen	not seen
Lymph	133	1.7	not seen	not seen

blq, Below the lower limit of quantification.

concentrations of radioactivity were still observed in the bile ducts, small intestine, kidney cortex, kidney medulla and urine. At time points ≥ 24 h, concentrations of radioactivity had fallen below the limit of detection in most tissues.

Urinary, biliary and faecal excretion of radioactivity in rat and dog

The urinary and faecal elimination of [^{14}C] UK-240,455 has been investigated in both male and female rats and dogs following i.v. administration. The data obtained in these studies are summarized in table 5.

Following i.v. administration to rats, the majority of radiolabel was eliminated in the urine (59–70%), the remainder of the dose being excreted in the faeces. The

Table 5. Percentage of the dose administered recovered in the urine and faeces of rats (2 mg kg⁻¹) and dogs (2 mg kg⁻¹) following intravenous administration of [¹⁴C] dose.

	Percentage of administered radioactivity			
	Rat (mean ± SD)		Dog	
	Male (n = 3)	Female (n = 3)	Male (n = 1)	Female (n = 1)
Urine				
0–12	52.6 ± 3.4	60.7 ± 13.8	54.8	57.1
12–24	4.7 ± 0.7	5.9 ± 2.6	3.0	0.1
Total excreted in urine (0–144 h)	59.5 ± 3.2	69.7 ± 8.7	60.1	58.3
Faeces				
0–12	15.0 ± 9.2	7.3 (n = 2)	0.1	0.1
12–24	19.0 ± 6.2	13.5 ± 9.8	10.8	n.s.
Total excreted in faeces (0–144 h)	35.2 ± 2.9	22.1 ± 4.5	28.6	23.4
Total in carcass	0.2 ± 0.1	0.2 ± 0.1	n.a.	n.a.
Total recovery (0–144 h)	94.9 ± 1.5	92.0 ± 4.3	88.7	81.7

blq, Below the limit of quantification; n.s., no sample; n.a., not applicable.

majority of the administered radioactivity was eliminated in the first 24 h post-dose. Comparison of the male and female rat excretion data indicates that there may be a sex difference in the routes of elimination with female rats showing a tendency to excrete more of the administered dose by the urinary route and less in the faeces.

In the dog, following i.v. administration the majority of the radiolabel is excreted in the urine (58–60%) with the remainder being excreted in the faeces, and again the majority of the administered dose is excreted in the first 24 h. In dog there is no evidence of any sex difference in the rates and routes of elimination of the radioactive dose.

The urine and faeces obtained from the above studies were analysed by hplc with radiochemical detection. Example radiochromatograms from these studies are shown in figure 4 and 5. The main drug-related component in the urine and faeces of rats and dogs is unchanged UK-240,455 and this accounts for the majority of the radioactivity excreted by these routes (> 95%). The identity of the UK-240,455 peak found in urine and faeces has been confirmed by co-chromatography and mass spectroscopy. In rats, a small proportion of the dose, approximately 3%, was eliminated as a metabolite in faeces. The identity of this metabolite is unknown (figure 4), but it is not the glucuronide conjugate of UK-240,455 previously seen in human plasma.

The biliary elimination of UK-240,455 has been evaluated following i.v. administration to male rats (1 mg, n = 3). The amount of compound in bile was evaluated by hplc determination of unlabelled drug. In rat, 23.2 ± 6.5% of the dose administered was excreted unchanged in the bile.

Discussion

Pharmacokinetics of UK-240,455

UK-240,455 is intended for constant rate infusion administration in man and was designed to have a short half-life to allow for rapid cessation of pharmacolo-

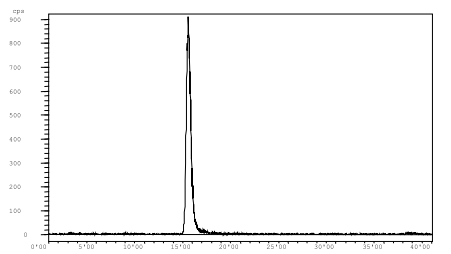
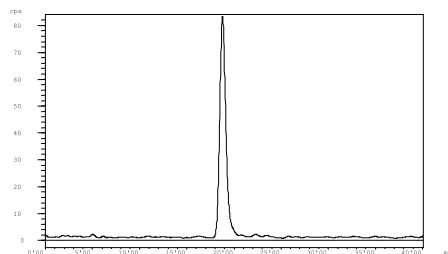
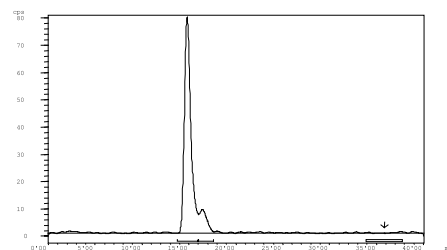
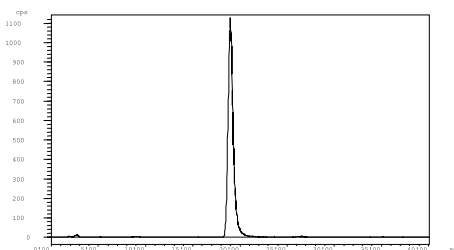
UK-240,455 standard spiked in urine**UK-240,455 standard spiked in faeces****Dosed Urine****Dosed Faeces**

Figure 4. Example radiochromatograms obtained in rat urine and faeces following i.v. administration of [^{14}C]-UK-240,455.

gical activity on termination of the maintenance infusion. The i.v. pharmacokinetics of UK-240,455 in rats, dogs and man are exemplified by a short half-life (0.4–1.4 h) primarily due to the relatively low volume of distribution of UK-240,455 in all species (0.4–0.8 l kg⁻¹). Overall, the volume of distribution of UK-240,455 in rat was lower than that seen in dog and man. This is believed to be due to the lower plasma-free fraction observed in this species (3 versus 13–15%). This lower free fraction restricts the distribution of UK-240,255 to tissues. This hypothesis is supported by an allometric relationship that exists for unbound volume of distribution in rat, dog and man (figure 6).

Clearances obtained for UK-240,455 are similar in rat and dog (12 and 13 ml min⁻¹ kg⁻¹, respectively) and higher than in man (6 ml min⁻¹ kg⁻¹). When plasma protein binding is taken into account, unbound clearances (clearance/fraction unbound) in rat, dog and man are 400, 87 and 46 ml min⁻¹ kg⁻¹. These values show an allometric relationship with bodyweight (figure 6) with an exponent of 0.62. Such an allometric relationship may be expected for a compound cleared largely by the renal route, where the rate will be related to organ blood flow and the value for the exponent is in keeping with the literature precedent (Boxenbaum 1982).

In dog, there is no evidence of any sex difference in pharmacokinetics. However, in rat, clearances in females are approximately 30–45% higher than those in males. The sex difference observed in the clearance of UK-240,455 is believed to be due to differences in active tubular secretion, the likely major

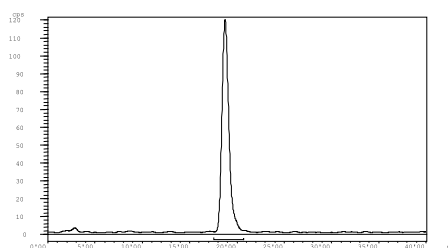
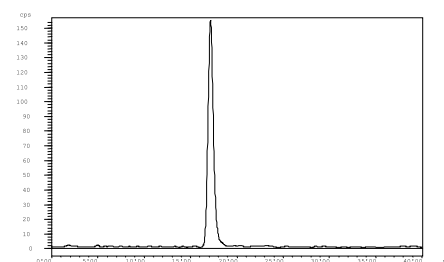
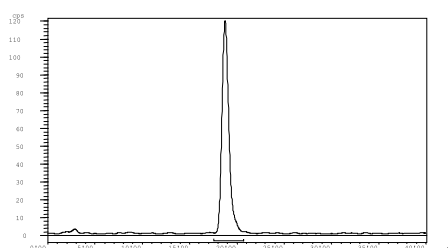
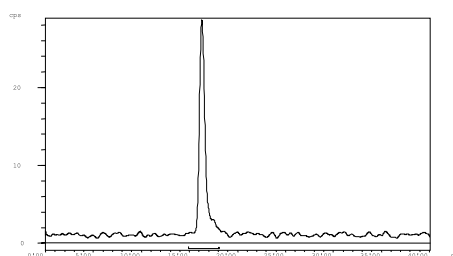
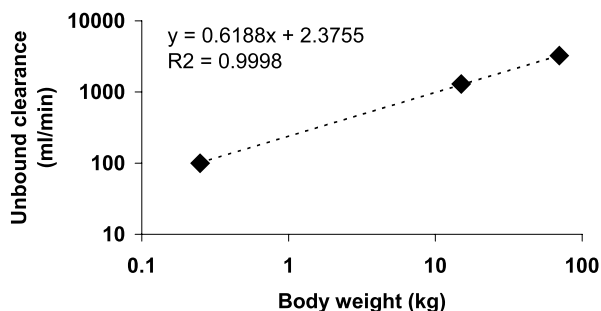
UK-240,455 standard spiked in urine**UK-240,455 standard spiked in faeces****Dosed Urine****Dosed Faeces**

Figure 5. Example radiochromatograms obtained in dog urine and faeces following i.v. administration of [^{14}C]-UK-240,455.

excretory route of this compound. This hypothesis is supported by the rat excretion balance data that indicate that the urinary excretion of UK-240,455 is greater in female than male rats. Zenerestat, an aldose reductase inhibitor (Tanaka *et al.* 1991) and an acidic metabolite of clentiazem (Hanada *et al.* 2001) show a similar sex difference in rat that is believed to be due to active tubular secretion. Evaluation of the acidic metabolite of clentiazem in rat (male and female) indicates that there is an active renal secretory mechanism (possibly involving the organic anion transport system) present in female rats that is either absent or relatively less active in males (Hanada *et al.* 2001). These data indicate that the sex difference seen in rat following i.v. administration of UK-240,455 is likely to be due to a difference in active tubular secretion by the kidney.

Following oral administration, the elimination half-lives in rat and dog are significantly longer than those seen following i.v. dosing (table 1 and figure 2). The increased half-life of UK-240,455 following oral administration indicates that absorption of the compound from the intestine is rate limiting and that the apparent elimination half-life of UK-240,455 following oral administration is a reflection of the absorption half-life. UK-240,455 is a relatively soluble compound (2 mg ml^{-1} in water). Therefore, it is unlikely that intestinal absorption of this compound is limited by the dissolution rate of UK-240,455 in the intestine. It is more likely that UK-240,455 poorly permeates across the intestine in both rats and dogs. This hypothesis is supported by data obtained in Caco-2 studies that indicate that this compound is poorly fluxed in the apical to basolateral direction

Unbound clearance



Volume of distribution

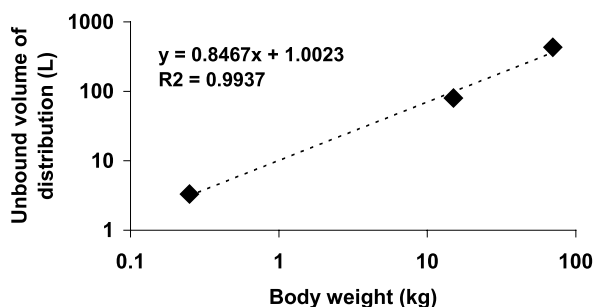


Figure 6. Allometric relationships observed for UK-240,455 (rat, dog and man).

($P_{\text{apps}} = 1 \times 10^{-6} \text{ cm}^{-1}$) across this colonic cell monolayer. It is also possible that the poor bioavailability is exacerbated by efflux transporters in the intestine. This hypothesis is supported by the increased permeability of UK-240,455 when permeability in Caco-2 cells is measured in the basolateral to apical direction ($P_{\text{apps}} = 3 \times 10^{-6} \text{ cm}^{-1}$).

Overall oral bioavailability of UK-240,455 in rats and dogs is lower than would be predicted given the low-to-moderate clearances of this compound (7 and $31 \pm 16\%$, respectively). Given the increase in apparent half-life in rat and dog and the extrapolation of this half-life to infinity, it is possible that these bioavailabilities may be an overestimate. However, these data are in keeping with the poor flux of this molecule seen in Caco-2 experiments. Oral bioavailabilities in dogs are higher than those in rats. These data may indicate that a portion of the UK-240,455 oral dose is absorbed by a paracellular route, particularly in dogs where the paracellular pores in the intestine are larger and more frequent than in rats (He *et al.* 1998). As rat is believed to be a better predictor of oral bioavailability in man than dog (Beaumont *et al.* 2000, van de Waterbeemd and Smith 2001), UK-240,455 is likely to have low oral bioavailability in man (limited by the poor intestinal permeability of the compound).

Clearance of UK-240,455

Following i.v. administration of [^{14}C]-UK-240,455 in rats and dogs, UK-240,455 is predominately excreted in urine as unchanged drug (58–68%). The

remainder of the dose is excreted in the faeces. Analysis of faeces data indicates that again the majority of the radioactivity excreted by this route was unchanged drug. In man, hplc quantification of unchanged UK-240,455 in urine indicates that urinary excretion is also a major route of clearance in man with 61% of the dose eliminated by this route.

UK-240,455 contains both a quinoxalinedione, which can tautomerize to yield a hydroxyl group, and an aliphatic hydroxyl group both of which can undergo glucuronidation and/or sulphation. It is possible that the unchanged compound seen in faeces of rat may result from a conjugate of UK-240,455 hydrolysed in the intestine by gut microflora. Bile duct cannulated rat experiments indicate that the majority of drug-related material in bile is unchanged UK-240,455. These data suggest that UK-240,455 is eliminated without metabolism into the intestine via the bile in the rat. In man, there is evidence of a glucuronide metabolite circulating in plasma. This metabolite was not detected in rat or dog plasma. These data indicate that in the preclinical species UK-240,455 is probably eliminated unchanged in bile by an active hepatic transport mechanism. The location (sinusoidal and/or cannicular) or identity of this putative transporter is unknown. In man, a proportion of the dose undergoes glucuronidation. However, it is likely that clearance via active hepatic transport will also occur in man. Further investigations are required to understand why the UK-240,455 glucuronide conjugate is only formed in man. Initial *in vitro* studies in rat, dog and human microsomes (fortified with uridine 5' diphosphoglucuronic acid) and hepatocytes did not provide evidence of the formation of this glucuronide. At present, it is not clear why this glucuronide conjugate is only seen to circulate in man, but it could be due to species differences in its formation by uridine 5' diphosphoglucuronyl transferase or in glucuronide degradation by β -glucuronidase.

The urinary clearance of UK-240,455 has been calculated in rats, dogs and man (table 1): 7, 5 and 3–4 ml min⁻¹ kg⁻¹, respectively. Correcting these values for plasma protein binding yields urinary clearances for unbound drug of 233, 33 and 23–31 ml min⁻¹ kg⁻¹ in rat, dog and man, respectively. Comparison of these unbound urinary clearances with glomerular filtration rates (GFR) in rat, dog and man (5.2, 6.1 and 1.8 ml min⁻¹ kg⁻¹, respectively; Davies and Morris (1993)) indicate that in all cases they are significantly larger. As only unbound drug can be cleared by glomerular filtration, these data indicate that an active mechanism is responsible for the clearance of UK-240,455 by the kidney. This is likely to be active renal tubular secretion. The transporter responsible for elimination of UK-240,455 into the urine is not known.

Examination of the major excretory organs in the WBA data generated in the rat confirm that both the kidney (radioactivity detected in the kidney and urine) and liver (radioactivity seen in liver, bile and intestines) have a role in the clearance of UK-240,455.

CNS and CSF penetration of UK-240,455

UK-240,455 will be required to access the brain, by rapidly penetrating the BBB, in order to achieve therapy in the target patient population. Therefore, accurate evaluation of this compound's brain penetration is essential in understanding the likely concentrations in the effect compartment and to predicting likely efficacious doses in animal and human studies. This is particularly important

for a molecule like UK-240,455, which has been shown to be relatively polar ($\log D_{7.4 \text{ octanol}} = 0.1$) and has poor permeation across a Caco-2 cell monolayer.

UK-240,455 is designed to act at the glycine site of the NMDA receptors. Therefore, the brain ECF concentrations of this compound would be the most relevant biophase to evaluate in order to understand the brain penetration of this molecule. However, the ECF compartment cannot be readily accessed although microdialysis has been used to obtain concentration data in this compartment (Hammarlund-Udenaes 2000). Obtaining quantitative data that accurately reflect ECF concentrations using this technique is time consuming and requires complex zero net flux techniques (de Lange *et al.* 1997). CSF concentrations are generally considered as representative of ECF concentration within the brain and CSF is readily accessible by needle puncture of the cisterna magna (van de Waterbeemd and Smith 2001). As such, CSF concentration data were used to profile the likely ECF concentrations of this compound in animals and man.

The CSF concentrations of UK-240,455 have been assessed in rat. The CSF:plasma concentration ratio was 4.3 ± 2.1 . Comparison of this values with that obtained for the free fraction of this compound in rat plasma (3%) shows that they are essentially the same and indicate that the free concentration in the plasma is equivalent to the CSF concentration. Unless the drug is entering the CSF by an active transport mechanism, the maximum CSF concentrations achievable are equal to the free concentrations in plasma (van de Waterbeemd and Smith 2001). These data indicate that despite the relatively polar nature of UK-240,455, the BBB penetration of this molecule is equal to the optimum that can be achieved for a compound that crosses the BBB by passive diffusion. Similar observations have been made with other polar compounds including atropine, tacrine and oxyphenbutazone, all with $\log D$ values in the region of zero and with essentially equal CSF and free plasma concentrations (van de Waterbeemd and Smith 2001).

The data obtained in these studies also demonstrate that UK-240,455 rapidly penetrates the BBB, as CSF concentrations are equal to the free concentration in plasma at the earliest time point measured in rats (0.25 h).

CNS concentrations were also evaluated during these studies and CNS:plasma concentration ratios were low ($0.7 \pm 0.15\%$). The CNS concentration data obtained by hplc analysis of rat brain homogenates are in keeping with the data obtained in rat WBA studies (1.9%) and indicates that UK-240,455 has only limited affinity for CNS tissue in keeping with its hydrophilic nature.

Analysis of the UK-240,455 brain penetration data indicate the importance of using either CSF data, or another measure of ECF concentrations to understand the brain penetration of a molecule. The CSF data obtained for this compound indicated that brain penetration of this molecule was maximal for a compound that crosses the BBB by passive diffusion. However, if only CNS concentration data were used to evaluate the ability of this molecule to penetrate the BBB, it is likely that this compound would not have been selected as these figures appear poor. A similar conclusion would be drawn if only WBA data were used to evaluate the CNS penetration of this compound. Analysis of the WBA data indicates that the brain has the lowest affinity of all tissues in the rat for UK-240,455 (1.9% of the concentrations in plasma). The affinity for CNS tissue, which is largely composed of lipid, is more likely to reflect lipophilicity and therefore cannot be considered as a measure of the free or efficacious concentrations of drug.

WBA data also show that the testes have low concentrations of UK-240,455. This tissue also has a vascular endothelial barrier similar to the BBB, and again it is possible that UK-240,455 readily penetrates this tissue but has a poor affinity for the tissue itself. These data illustrate a limitation of WBA in evaluating the tissue distribution of a compound as it can only detect total tissue drug concentrations but cannot separate concentrations in the effect compartment (e.g. ECF) from those bound non-productively to tissue components.

The observation that UK-240,455 rapidly penetrates the BBB achieving its maximum theoretical concentration in CSF (free concentration in plasma = concentration in ECF) within 0.25 h of administration is at odds with the observed oral absorption of this compound, which is poor (in rat it is estimated that <10% of the compound administered is absorbed from the intestinal tract) due to poor intestinal permeability. These data may suggest that the BBB or blood CSF barrier is more permeable than the barriers to absorption seen in the intestine. Alternatively, Caco-2 data indicate that this molecule may be a substrate for an active efflux transporter that reduces the apparent permeability of this compound across the cell monolayer. It is therefore possible that this difference in apparent permeability may be due to a difference in the type or number of efflux transporter expressed in the intestine or the BBB.

In conclusion, UK-240,455 has a short half-life in rat, dog and man, which indicates that following termination of infusion, concentrations of drug in plasma will fall rapidly. This is advantageous for a NMDA glycine site antagonist where the therapeutic ratio may be narrow, meaning that any side-effects mediated by this compound will potentially disappear rapidly when the infusion is discontinued. Data obtained in the rat indicate that this compound penetrates the BBB rapidly and effectively by passive diffusion. This is also an important feature for a compound designed to act rapidly from the ECF in the brain.

UK-240,455 is extensively cleared as unchanged drug, probably by carrier-mediated processes in the kidney and liver. There is evidence that in man this compound undergoes some glucuronidation. However, there is no evidence for this clearance mechanism in either rat or dog. This potentially human-specific metabolite could cause development issues for this compound as neither of the toxicology species (rat and dog) have been exposed to this circulating metabolite. In general, glucuronide metabolites are unlikely to be active and as such may not represent the same risk as metabolites formed by phase I enzyme systems, e.g. P450 (draft PhRMA position paper, Metabolites in Safety Testing).

Comparison of the pharmacokinetics of UK-240,455 in rats, dogs and man shows that an allometric relationship exists for clearance and volume of distribution when plasma protein binding differences are accounted for. This supports the view that allometry can be used to predict the pharmacokinetic of compounds cleared by active hepatic or renal process from animals to man and is in keeping with previous observations (Walker *et al.* 1999).

Acknowledgements

The authors gratefully acknowledge the assistance of Dr Alan Stobie and colleagues, Discovery Chemistry, Pfizer Global Research and Development, for supplying UK-240,455.

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Identification of coenzyme A-related tolmetin metabolites in rats: relationship with reactive drug metabolites

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Received 12 July 2002

1. It has recently been proposed that acyl coenzyme A thioesters (acyl-CoAs) of xenobiotic carboxylic acids are electrophilic, reactive metabolites that may react with proteins.

2. The primary objective was to investigate the reactivity of the tolmetin acyl coenzyme A thioester (Tol-CoA). The second objective was to identify and quantify tolmetin (Tol) metabolites *in vivo* that were formed via Tol-CoA, e.g. the glycine (Tol-Gly) and taurine (Tol-Tau) conjugates. This finding would be indicative of Tol-CoA formation and thus of other acyl-CoA-related reactions that might occur, e.g. covalent binding to proteins.

3. In order to study the chemical reactivity, Tol-CoA (0.5 mM) was incubated with glutathione (5 mM) in a 0.1 M phosphate buffer (pH 7.4) at 37°C. Tol-CoA reacted rapidly with glutathione *in vitro* to form the *S*-acyl glutathione conjugate at a rate of $14.9 \pm 0.7 \mu\text{M min}^{-1}$ (mean $\pm$ SD, $n = 3$) from 0 to 10 min. Compared with acyl-CoAs of other xenobiotic carboxylic acids, naproxen and clofibric acid, the rate by which Tol-CoA reacted with glutathione was high.

4. Following administration of ^3H -Tol (100 mg kg^{-1} , $200 \mu\text{Ci kg}^{-1}$, p.o.) to male Sprague–Dawley rats, Tol-Tau and Tol-Gly were identified in urine by electrospray ionization MS-MS in both positive- and negative-ion modes. The conjugates were only formed at trace levels ($< 0.5\%$). However, the presence of Tol-Tau and Tol-Gly showed the reactive Tol-CoA was formed *in vivo*.

Introduction

A major metabolic pathway of drugs that possess a carboxylic acid functional group is glucuronidation. In addition to hydrolysis and isomerization reactions (Iwaki *et al.* 1999), acyl glucuronides have been reported to be reactive metabolites that may react with proteins in plasma (Benet *et al.* 1993, Smith *et al.* 1986) and in the liver (Bailey and Dickinson 1996). The presence of these covalent protein adducts has been associated with serious hypersensitivity reactions (Zia-Amirhosseini *et al.* 1995) and hepatotoxicity (Zimmerman 1994).

In addition to acyl glucuronides, recent reports suggest that acyl coenzyme A thioesters (acyl-CoAs) also are reactive metabolites that may covalently modify endogenous proteins (Sallustio *et al.* 2000, Grillo and Benet 2002, Olsen *et al.* 2002). Recent studies on clofibric acid (Grillo and Benet 2002) and naproxen (Olsen *et al.* 2002) have shown that acyl-CoAs are electrophilic metabolites that are markedly more reactive than the respective acyl glucuronides in the reaction with the thiol of glutathione.

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Acyl-CoAs are formed by acyl-CoA synthetases, which are predominantly located in the liver and, to a lesser extent, in the kidney (Brugger *et al.* 2001). Since acyl-CoAs are not excreted from the cells, mechanistic studies on acyl-CoA derivatives therefore require analysis of tissue homogenates, which would be difficult to perform in humans. However, evaluation of acyl-CoA metabolism may be obtained by analysis of acyl-CoA-mediated metabolites in the urine and faeces. Taurine and glycine conjugation requires initial formation of the acyl-CoA precursor before *N*-acylation of the amino group, a reaction catalysed by an amino acid *N*-acyltransferase (Hutt and Caldwell 1990). Thus, excretion of glycine and taurine conjugates may be considered as indirect evidence for the drug to undergo acyl-CoA-dependent metabolism. Alternatively, identification of acyl-carnitine esters, as reported for trimethyl acetic acid (Melegh *et al.* 1990), can be employed to investigate intracellular acyl-CoA formation.

Tolmetin acyl glucuronide (Tol-GlcU) has been reported to acylate plasma proteins in humans (Hyneck *et al.* 1988, Zia-Amirhosseini *et al.* 1994). In the current study, the metabolism of tolmetin (Tol) was further explored with respect to reactive drug metabolites. First, the *in vitro* reactivity of the tolmetin acyl-CoA (Tol-CoA) towards glutathione was determined and compared with other acyl-CoAs. A second objective was to identify and quantify the tolmetin–glycine (Tol-Gly) and tolmetin–taurine conjugates (Tol-Tau) in rat, which would indirectly indicate if Tol-CoA was formed *in vivo* and thus could be indicative of other acyl-CoA-related reactions that might occur, e.g. covalent binding to proteins.

Materials and methods

Chemicals

Hionic Fluor and Soluene[®]-350 were purchased from Packard (Meriden, CT, USA). ³H-Tol (specific activity 80.8 µCi mmol⁻¹, 1590 Ci ml⁻¹ ethanol) was obtained from the Department of Isotope Chemistry, Novo Nordisk A/S. The position of the ³H label was in an unknown position of the benzene ring system. The radiochemical purity was >97%.

Unlabeled Tol (the sodium salt), glutathione, anhydrous tetrahydrofuran and coenzyme A (trilithium salt) were purchased from Sigma Chemical Co. (St Louis, MO, USA). *N,N*-carbonyldiimidazole was purchased from Fluka (Buchs, Switzerland). All other solvents were of analytical grade or HPLC grade.

Synthesis of Tol-CoA

The synthesis was essentially performed as described by Kawaguchi *et al.* (1981). Briefly, Tol was purchased as the sodium salt and converted to the respective carboxylic acid before synthesis. All steps in the synthesis were performed under a nitrogen atmosphere. Tol (0.09 mmol) was dissolved in 1 ml anhydrous tetrahydrofuran to which *N,N*-carbonyldiimidazole was added (0.20 mmol dissolved in 1 ml anhydrous tetrahydrofuran). After 30 min, formation of the imidazole intermediate was stopped by addition of 0.5 ml water, which hydrolysed the remaining *N,N*-carbonyldiimidazole. A total of 0.02 mmol CoA (trilithium salt) dissolved in 0.5 ml water was added to the imidazole intermediate and the mixture was stirred for 1 h at room temperature. Subsequently, the pH was adjusted to 2–3 and the synthesis mixture extracted with 2 × 2 ml heptane. The acyl-CoA (in the aqueous phase) was purified using preparative HPLC (Tracy and Hall 1991). Electrospray ionization (ESI) MS-MS of the purified product ([M + H]⁺ at *m/z* 1007) showed the following characteristic fragment ions: *m/z* 428 ([adenosine diphosphate + H]⁺, 27%), *m/z* 500 ([M—adenosine triphosphate + H]⁺, 100%), *m/z* 580 ([M—adenosine diphosphate + H]⁺, 57%) and *m/z* 598 ([580 + H₂O]⁺, 58%).

In vitro reactivity of Tol-CoA

Characterization of the reactivity of Tol-CoA was performed as suggested in the literature (Shore *et al.* 1995, Grillo and Benet 2002, Olsen *et al.* 2002). Briefly, 0.5 mM Tol-CoA was incubated with 5 mM glutathione in a 0.1 M phosphate buffer (pH 7.4) at 37°C. The initial rate of formation of the *S*-acyl-glutathione conjugate was used as a measure of the reactivity. HPLC analysis of the *S*-acyl-

glutathione conjugate was performed as described (Olsen *et al.* 2002) for the reaction of naproxen acyl-CoA with glutathione except that the UV detection wavelength was 320 nm. Identification of the *S*-acyl glutathione conjugate was performed using LC-MS-MS ($[M+H]^+$ at m/z 547), which resulted in the characteristic loss of m/z 129 ([pyroglutamyl moiety—H] $^-$, 100%) as reported (Grillo and Benet 2002, Olsen *et al.* 2002). Other fragment ions were produced at m/z 529 ($[M+H-H_2O]^+$, 22%), m/z 472 ($[M+H-glycine]^+$, 33%), m/z 411 ($[M+H-glycine-COOH-NH_2]^+$, 47%), m/z 400 ($[M+H-pyroglutamic\ acid]^+$, 25%), m/z 382 ($[M+H-pyroglutamic\ acid-H_2O]^+$, 41%), m/z 343 ($[tolmetin-S-CH_2(CO)CHNH_2]^+$, 23%) and m/z 240 ($[tolmetin+H-H_2O]^+$, 72%).

Animals

Three male Sprague-Dawley rats (200 g $\pm$ 20 g) were acclimatized for 2 days before dose administration. The rats had free access to water before and after dose administration. The animals were administered 100 mg kg $^{-1}$ ^3H -Tol (200 μ Ci kg $^{-1}$) p.o. by gavage (5 ml kg $^{-1}$) and were placed individually in metabolism cages, and urine samples were collected in 50-ml containers at 0–6, 6–24, 24–48 and 48–72 h post-dose. In each container, 300 μ l concentrated phosphoric acid (85%) was added in advance to prevent degradation of the acyl glucuronide. Faeces samples were collected at 0–24, 24–48 and 48–72 h. The animals were killed under carbon dioxide gas and the metabolism cages were washed with water followed by methanol. The samples were stored at -20°C until analysis.

Dose solutions were prepared in a 0.1 M potassium phosphate buffer (pH 7.4). A ^3H -Tol stock solution in ethanol was evaporated to dryness, redissolved in 0.1 M potassium phosphate buffer (pH 7.4) and mixed with a stock solution of non-radiolabelled Tol in 0.1 M potassium phosphate buffer (pH 7.4) to give a final concentration of radioactivity corresponding to 40 μ Ci ml $^{-1}$ and non-labelled Tol corresponding to 20 mg ml $^{-1}$.

Measurement of total radioactivity in urine, faeces and cage wash

Measurement of total radioactivity in urine, faeces and cage wash was performed in duplicate. Urine, faeces and cage wash were treated as described below.

Urine and cage wash. An aliquot of the sample (50–200 μ l urine, 1000 μ l cage wash) was weighed into a scintillation vial. A total of 3 ml (10 ml for cage wash) of scintillation cocktail (Hionic Fluor) were added and the radioactivity was measured by liquid scintillation counting on a TriCarb 2900 CA scintillation counter (Packard Instrument, Meriden, CT, USA).

Faeces. The faeces samples were homogenized in water (1:1, w/v) before analysis. A total of 20–60 mg homogenate were weighed into a scintillation vial and mixed with 1.0 ml Soluene $^{\text{®}}$ -350 and the sample was heated to 40°C for 1–2 h. A total of 0.5 ml 2-propanol, 0.2 ml hydrogen peroxide (30% v/v solution) and 0.1 ml glacial acetic acid were then added and the mixture heated to 40°C for 1–2 h. Finally, 10 ml liquid scintillation cocktail (Hionic-Fluor) were added and the radioactivity measured using liquid scintillation counting.

Metabolic profiling and metabolite identification

Metabolic profiling was performed for urine and faeces samples. Urine samples were added to an equal volume of acetonitrile to dissolve potentially precipitated metabolites and to desalt the samples. Subsequently, the samples were centrifuged (3000g for 5 min). Faeces samples (2 g) were extracted with 3 $\times$ 6 ml methanol for 5 min. The methanolic fractions were combined and extracted with hexane to remove lipids before evaporation under nitrogen gas to near dryness.

For profiling and metabolite identification, HPLC was performed using a Luna column (5 μ m, C-18 (2), 250 $\times$ 4.6 mm i.d.) obtained from Phenomenex (Torrance, CA, USA). The mobile phases consisted of mobile phase A: 0.2% formic acid, and mobile phase B: 0.2% formic acid in acetonitrile. A linear gradient from 10 to 90% B from 0 to 80 min was performed using a flow rate of 1 ml min $^{-1}$. For analysis of the tolmetin-*O*-sulphate conjugate, the mobile phases were altered to: A. 10 mM ammonium formate (pH 3.5), and B. 10 mM ammonium formate (pH 3.5) in 80% acetonitrile. Other chromatographic conditions were as described above.

For metabolite profiling, 200 μ l urine sample or 100 μ l faeces extract were loaded onto the chromatographic column and the samples analysed on a Merck HPLC system (Darmstadt, Germany) (gradient pump L-7100, autosampler L-720, column oven L-7300, UV detector L-7400) equipped with a radiochemical detector from Packard Instruments (Flo-one 150 TR, Greve, Denmark). The limit of quantification (signal:noise ratio 10) of Tol-Gly and Tol-Tau was 0.02 and 0.04 mM, respectively. The precision of the HPLC method, expressed as the relative standard deviation ($n=6$), was 0.4%.

For metabolite identification, urine samples and faeces extracts were loaded onto an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA), in which the outlet was connected to a Bruker LC-Esquire Mass Spectrometer (Bruker, Bremen, Germany) and a Gilson FC 204 fraction collector

(Gilson, Inc., Middleton, WI, USA), respectively. After the chromatographic column, the flow was split 1:4 to the mass spectrometer and fraction collector, respectively. A total of 0.5-min fractions were collected and used for MS-MS analysis in order to characterize metabolites. MS-MS analysis was performed by direct infusion ($5 \mu\text{l min}^{-1}$) of the collected fractions into the mass spectrometer. A total of 200 μl of the fractions were used for scintillation counting to confirm that the radioactivity profile was identical with that obtained for the radiochemical detection.

Mass spectrometer settings

The Bruker LC-Esquire Mass Spectrometer was equipped with an electrospray source. MS settings were used as follows: spray voltage ± 3500 V (depending on the ion mode); voltage of skimmer 1, 15 V; temperature of heated capillary 350°C , trap drive 50.

Results

In vitro reactivity of Tol-CoA

The ability of Tol-CoA to react with the thiol group of glutathione was used to characterize the reactivity. As shown in figure 1, time-dependent formation of *S*-acyl-glutathione conjugate was rapid and reached a maximum after approximately 120 min. The linear range of the profile was short and the initial rate of formation of *S*-acyl-glutathione conjugate was calculated from this linear range to be $14.9 \pm 0.7 \mu\text{M min}^{-1}$ (mean $\pm$ SD, $n = 3$).

Metabolism of Tol in rat

Three male Sprague–Dawley rats were administered Tol (p.o., 100 mg kg^{-1} , $200 \mu\text{Ci kg}^{-1}$), and urine and faeces were collected from 0 to 72 h. Based on measurements of the total radioactivity from 0 to 72 h in urine, faeces and cage wash, the total recovered dose was $105\% \pm 1.7\%$ (mean $\pm$ SD, $n = 3$). The radioactivity was primarily excreted in the urine from 0 to 24 h ($90.2\% \pm 1.7\%$), whereas $10.5\% \pm 1.1\%$ was excreted in faeces from 0 to 24 h.

Tol metabolites in urine samples and faeces extracts were detected and quantified by means of HPLC with radiochemical detection, and a representative

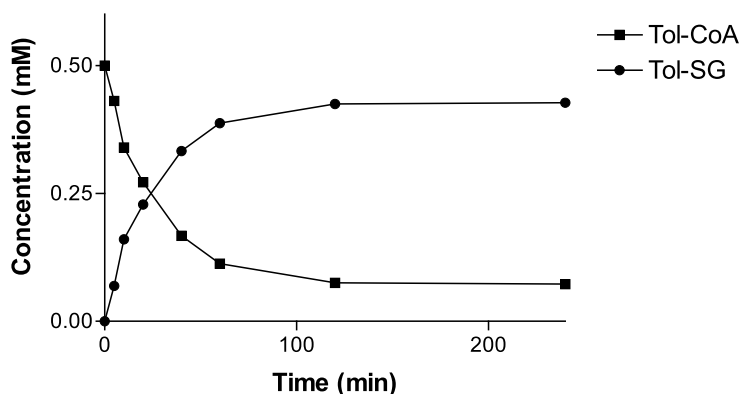


Figure 1. Time-dependent formation of *S*-acyl-glutathione conjugate (Tol-SG, circles) following reaction of 0.5 mM Tol-CoA (squares) with 5 mM glutathione (circles). Incubations were performed in a 0.1 M phosphate buffer (pH 7.4) at 37°C . Values are expressed as the mean $\pm$ SD of three independent experiments.

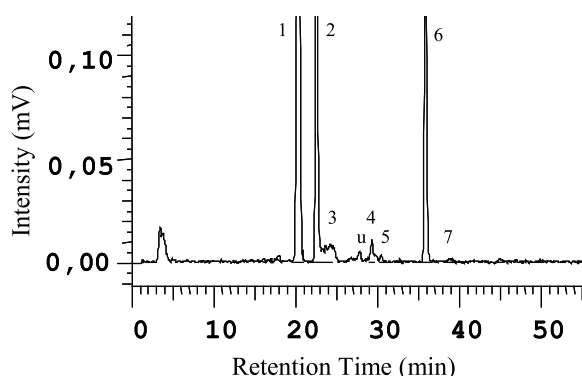


Figure 2. Metabolic profile of a 0–6-h urine sample following administration of ^3H -Tol (100 mg kg^{-1} , $200\text{ }\mu\text{Ci kg}^{-1}$, p.o.) to the rat. HPLC analysis with radiochemical detection was performed using a Phenomenex Luna (C-18) column ($5\text{ }\mu$, $250\text{ mm} \times 4.6\text{ mm i.d.}$). Mobile phases A and B consisted of 0.2% formic acid and 0.2% formic acid in acetonitrile, respectively. A linear gradient from 10 to 90% B over 80 min was performed using a flow rate of 1 ml min^{-1} . Peaks were identified as follows: 1, Tol-OH; 2, Tol-COOH; 3, Tol-O-SO₃H; 4, Tol-GlcU; 5, Tol-Gly; 6, Tol; and 7, Tol-Tau. An unknown metabolite (u) eluted after 27.5 min.

chromatogram is shown in figure 2. In order to analyse the tolmetin-*O*-sulphate ester (Tol-*O*-SO₃H), the mobile phase was altered from acetonitrile–water solutions containing 0.2% formic acid to acetonitrile–water solutions containing 10 mM ammonium formate (pH 3.5). In this chromatographic system (data not shown), Tol-*O*-SO₃H eluted before Tol and other Tol metabolites and Tol-*O*-SO₃H did not coelute with other minor metabolites as seen in figure 2 (data not shown).

The same chromatographic conditions as used for the quantitative analysis were used for metabolite identification. Structure elucidation was performed by means of LC-MS and subsequent MS-MS analysis of the simultaneously collected fractions that contained radiolabelled compounds. MS-MS fragmentation of Tol metabolites was obtained using electrospray ionization (ESI) in positive-ion mode (table 1). An MS-MS spectrum of the glycine conjugate is shown in figure 3 and

Table 1. Fragment ions of Tol metabolites from off-line MS-MS analysis of collected fractions that contained radioactivity.

Compound	Retention time (min)	$[\text{M} + \text{H}]^+$ (m/z)	MS-MS ^a (m/z , (%))
Tol-OH	20	274	135 (100), 94 ^b (6)
Tol-COOH	22	288	270 ^c (21), 242 (24), 167 (10), 149 (100), 94 ^b (55)
Tol- <i>O</i> -SO ₃ H	24	354	274 (23), 256 ^c (93), 215 (67), 135 ^d (16)
Tol-GlcU	29	434	416 ^c (100), 258 ^c (72)
Tol-Gly	30	315	240 (100), 212 (24), 179 (13), 151 (10), 119 (23)
Tol	35	258	240 (2), 119 (100), 94 ^b (4)
Tol-Tau	39	365	240 (100), 212 (26), 119 (19)

^a See figure 4 for the assignment of fragment ions other than ^b m/z 94 ($[\text{N-methyl-pyrrole-CH}_2]^+$) and ^closses of water (m/z 18). ^dThe same fragment ion as seen for Tol-OH. ^eMS-MS-MS of m/z 258 produced a spectrum identical to the MS-MS spectrum of Tol.

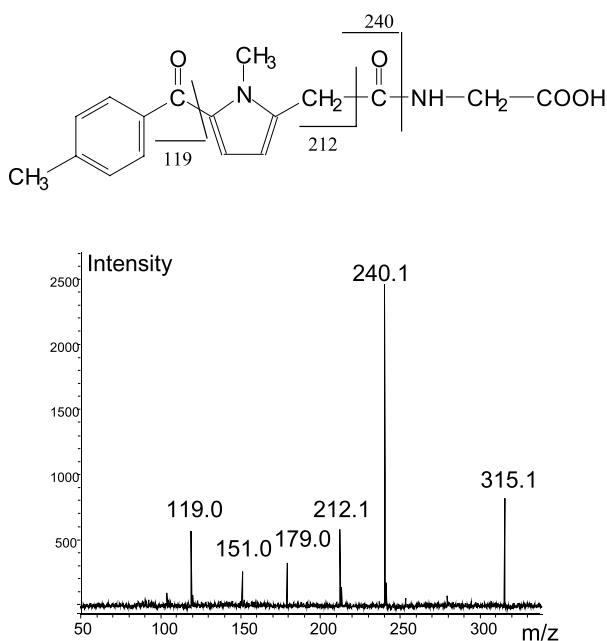


Figure 3. ESI-MS-MS spectrum of Tol-Gly ($[M + H]^+$ at m/z 315). Assignment of fragment ions not indicated in the figure: m/z 179 ($[M + H - CH_3(C_6H_4) - COOH]^+$), m/z 151 ($[M + H - CH_3(C_6H_4)CO - COOH]^+$).

structures of the identified metabolites and assignment of MS-MS fragments are shown in figure 4. As seen from table 1, the characteristic fragment ion of Tol at m/z 119 ($[CH_3 - C_6H_4 - CO]^+$) was also detected for Tol-Gly ($[M + H]^+$ at m/z 315) and Tol-Tau ($[M + H]^+$ at m/z 365). Moreover, MS-MS fragmentation of m/z 315 and 365, respectively, resulted in fragment ions at m/z 240 ($[tolmetin - H_2O + H]^+$) and m/z 212 ($[tolmetin - H_2O - CO + H]^+$), which was consistent with the structures of Tol-Gly and Tol-Tau (figure 4).

Further evidence on the structures of Tol-Gly, Tol-Tau and Tol-GlcU came from ESI-MS-MS experiments in negative-ion mode. MS-MS fragmentation of Tol-Tau at m/z 363 ($[M - H]^-$) gave one dominant fragment ion at m/z 124 (deprotonated taurine, 100%) and MS-MS fragmentation of Tol-Gly at m/z 313 ($[M - H]^-$) gave one characteristic ion at m/z 238 (100%) corresponding to $[M - H]^-$ of tolmetin with a loss of water. Fragmentation of Tol-GlcU ($[M - H]^-$ at m/z 432) produced fragment ions at m/z 175 ($[glucuronic\ acid - H_2O - H]^-$, 100%) and m/z 193 ($[glucuronic\ acid - H]^-$, 96%).

The metabolic profile showed that tolmetin mainly underwent oxidative metabolism at the 4-phenyl methyl group to the respective hydroxy (Tol-OH) and the carboxy-metabolite (Tol-COOH), which accounted for 36 and 32% of the total administered dose, respectively. In addition, Tol-GlcU (1.5%), Tol-O-SO₃H (2.2%), Tol-Gly (< 0.5%) and Tol-Tau (< 0.5%) were found as minor metabolites (see table 2 for a summary of the quantitative data). As seen from the chromatogram shown in figure 2, the concentration of Tol-Gly and Tol-Tau was close to the limit of detection (signal:noise ratio 3) for the radiochemical detection. With the sensitivity of the HPLC method, it would be possible to quantify the glycine and

Table 2. Quantitative analysis of Tol metabolites, expressed as the percentage dose, following oral administration (100 mg kg^{-1} , $200 \mu\text{Ci kg}^{-1}$).

Compound	Urine 0–6 h (%)	Urine 6–24 h (%)	Faeces 0–24 h (%)	Total excretion (%)
Tol-OH	18.1 ± 4.5	15.6 ± 7.4	2.2 ± 0.2	35.9 ± 4.3
Tol-COOH	7.4 ± 5.4	18.1 ± 1.8	6.5 ± 1.2	32.3 ± 4.9
Tol-O-SO ₃ H	1.4 ± 0.4	0.8 ± 0.9	N.D.	2.2 ± 1.3
Tol-GlcU	0.9 ± 0.1	0.7 ± 0.4	N.D.	1.5 ± 0.4
Tol-Gly	< 0.5%	N.D.	N.D.	< 0.5%
Tol	9.3 ± 3.0	12.6 ± 4.7	1.2 ± 0.6	23.1 ± 3.1
Tol-Tau	< 0.5%	N.D.	N.D.	< 0.5%

Tol and metabolites accounted for 95% of the total given dose. Values as the mean $\pm$ S.D. ($n = 3$). N.D., not detectable.

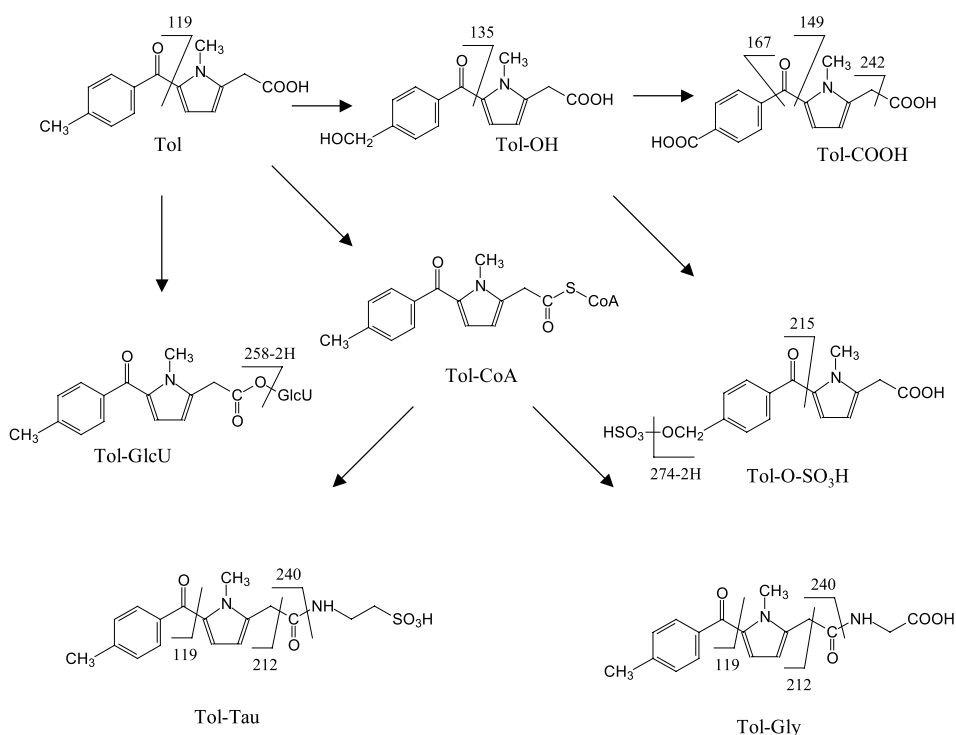


Figure 4. Proposed scheme for the metabolic pathways of Tol in rat. Characteristic MS-MS fragment ions are also shown (see table 1).

taurine conjugates at levels higher than approximately 0.5% of the administered dose.

Discussion

The main objectives of the present studies were to investigate the *in vitro* reactivity of Tol-CoA and to examine if this metabolite was formed *in vivo* in rats. Since acyl-CoAs are not excreted from cells (Mathews and van Holde 1997), we studied the formation of glycine and taurine conjugates which are formed via the respective acyl-CoA precursor. Urinary excretion of the glycine and taurine conjugates was low and accounted for less than 0.5% of the administered dose.

Nonetheless, the finding that both metabolites are formed is of mechanistic significance since formation occurs via the reactive Tol-CoA intermediate.

Tol-CoA formation may have been mediated by the medium-chain acyl-CoA synthetase since glycine and taurine conjugates are formed in the mitochondrial matrix in which the medium acyl-CoA synthetase and the *N*-acyltransferase are located (Hutt and Caldwell 1990). In addition, Tol-CoA may have been formed via the long-chain acyl-CoA synthetase, which is involved in the formation of other xenobiotic acyl-CoAs, e.g. ibuprofen acyl-CoA. The long-chain acyl-CoA synthetase is located in microsomes, peroxisomes and the outer mitochondrial membrane (Knights 1998) and, thus, Tol-CoA may have been formed outside of the mitochondria and subsequently transported into the mitochondrial matrix in which *N*-acylation of glycine or taurine took place. Transportation of acyl-CoAs into the mitochondria occurs via the corresponding acyl-carnitine ester, which in this study was not identified for Tol.

Hence, various acyl-CoA synthetases located in different cell compartments may be involved in the formation of Tol-CoA and, therefore, Tol-CoA may only in part be available for the amino acid conjugation process in the mitochondria. Furthermore, formation of amino acid conjugates may be limited by the availability of amino acid (Gregus *et al.* 1998) and, therefore, the amount of excreted glycine and taurine conjugates may not reflect the intracellular concentration of an acyl-CoA. However, identification of Tol-Gly and Tol-Tau may be used as indirect evidence for Tol-CoA formation.

The study on the *in vitro* reactivity of Tol-CoA (figure 1) showed that this metabolite rapidly reacted with glutathione. The rationale for conducting this study was that if the acyl-CoA thioester reacted with glutathione, then Tol-CoA might also react with nucleophilic groups such as those found in proteins (e.g. -SH, -NH₂, -OH). Previous studies for characterizing the *in vitro* reactivity with glutathione have been performed on acyl glucuronides and acyl-CoAs of clofibrilic acid (Shore *et al.* 1995, Grillo and Benet 2002) and naproxen (Olsen *et al.* 2002) under similar incubation conditions as employed here. In these latter studies, it was demonstrated that the acyl-CoAs of naproxen (Olsen *et al.* 2002) and clofibrilic acid (Shore *et al.* 1995, Grillo and Benet 2002) reacted more rapidly with glutathione as compared with the corresponding acyl glucuronides, i.e. acyl-CoAs are electrophilic metabolites that are more reactive than the corresponding acyl glucuronides.

The present results from our studies on *in vitro* reactivity showed that Tol-CoA was more reactive than that reported for the naproxen acyl-CoA (Olsen *et al.* 2002) and the clofibrilic-CoA (Grillo and Benet 2002). Examination of the naproxen acyl-CoA data reveals that the formation of *S*-acyl glutathione conjugate following the reaction of 0.5 mM naproxen-CoA with glutathione (5 mM) at pH 7.4 was linear from 0 to 5 h with an approximately rate of 1 $\mu\text{M min}^{-1}$ (Olsen *et al.* 2002). In addition, 1 mM clofibrilic-CoA (Grillo and Benet 2002) reacted with 5 mM glutathione at physiological pH resulted in a linear formation of *S*-acyl glutathione conjugate from 0 to 4 h that was 0.8 $\mu\text{M min}^{-1}$. In the current study on Tol-CoA, the linear range was shorter than 10 min and the initial rate of formation was 15 $\mu\text{M min}^{-1}$, i.e. the *in vitro* reactivity of Tol-CoA was comparably higher than that determined for other acyl-CoAs reported in the literature.

Our results are consistent with structure-reactivity relationships proposed by Benet *et al.* (1993) and by Grillo and Benet (2002), who reported that both acyl

glucuronides and *S*-acyl thioesters of aryl-acetic acid derivatives (e.g. Tol) are more reactive than 2-aryl-propionic acid derivatives (e.g. naproxen) and 2-methyl-2-phenoxy propionic acid derivatives (e.g. clofibrilic acid). Thus, it may be possible to predict the *in vivo* reactivity of an acyl-CoA towards proteins based on the structure if there is a correlation between the *in vitro* reactivity towards glutathione and the chemical reactivity towards proteins. However, the consequences of such reactivity with respect to toxicity depend on numerous factors such as the levels of formation of the reactive metabolite and the proteins that are modified (Qiu *et al.* 1998, 2001). In addition, stability of drug-protein adducts is another factor to consider in order to address the consequences of the presence of covalently modified proteins.

Tol primarily underwent oxidative metabolism, which is in agreement with previous findings (Hashimoto *et al.* 1979). Apart from the Tol-Gly and Tol-Tau, two other new metabolites in rat were identified here: Tol-GlcU and the sulphate ester of the hydroxy-metabolite (Tol-O-SO₃H). Tol-GlcU is a common metabolite in humans (Hyneck *et al.* 1987, Zia-Amirhosseini *et al.* 1994) and monkeys (Grindel 1981), and as a consequence the presence of Tol-GlcU in plasma has been associated with formation of covalent proteins adducts in humans (Hyneck *et al.* 1988, Zia-Amirhosseini *et al.* 1994). Formation of Tol-GlcU in the rat has not been reported before, which is probably due to the low amounts excreted in the urine (1.5%). This low urinary excretion is in agreement with reports on zomepirac (Wang and Dickinson 2000), which is structurally similar to Tol. The zomepirac acyl glucuronide has been reported primarily to undergo biliary excretion in rat (King and Dickinson 1996). In general, compounds with a molecular weight greater than 350 g mol⁻¹ (Tol-GlcU: 433 g mol⁻¹) are mainly eliminated in the bile of rats whereas the threshold is higher in man (about 500 g mol⁻¹). Therefore, Tol-GlcU to a certain degree may have been eliminated in the bile into the intestine in which it is hydrolysed to tolmetin and reabsorbed or excreted in the faeces.

In conclusion, the current study has shown that Tol-CoA was a reactive metabolite that may react with nucleophilic sites in proteins to form drug-proteins adducts. Furthermore, we identified the glycine and taurine conjugates as minor metabolites excreted in the urine. Since these conjugates are formed via Tol-CoA, this provides indirect evidence that the reactive acyl-CoA is formed *in vivo*, presumably in the liver, indicating that some Tol-protein adducts, if present, may arise from the reaction of the reactive Tol-CoA with proteins.

Acknowledgement

Steen Karsk Johansen at Novo Nordisk A/S (Department of Isotope Chemistry) is greatly acknowledged for synthesis of radiolabelled tolmetin.

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Functional characterization of three human cytochrome P450 2E1 variants with amino acid substitutions

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Received 1 November 2002

1. Cytochrome P450 (P450) 2E1 is a hepatic enzyme of importance for the metabolism of xenobiotics such as drugs and environmental toxicants. Genetic polymorphisms of *CYP2E1* in 5'-flanking and coding regions have been found previously in Caucasian and Chinese populations.

2. In order to investigate the effects of amino acid substitutions on the function of CYP2E1, the enzymes of all known CYP2E1 variants in the coding region (CYP2E1.2, CYP2E1.3 and CYP2E1.4) with Arg76His, Val389Ile and Val179Ile substitutions, respectively, as well as the wild-type CYP2E1 (CYP2E1.1) were expressed in COS-1 cells, and their chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities were determined.

3. The protein level of CYP2E1.2 was reduced to 29% compared with that of CYP2E1.1. The profiles of the level of activity relative to CYP2E1.1 for chlorzoxazone 6-hydroxylation (300 µM substrate) and 4-nitrophenol 2-hydroxylation (150 µM substrate) were very similar.

4. Although the K_m values were not significantly different among wild-type and variant CYP2E1s in any oxidation metabolism, the V_{max} and V_{max}/K_m of CYP2E1.2 on the basis of the CYP2E1 protein level were 2.7–3.0-fold higher than those of CYP2E1.1. In contrast, the levels of CYP2E1 protein and catalytic activity of CYP2E1.3 and CYP2E1.4 were not affected by the corresponding amino acid substitutions.

5. The findings suggest that Arg76 is closely associated with the function of CYP2E1, and that the genetic polymorphism of *CYP2E1* is one cause of interindividual differences in the toxicity of xenobiotics.

Introduction

Cytochrome P450 (P450) comprises a family of enzymes that catalyse the oxidation of a variety of xenobiotic chemicals such as drugs, toxic chemicals and carcinogens, as well as endobiotic chemicals including steroids, fatty acids and vitamins. The *CYP2E1* gene has been localized to human chromosome 10 and is composed of nine exons (Umeno *et al.* 1988). This P450 form is expressed mainly in the liver, and expression is induced by ethanol (Gonzalez 1990, Nelson *et al.* 1996). CYP2E1 has been known to metabolize compounds of relatively small molecular weight such as acetaminophen (analgesic), halothane (anaesthetic drug), haloalkane (organic solvent) and short-chain nitrosamines (Guengerich and

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Table 1. Characterization of *CYP2E1* alleles examined.

Allele	Protein	Nucleic acid change	Amino acid substitution	Reference
<i>CYP2E1*1A</i> ^a	CYP2E1.1 ^a	none	none	Umeno <i>et al.</i> (1988)
<i>CYP2E1*2</i>	CYP2E1.2	227G > A	Arg76His	Hu <i>et al.</i> (1997)
<i>CYP2E1*3</i>	CYP2E1.3	1165G > A	Val389Ile	Hu <i>et al.</i> (1997)
<i>CYP2E1*4</i>	CYP2E1.4	535G > A	Val179Ile	Fairbrother <i>et al.</i> (1998)

^a Wild-type.

Shimada 1991, Rendic and Di Carlo 1997). It has been suggested that there is a large interindividual variation in protein and catalytic activity levels of CYP2E1 in human liver microsomes as well as other P450 forms (Yoo *et al.* 1988, Shimada *et al.* 1994). Previous *in vivo* studies of a Caucasian population using chlorzoxazone as a probe drug reported a several-fold variation in clearance with a normal distribution of activities and suggested that such variation was caused in part by the genetic polymorphism of *CYP2E1*, although less extensively than observed in *in vitro* studies (Kim and O'Shea 1995, Lucas *et al.* 1995).

Several *CYP2E1* allelic variants were detected using restriction fragment polymorphism analysis or DNA sequencing, and their relevance to CYP2E1 activity, disease susceptibility and xenobiotic toxicity has been widely investigated (Song 1996, Hu *et al.* 1997, 1999, Fairbrother *et al.* 1998, Novak and Woodcroft 2000). *CYP2E1*1C* and *CYP2E1*1D* have an insertion of a repeated nucleotide sequence in the 5'-flanking region, and it is supposed that individuals with *CYP2E1*1D* show a remarkable increase in CYP2E1 activity after drinking alcohol (McCarver *et al.* 1998, Hu *et al.* 1999). In the coding region, three allelic variants termed *CYP2E1*2*, *CYP2E1*3* and *CYP2E1*4* with amino acid substitutions in exons 2, 7 and 4, respectively, have been found in Caucasian and Chinese populations (table 1), and the frequencies have been estimated as 2.4–2.6% (Hu *et al.* 1997, Fairbrother *et al.* 1998). One of them, which causes the Arg76His substitution (*CYP2E1*2*), has been reported to reduce CYP2E1 protein and catalytic activity levels. However, very little has been reported about the effects of genetic polymorphism of *CYP2E1* on the detailed enzymatic properties or about comparisons among the identified allelic variants with amino acid substitutions. Because CYP2E1 metabolizes several organic solvents and several procarcinogens, the genetic polymorphism of this P450 has been regarded as an important environmental risk factor similar to that of glutathione *S*-transferase (Brockmoller *et al.* 1996, Kiss *et al.* 2000, Tan *et al.* 2000).

The purpose of the current study was to investigate whether amino acid substitutions in CYP2E1 affect enzyme function. To achieve this, the wild-type and variant CYP2E1 cDNAs were constructed, and the corresponding CYP2E1 proteins were transiently expressed in COS-1 cells. The enzymatic properties of CYP2E1 proteins were examined by kinetic analysis using chlorzoxazone and 4-nitrophenol as substrates.

Materials and methods

Materials

Human adult normal liver mRNA was purchased from the BioChain Institute (Hayward, CA, USA). SuperScript first-strand synthesis system, Platinum *Pfx* DNA polymerase, pENTR/D-TOPO vector, pcDNA-DEST40 mammalian expression vector, Lipofectamine 2000 and OPTI-MEM

medium were purchased from Invitrogen (Carlsbad, CA, USA). A QuikChange Multi site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA, USA). COS-1 cells were obtained from the Japanese Collection of Research Biosource (Osaka, Japan). Dulbecco's modified Eagle's medium and foetal bovine serum were purchased from Sigma (St Louis, MO, USA). Chlorzoxazone and 6-hydroxychlorzoxazone were purchased from Ultrafine Chemicals (Manchester, UK). 4-Nitrophenol, 4-nitrocatechol and salicylamide were obtained from Wako Pure Chemical Industries (Osaka, Japan). NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). ABI BigDye terminator cycle sequencing reaction kit v.2.0 was obtained from Applied Biosystems (Foster City, CA, USA), rabbit anti-human CYP2E1 antibody (purified human liver CYP2E1 as immunogen) was from Research Diagnostics (Flanders, NJ, USA), and peroxidase-conjugated donkey anti-rabbit immunoglobulin and ECL-plus reagents were from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals and reagents were of the highest quality commercially available.

Construction of CYP2E1 plasmids

The mRNA from normal human livers was reversed-transcribed to cDNA using a SuperScript first-strand synthesis system. The cDNA of CYP2E1*1 was amplified by polymerase chain reaction (PCR) from 50 ng of single-stranded cDNA template using the forward primer 5'-CACC-ATGTCCTGCCCTCGGA-3' and the reverse primer 5'-TCATGAGCGGGGAATGACAC-3'. The underlined sequence was introduced to perform directional TOPO cloning. The amplification mixture (50 µl) contained 1 unit Platinum *Pfx* DNA polymerase, 1 × *Pfx* amplification buffer, 1 mM MgSO₄, 0.4 mM dNTPs and 0.3 µM each of forward and reverse primers. The cycling parameters were: denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s and 68 °C for 2 min, and termination by a 7-min extension at 68 °C. The PCR products were cloned into the pENTR/D-TOPO vector and sequenced in both the forward and reverse directions using the ABI BigDye terminator cycle sequencing reaction kit v.2.0 on the DNA analyser ABI Prism 3700 (Applied Biosystems). The plasmid carrying the CYP2E1*1 cDNA was used as a template to generate point mutations in the target regions. Three single variant CYP2E1s (CYP2E1*2, CYP2E1*3, CYP2E1*4) were constructed with the QuikChange Multi site-directed mutagenesis kit according to the manufacturer's instructions using the 5'-phosphorylated primers listed in table 2. The primers for the respective mutations were MP-G227A for CYP2E1*2, MP-G1165A for CYP2E1*3 and MP-G535A for CYP2E1*4. All CYP2E1 plasmids were sequenced to confirm a successful mutagenesis. The wild-type and variant CYP2E1 cDNAs were subsequently subcloned into the pCDNA-DEST40 mammalian expression vector.

Expression of CYP2E1 enzymes

COS-1 cells, an African green monkey kidney cell line, were cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum under 5% CO₂ at 37 °C. Cells (5.5×10^4 cm⁻²) were seeded in a 10-cm culture dish 1 day before transfection. On the following day, the culture medium was replaced with OPTI-MEM medium, and the CYP2E1 expression plasmids (15 µg DNA) were transfected using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h, the cells were washed twice with ice-cold phosphate-buffered saline and harvested in 100 mM potassium phosphate buffer (pH 7.4) containing 250 mM sucrose. Microsomes from COS-1 cells were prepared by sequential centrifugation according to a standard procedure described by Ekins *et al.* (1999). After centrifugation, the microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol and stored at -80 °C until use. The protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Quantification of CYP2E1 protein levels

Microsomal proteins (20 µg) from COS-1 cells were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970) and electrotransferred to a polyvinylidene fluoride

Table 2. Primers used for site-directed mutagenesis.

Primer	Sequence ^a	Location ^b
MP-G227A	5'-GTGGGGCTCGCAGCATGGTGGTGATG-3'	214–240 (27)
MP-G1165A	5'-CCAAGGGCACAGTCATAGTGCCAACTCTGG-3'	1151–1180 (30)
MP-G535A	5'-CGCGCCCTGCAACATCATAGCCGACATC-3'	522–549 (28)

All primers were 5'-phosphorylated.

^a Bold letters are the mutations produced by PCR-based site-directed mutagenesis.

^b Values in parentheses are the number of bases (mer).

sheet (Bio-Rad Laboratories, Hercules, CA, USA) as described by Towbin *et al.* (1979). The sheet was incubated with rabbit anti-human CYP2E1 antibody as the primary antibody and then with peroxidase-conjugated donkey anti-rabbit immunoglobulin as the secondary antibody. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (ECL-plus reagents), and the band densities were determined using microsomes from insect cells expressing human CYP2E1 (Gentest, Woburn, MA, USA) as a standard with the bioimaging analyser Typhoon 9400 (Amersham Biosciences).

Assay for CYP2E1-dependent enzymatic activities

Chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities were determined by high-performance liquid chromatography (HPLC) as described previously with some modifications (Tassaneeyakul *et al.* 1993, Lucas *et al.* 1996). The incubation mixture contained chlorzoxazone (20–1000 μM) or 4-nitrophenol (10–500 μM) as a substrate, the microsomal fraction of COS-1 cells (500 μg protein ml^{-1}) and an NADPH-generating system (1 mM NADP⁺, 10 mM glucose 6-phosphate, 2 U ml^{-1} glucose 6-phosphate dehydrogenase, 5 mM MgCl_2) in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 500 μl . Both substrates were dissolved in methanol (final concentration in the reaction medium, 0.5% v/v). The reaction was initiated by the addition of the NADPH-generating system after a 2-min pre-incubation at 37 °C. After incubation for 20 min (chlorzoxazone 6-hydroxylation) or 30 min (4-nitrophenol 2-hydroxylation) at 37 °C, the reaction was terminated by the addition of 3 ml ethyl acetate. Samples for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities were spiked with internal standards (1500 pmol 4-nitrophenol and 1000 pmol salicylamide, respectively) and vigorously vortexed for 2 min. After centrifugation at 2000g for 15 min, the organic phase was evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residues were dissolved in 200 μl methanol-water (50:50, v/v) and analysed by HPLC.

HPLC analysis was performed using a Shimadzu SCL-10A system controller (Kyoto, Japan) consisting of two or three LC-10AD pumps, an SIL-10A autoinjector with a sample cooler, an SPD-10AV UV-Vis detector, a CTO-10A column oven, a DGU-14A degasser and a C-R7A chromatopac integrator. The samples were cooled at 4 °C and 50- μl aliquots were injected into an Inertsil ODS-80A column (150 $\times$ 4.6 mm i.d.; GL Sciences, Tokyo, Japan). The column was kept at 40 °C. The product (6-hydroxychlorzoxazone) in the assay for chlorzoxazone 6-hydroxylation activity was eluted isocratically with 80 mM acetic acid–acetonitrile (82:18, v/v) at a flow rate of 1.0 ml min^{-1} . UV detection was performed at 295 nm. In the assay for 4-nitrophenol 2-hydroxylation activity, the product (4-nitrocatechol) was eluted isocratically with 20 mM triethylamine–acetonitrile–methanol (78:12:10, v/v/v) at a flow rate of 1.0 ml min^{-1} . The pH of the aqueous portion was adjusted to 3.0 with phosphoric acid. UV absorption was monitored at 250 nm. The standard curve samples for both assays were prepared in the same manner as the incubation samples. The intra- and interday variation coefficients did not exceed 6% in any of the assays.

Data analysis

Kinetic parameters such as K_m and $V_{\max}$ were estimated using the computer program Prism v.3.0 (GraphPad Software, San Diego, CA, USA) designed for non-linear regression analysis of a hyperbolic Michaelis–Menten equation. Intrinsic clearances were determined as the ratio $V_{\max}/K_m$. All reported values are the mean $\pm$ SD of three separate experiments derived from independent preparations. Statistical comparisons were made with Dunnett's *post-hoc* test using the computer program StatView v.5.0 (SAS Institute, Cary, NC, USA), and differences were considered to be statistically significant when $p < 0.05$.

Results

Expression of wild-type and variant CYP2E1s in COS-1 cells

The cDNA-encoding CYP2E1*1 was cloned into the pENTR/D-TOPO vector, and the corresponding mutations of each variant CYP2E1 were introduced into the wild-type CYP2E1 plasmid using PCR-based site-directed mutagenesis. The wild-type and variant CYP2E1 cDNAs were subsequently inserted into the pcDNA-DEST40 mammalian expression vector and transiently transfected into COS-1 cells. For comparison, cells transfected with pcDNA3.1 as a null vector were used as a negative control. The expression levels of CYP2E1 protein in the microsomal fraction of transfected cells were examined by immunoblotting (figure 1). All constructs except the negative control yielded immunodetectable

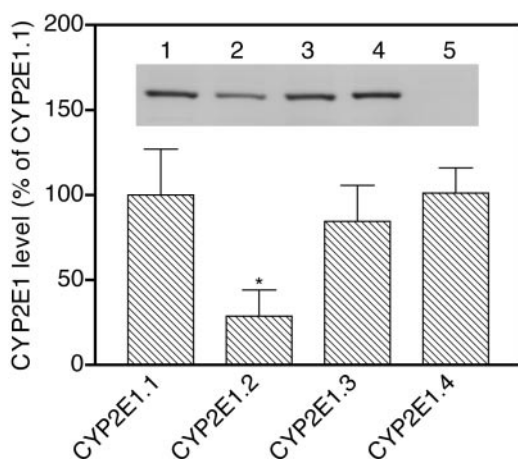


Figure 1. CYP2E1 protein levels in microsomes from COS-1 cells expressing wild-type and variant CYP2E1s. Experimental conditions are described in the Materials and methods. The results are expressed as a percentage of levels (pmol mg^{-1} protein) of CYP2E1.1. The level of CYP2E1.1 was 3.49 ± 0.96 $\text{pmol CYP2E1 mg}^{-1}$ protein. Each bar represents the mean $\pm$ SD of three separate experiments derived from independent preparations. * $p < 0.01$ versus CYP2E1.1. (inset) Immunoblotting of pooled microsomes from three independent preparations. Lanes 1, CYP2E1.1; 2, CYP2E1.2; 3, CYP2E1.3; 4, CYP2E1.4; 5, negative control.

CYP2E1 protein. The expressed protein level of CYP2E1.1 was 3.5 pmol mg^{-1} protein. The expression levels of CYP2E1.3 and CYP2E1.4 were comparable with that of CYP2E1.1, whereas the level of CYP2E1.2 was significantly reduced to 29% compared with that of CYP2E1.1. The profile for the relative expression levels of the CYP2E1 protein was reproducible in three independent transfections.

Enzymatic properties of wild-type and variant CYP2E1s

Chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities in microsomes from COS-1 cells expressing wild-type and variant CYP2E1s were then examined. No activity in microsomes of the negative control was detected with any substrate (data not shown). Figures 2 and 3 show the levels relative to the activity of CYP2E1.1 at single substrate concentrations ($300 \mu\text{M}$ for chlorzoxazone 6-hydroxylation and $150 \mu\text{M}$ for 4-nitrophenol 2-hydroxylation) on the basis of the protein and CYP2E1 levels, respectively. Chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities of CYP2E1.1 on the basis of the microsomal protein level were 25 and $33 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein, respectively. The activities of CYP2E1.2 were reduced to 70–72% compared with those of CYP2E1.1, whereas the activities of CYP2E1.3 and CYP2E1.4 were not significantly different. On the other hand, chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities of CYP2E1.1 on the basis of the CYP2E1 protein level were 7.5 and $9.7 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ CYP2E1, respectively. In both oxidation reactions, the activities of CYP2E1.2 were significantly higher than those of CYP2E1.1, whereas the activities of CYP2E1.3 and CYP2E1.4 exhibited similar levels to those of CYP2E1.1.

To obtain further information on the enzymatic properties of variant CYP2E1s as well as the wild-type CYP2E1, Michaelis–Menten kinetics for chlorzoxazone 6-

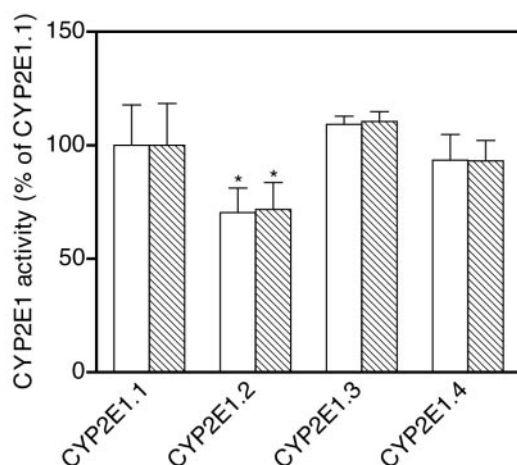


Figure 2. CYP2E1-dependent enzymatic activities based on the microsomal protein level in microsomes from COS-1 cells expressing wild-type and variant CYP2E1s. The substrate concentrations used were 300 μM for chlorzoxazone 6-hydroxylation and 150 μM for 4-nitrophenol 2-hydroxylation. The experimental conditions are described in the Materials and methods. The results are expressed as a percentage of the activity ($\text{pmol min}^{-1} \text{mg}^{-1}$ protein) of CYP2E1.1. The activities of CYP2E1.1 for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation were 25.3 ± 4.5 and $32.8 \pm 6.0 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein, respectively. Each bar represents the mean $\pm$ SD of three separate experiments derived from independent preparations. Open bar, chlorzoxazone 6-hydroxylation; hatched bar, 4-nitrophenol 2-hydroxylation. * $p < 0.05$ versus CYP2E1.1.

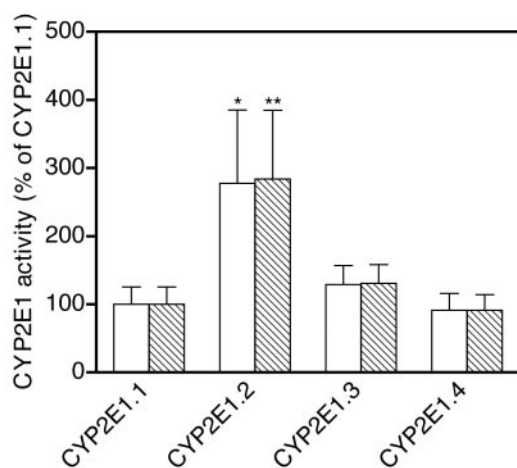


Figure 3. CYP2E1-dependent enzymatic activities based on the CYP2E1 protein level in microsomes from COS-1 cells expressing wild-type and variant CYP2E1s. The substrate concentrations used were 300 μM for chlorzoxazone 6-hydroxylation and 150 μM for 4-nitrophenol 2-hydroxylation. Experimental conditions are described in the Materials and methods. The results are expressed as a percentage of the activity ($\text{pmol min}^{-1} \text{pmol}^{-1}$ CYP2E1) of CYP2E1.1. The activities of CYP2E1.1 for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation were 7.51 ± 1.92 and $9.73 \pm 2.46 \text{ pmol min}^{-1} \text{pmol}^{-1}$ CYP2E1, respectively. Each bar represents the mean $\pm$ SD of three separate experiments derived from independent preparations. Open bar, chlorzoxazone 6-hydroxylation; hatched bar, 4-nitrophenol 2-hydroxylation. * $p < 0.05$, ** $p < 0.01$ versus CYP2E1.1.

hydroxylation and 4-nitrophenol 2-hydroxylation were examined. The calculated kinetic parameters are summarized in tables 3 and 4, respectively. The K_m values of wild-type and variant CYP2E1s for chlorzoxazone 6-hydroxylation ranged from 250 to 300 μM , and a significant difference was not observed between wild-type and variant CYP2E1s. The $V_{\max}$ and $V_{\max}/K_m$ of wild-type and variant CYP2E1s on the basis of the microsomal protein level were 34–55 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein and 130–190 $\text{nl min}^{-1} \text{mg}^{-1}$ protein, respectively. The $V_{\max}$ of CYP2E1.2 was significantly lower than that of CYP2E1.1, although no significant difference in $V_{\max}/K_m$ values was observed among wild-type and variant CYP2E1s. When the activities were normalized to CYP2E1 protein levels, the $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.1 were 15 $\text{pmol min}^{-1} \text{pmol}^{-1}$ CYP2E1 and 51 $\text{nl min}^{-1} \text{pmol}^{-1}$ CYP2E1, respectively. The $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.2 were significantly higher compared with those of CYP2E1.1 (2.7- and 2.9-fold, respectively). The K_m of wild-type and variant CYP2E1s for 4-nitrophenol 2-hydroxylation had similar values ranging from 120 to 140 μM . The $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.1 on the basis of the microsomal protein level were 63 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein and

Table 3. Kinetic parameters for chlorzoxazone 6-hydroxylation by microsomes from COS-1 cells expressing wild-type and variant CYP2E1s.

Variant	K_m^a	$V_{\max}$		$V_{\max}/K_m$	
		Protein ^b	P450 ^c	Protein ^d	P450 ^e
CYP2E1.1	283 $\pm$ 13	48.9 $\pm$ 9.0	14.5 $\pm$ 3.9	173 $\pm$ 24	51.2 $\pm$ 12.3
CYP2E1.2	272 $\pm$ 19	33.7 $\pm$ 3.7*	39.4 $\pm$ 14.8*	125 $\pm$ 22	146 $\pm$ 61*
CYP2E1.3	297 $\pm$ 13	54.8 $\pm$ 2.1	19.3 $\pm$ 4.3	185 $\pm$ 10	64.8 $\pm$ 13.7
CYP2E1.4	247 $\pm$ 18	42.9 $\pm$ 4.3	12.4 $\pm$ 3.1	175 $\pm$ 29	51.0 $\pm$ 15.7

Each value is the mean $\pm$ SD of three separate experiments derived from independent preparations.

^a μM .

^b $\text{pmol min}^{-1} \text{mg}^{-1}$ protein.

^c $\text{pmol min}^{-1} \text{pmol}^{-1}$ CYP2E1.

^d $\text{nl min}^{-1} \text{mg}^{-1}$ protein.

^e $\text{nl min}^{-1} \text{pmol}^{-1}$ CYP2E1.

* $p < 0.05$ versus CYP2E1.1.

Table 4. Kinetic parameters for 4-nitrophenol 2-hydroxylation by microsomes from COS-1 cells expressing wild-type and variant CYP2E1s.

Variant	K_m^a	$V_{\max}$		$V_{\max}/K_m$	
		Protein ^b	P450 ^c	Protein ^d	P450 ^e
CYP2E1.1	141 $\pm$ 11	63.3 $\pm$ 9.8	18.9 $\pm$ 5.0	453 $\pm$ 87	133 $\pm$ 32
CYP2E1.2	124 $\pm$ 13	43.3 $\pm$ 8.9*	51.2 $\pm$ 22.6*	346 $\pm$ 43	404 $\pm$ 139**
CYP2E1.3	129 $\pm$ 16	67.8 $\pm$ 3.3	23.7 $\pm$ 6.0	524 $\pm$ 46	183 $\pm$ 34
CYP2E1.4	120 $\pm$ 10	54.9 $\pm$ 4.8	15.9 $\pm$ 3.8	460 $\pm$ 59	134 $\pm$ 37

Each value is the mean $\pm$ SD of three separate experiments derived from independent preparations.

^a μM .

^b $\text{pmol min}^{-1} \text{mg}^{-1}$ protein.

^c $\text{pmol min}^{-1} \text{pmol}^{-1}$ CYP2E1.

^d $\text{nl min}^{-1} \text{mg}^{-1}$ protein.

^e $\text{nl min}^{-1} \text{pmol}^{-1}$ CYP2E1.

* $p < 0.05$, ** $p < 0.01$ versus CYP2E1.1.

450 nl min⁻¹ mg⁻¹ protein, respectively. The $V_{\max}$ of CYP2E1.2 based on the microsomal protein level was significantly reduced to 68% that of CYP2E1.1; however, there was no significant difference in the $V_{\max}/K_m$ values of any variant CYP2E1. Based on the CYP2E1 protein level, the $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.1 were 19 pmol min⁻¹ pmol⁻¹ CYP2E1 and 130 nl min⁻¹ pmol⁻¹ CYP2E1, respectively. The $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.2 were significantly increased 2.7–3.0-fold compared with those of CYP2E1.1. However, no significant difference in the $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.3 and CYP2E1.4 based on the CYP2E1 protein level was observed in any oxidation metabolism of chlorzoxazone and 4-nitrophenol.

Discussion

CYP2E1 is involved in the metabolism of several xenobiotics, and large interindividual variation in the activity both *in vivo* and *in vitro* has been reported (Yoo *et al.* 1988, Shimada *et al.* 1994, Kim and O'Shea 1995, Lucas *et al.* 1995). Although the underlying mechanisms for this interindividual diversity are complex, genetic polymorphisms in *CYP2E1* been considered to be at least partly responsible (Kim and O'Shea 1995, Lucas *et al.* 1995, Song 1996). Since *CYP2E1* polymorphisms have been suggested to be closely associated with the incidence of several cancers, it may be an important risk factor for susceptibility to toxicants (Song 1996, Tanaka *et al.* 2000). The functional evaluation of polymorphic CYP2E1 variants should provide important information for predicting the *in vivo* metabolic activation and inactivation of xenobiotics. In the present study, the enzymatic activities in microsomes from COS-1 cells expressing wild-type and three variant CYP2E1s were investigated by kinetic analysis. To this end, we used chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation as probes for CYP2E1.

Determination of the CYP2E1 protein levels in microsomes from COS-1 cells expressing variant CYP2E1s indicated that the levels of CYP2E1.3 and CYP2E1.4 are comparable with that of CYP2E1.1. In contrast, the expressed level of CYP2E1.2 was significantly reduced compared with that of CYP2E1.1. To examine this phenomenon further, an *in vitro* transcription/translation assay of the CYP2E1 expression plasmids using rabbit reticulocyte lysate and fluorescent labelling lysine-tRNA was performed in a preliminary study. The transcription and translation rates for each expression plasmid of variant CYP2E1 were very similar to those for CYP2E1*1. Hu *et al.* (1997) have reported that CYP2E1*2 cDNA reduces the expression level of protein (37% of CYP2E1*1 cDNA) but not mRNA in an expression system using COS-1 cells. The expression pattern of CYP2E1 obtained in this study agreed with that of a previous report, so it is possible that the reduction in the protein levels of CYP2E1.2 is due to an unstable or improper conformation of the protein rather than transcription/translation or a low transfection efficiency, as suggested for CYP2D6*10 (Johansson *et al.* 1994, Fukuda *et al.* 2000).

It has been suggested that chlorzoxazone and 4-nitrophenol are representative probes for CYP2E1. The enzymatic activities in this study were analysed in two ways: as pmol min⁻¹ mg⁻¹ protein (on the basis of the microsomal protein level) to take the efficiency of the mutant expression into account, and as pmol min⁻¹ pmol⁻¹ CYP2E1 (on the basis of the CYP2E1 protein level) to assess

the function of each mutant protein. All of the variant CYP2E1s were capable of oxidating chlorzoxazone and 4-nitrophenol as well as wild-type CYP2E1 at the substrate concentrations examined; however, a notable difference was observed for one variant of CYP2E1. Both oxidation activities of CYP2E1.2 on the basis of the microsomal protein level were significantly lower than those of CYP2E1.1, whereas the activities of CYP2E1.2 on the basis of the CYP2E1 protein level inversely exhibited higher values compared with those of wild-type and the other two variant CYP2E1s. Hu *et al.* (1997) also reported that chlorzoxazone 6-hydroxylation activity of CYP2E1.2 based on the microsomal protein level was reduced to 36% that of CYP2E1.1, whereas the level of CYP2E1.3 was comparable with that of CYP2E1.1. Therefore, the difference in CYP2E1-dependent enzymatic activities at the microsomal protein level among wild-type and variant CYP2E1s may be due to the level of protein expression. Interestingly, the profile relative to the activity of CYP2E1.1 for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation at single substrate concentrations was very similar in any unit term, suggesting that the binding site of CYP2E1 for these substrates is the same.

To characterize further the enzymatic properties of wild-type and variant CYP2E1s, we performed kinetic analyses for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation. We confirmed that the kinetics for these oxidation metabolisms in microsomes from insect cells expressing wild-type CYP2E1 fit the single enzyme model with a typical Michaelis–Menten equation, and that the $K_m = 560 \mu\text{M}$ for chlorzoxazone 6-hydroxylation and $350 \mu\text{M}$ for 4-nitrophenol 2-hydroxylation. On the other hand, previous studies have estimated that the K_m values for chlorzoxazone 6-hydroxylation in wild-type CYP2E1 expressed in insect cells and human B-lymphoblastoid cells were 660 and $81 \mu\text{M}$, respectively (Fairbrother *et al.* 1998, Shimada *et al.* 1999). Thus, the K_m of CYP2E1.1 obtained in this study differed from the values in other studies. The possibility has to be considered that the discrepancy was due to the difference in the heterologous cell expression system. The K_m values of wild-type and variant CYP2E1s were similar for both oxidation reactions, whereas the $V_{\max}$ values of CYP2E1.2 based on the CYP2E1 protein level were significantly higher than those of CYP2E1.1. Consequently, the $V_{\max}/K_m$ of CYP2E1.2 exhibited the highest value among the CYP2E1 proteins examined. However, the kinetic parameters of CYP2E1.3 and CYP2E1.4 were not affected by the corresponding amino acid substitutions (Val389Ile and Val179Ile, respectively) in either of the two enzyme assays. Therefore, we must consider the possibility that the Arg76His substitution influences the catalytic activity of CYP2E1.

Because CYP2E1 has not been successfully crystallized, no detailed alignment and homology models for the localization of amino acids in the three-dimensional structure of the enzyme are available. However, Hu *et al.* (1997) have predicted that the Arg76 of human CYP2E1 locates in the β 1-sheet using the crystal structures of bacterial P450s (CYP101, CYP102 and CYP108) as templates. Arg76 of CYP2E1 is conserved in all mammals (Song *et al.* 1986, Khani *et al.* 1988, Freeman *et al.* 1992, Komori *et al.* 1992), possibly indicating an important function for this amino acid residue. CYP2E1 has been reported to play a role in the toxicity of therapeutic drugs such as acetaminophen and halothane, and environmental chemicals including benzene and nitrosamines (Novak and Woodcroft 2000, Tanaka *et al.* 2000). These toxicities have been generally thought to

result from lipid peroxidation and free radical generation (Albano *et al.* 1996, Dupont *et al.* 2000). Furthermore, several studies have implicated CYP2E1 in the increased incidence of liver disease and cancer in diabetics, alcoholics and obese individuals (Lieber 1988, Song 1996). Therefore, we consider that the Arg76His substitution is an important marker for evaluating CYP2E1-mediated toxicity. However, it has been also suggested that the coding regions of the *CYP2E1* gene are well conserved among different ethnic groups (Ronis *et al.* 1996). The factors regulating CYP2E1 expression in the 5'-flanking region may further influence the interindividual difference in xenobiotic toxicity.

In conclusion, we have expressed amino acid-substituted enzymes of three variant CYP2E1s as well as wild-type CYP2E1 in COS-1 cells, and examined their enzymatic properties using chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation. The protein level of CYP2E1.2 was remarkably low probably due to the decreased stability of the protein. The $V_{\max}$ and $V_{\max}/K_m$ of CYP2E1.2 with the Arg76His substitution on the basis of the CYP2E1 protein level were significantly higher than those of CYP2E1.1, whereas the levels of CYP2E1.3 and CYP2E1.4 were not affected by the corresponding amino acid substitutions. These results suggest that the amino acid at position 76 is a key residue in the CYP2E1 function, and that polymorphic alleles of *CYP2E1* partly lead to variations in the toxicity of clinically used drugs and environmental or abused chemicals.

Acknowledgements

The authors thank Reika Kou for secretarial assistance. This work was supported in part by a Grant-in-Aid for the Development of Innovative Technology (13309) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Action of quercetin on glycogen catabolism in the rat liver

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1. The influence of quercetin on glycogen catabolism and related parameters was investigated in the isolated perfused rat liver and subcellular systems.
2. Quercetin stimulated glycogenolysis (glucose release). This effect was already evident at a concentration of 50 μM maximal at 300 μM and declined at higher concentrations. Quercetin also stimulated oxygen consumption, with a similar concentration dependence.
3. Lactate production from endogenous glycogen (glycolysis) was diminished by quercetin without significant changes in pyruvate production.
4. Quercetin did not inhibit glucose transport into cells but decreased intracellular sequestration of [5- ^3H]glucose under conditions of net glucose release.
5. In isolated mitochondria, quercetin diminished the energy transduction efficiency. It also inhibited several enzymatic activities, e.g. the K^+ -ATPase/ Na^+ -ATPase of plasma membrane vesicles and the glucose 6-phosphatase of isolated microsomes.
6. No significant changes of the cellular contents of AMP, ADP and ATP were found. The cellular content of glucose 6-phosphate, however, was increased (3.12-fold).
7. Some of the effects of quercetin (glycogenolysis stimulation) can be attributed to its action on mitochondrial energy metabolism, as, for example, uncoupling of oxidative phosphorylation. However, the multiplicity of the effects on several enzymatic systems certainly produces an intricate interplay that also generates complex and apparently contradictory effects.

Introduction

The antioxidant activities of quercetin and other flavonoids are certainly among the most intensively investigated biological effects of these compounds (Bors *et al.* 1990, Kawada *et al.* 1998, Breinholt *et al.* 1999, Kawagoe and Nakagawa 2000). Besides these effects, quercetin, as well as other flavonoids, can exert a variety of short- and long-term metabolic effects on mammalian and tumour cells. Glycolysis inhibition, which has been described in tumour cells and rat testis, is one such effect (Suolinna *et al.* 1973, 1975, Trejo *et al.* 1995). This action has been related to a highly effective inhibition of the K^+ -ATPase/ Na^+ -adenosine-triphosphatase, a phenomenon that leads to low cellular concentrations of ADP and inorganic phosphate. These events, in turn, are likely to lead to a substantial

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inhibition of glycolysis, contributing to a reduction of tumour cell growth (Suolinna *et al.* 1975).

Quercetin, as with most natural flavonoids, is at least partly transformed in the liver (Manach *et al.* 1995, Morand *et al.* 1998). This relationship between xenobiotic exposure and the liver inevitably leads to the question of whether such substances can also affect liver metabolism in some way. For this reason, we decided to investigate the possible action of quercetin on glycogen catabolism and related parameters. In the liver, glycogen degradation leads to glucose release, but it is also accompanied by a glycolytic activity that results in lactate and pyruvate release (Kimmig *et al.* 1983). Most experiments in the present work were undertaken in the isolated perfused rat liver, an experimental system that allows the measurement of metabolic phenomena under conditions of preservation of the microcirculation and cell integrity. Experiments were also undertaken with isolated mitochondria and microsomes to obtain an explanation for the effects of quercetin on glycogen catabolism and related parameters.

Materials and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá (Kelmer-Bracht *et al.* 1984). Glucose, enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of the best available grade (98–99.8% purity) and were purchased from Merck (Darmstadt, Germany), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

Liver perfusion

Male Wistar rats (weighing 200–280 g) fed with a standard laboratory diet (Purina[®], São Paulo, Brazil) were used in all experiments. All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá. For the surgical procedure, rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg⁻¹). Haemoglobin-free, non-recirculating perfusion was undertaken according to the technique described by Scholz and Bücher (1965). After cannulation of the portal and cava veins, the liver was positioned in a Plexiglas chamber. The hepatic artery was closed (monovascular perfusion) and the bile duct left open. The antegrade flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, Villiers-le-Bel, France) and was adjusted to between 30 and 35 ml min⁻¹, depending on the liver weight. The perfusion fluid was Krebs–Henseleit–bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The composition of the Krebs–Henseleit–bicarbonate buffer was: 115 mM NaCl, 25 mM NaHCO₃, 5.8 mM KCl, 1.2 mM Na₂SO₄, 1.18 mM MgCl₂, 1.2 mM NaH₂PO₄ and 2.5 mM CaCl₂. Quercetin was dissolved into the perfusion fluid. Solubilization was achieved by the simultaneous addition of an equivalent amount of NaOH.

Samples of the effluent perfusion fluid were collected at 2-min intervals and analysed for their metabolite content. Lactate and pyruvate were assayed by means of standard enzymatic procedures using L-lactate dehydrogenase (Czok and Lamprecht 1974, Gutman and Wahlefeld 1974). Interference by quercetin (absorbance at 340 nm) was excluded by running blanks. The oxygen concentration in the outflowing perfusate was monitored polarographically, employing a Teflon-shielded platinum electrode adequately positioned in a Plexiglas chamber at the exit of the perfusate. Quercetin interferes with the glucose oxidase reaction and, for this reason, glucose was measured colorimetrically by means of an *o*-toluidine method (Dubowski 1962).

The hepatic contents of AMP, ADP, ATP and glucose 6-phosphate were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralized with K₂CO₃ and assayed by means of standard enzymatic procedures (Jaworek *et al.* 1974, Lamprecht and Trautschold 1974, Lang and Michal 1974).

Multiple-indicator dilution experiments

A mixture (100 μ l) containing [U - ^{14} C]sucrose (5 μ Ci), [^{3} H]water (20 μ Ci) and [5 - ^{3} H]glucose (10 μ Ci) was injected into the portal vein. The effluent perfusate was collected in 0.5–2.0-s fractions over 90 s. Radioactivity was measured by liquid scintillation, with isotope discrimination for the simultaneous determination of [U - ^{14} C]sucrose and total ^{3} H. [5 - ^{3} H]glucose was counted after elimination of [^{3} H]water by freeze drying, and the latter was computed from the difference between total ^{3} H and [5 - ^{3} H]glucose. The scintillation solution contained toluol/ethanol (10/2) and 5.0 g l $^{-1}$ of 1,5-diphenyloxazole plus 0.2 g l $^{-1}$ 2,2-*p*-phenyl-bis-5-phenyleneoxazole. The indicator dilution curves were normalized as: (amount in the effluent sample) $\times$ (second) $^{-1} \times$ (total amount injected) $^{-1}$.

Experiments with isolated mitochondria

Liver mitochondria were isolated by differential centrifugation according to Voss *et al.* (1961) using a sucrose-manitol isolation medium. Oxygen uptake by isolated mitochondria was measured polarographically at 25 °C as described by Voss *et al.* using an incubation medium containing 5 mM disodium phosphate, 10 mM tris(hydroxymethyl) aminomethane-HCl (Tris-HCl, pH 7.4), 0.2 mM ethylenediamine tetraacetic acid (EDTA), 10 mM potassium chloride, 250 mM mannitol and 50 mg% fatty-acid free bovine serum albumin. Mitochondria were added to the incubation for a final protein concentration around 2 mg ml $^{-1}$. Quercetin was added to the incubation medium as a solution in dimethylformamide. The rates of oxygen uptake were evaluated from the polarographic records. The respiratory control ratio (RC) and the ADP/O ratio were calculated according to Chance and Williams (1955). The mitochondrial ATPase activity was measured in intact and freeze-thawed disrupted mitochondria. The reaction medium contained 200 mM sucrose, 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.2 mM EGTA, 5 mM ATP and 0.5 mg ml $^{-1}$ mitochondrial protein. Phosphate release was measured according to Fiske and Subbarow (1925). Protein content was measured by the method of Lowry *et al.* (1955).

Experiments with microsomes

Microsomes were isolated by differential centrifugation according to Bygrave (1978). The pellet of the last 105 000g centrifugation, containing the microsomal fraction, was suspended in cold medium (4 °C) containing 250 mM sucrose and 2.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 6.8). The volume was adjusted to achieve a protein concentration of 10 mg ml $^{-1}$. The glucose 6-phosphatase activity was measured at 37 °C in a medium containing 100 mM KCl, 5 mM MgCl $_2$, 20 mM Tris-HCl (pH 7.2), 15 mM glucose 6-phosphate and 0.1–0.2 mg microsomal protein. Phosphate release was measured according to the method of Fiske and Subbarow (1925).

Experiments with plasma membrane vesicles

Liver plasma membrane vesicles were isolated essentially as described by Armstrong and Newman (1985). Some modifications in the original technique were done in order to adapt the procedures to our experimental conditions and to increase purity and recovery. The final membrane pellet ($\cong$ 4 mg protein ml $^{-1}$) was suspended in 0.02 M Tris, pH 8.0. This preparation was used for the assay of the membrane-bound adenosine-triphosphatases in the presence and absence of quercetin using two incubation media originally proposed by Song *et al.* (1969) and Schanne and Moore (1986) with only a few modifications. Incubation medium A contained: 0.02 M Tris (pH 7.4), 5 mM MgCl $_2$, 10 μ g ml $^{-1}$ oligomycin, 100 mM KCl, 1.0 mM ATP and membrane vesicles (0.07–0.15 mg protein ml $^{-1}$). Incubation medium B contained: 0.02 M Tris (pH 7.4), 5 mM MgCl $_2$, 10 μ g ml $^{-1}$ oligomycin, 50 mM KCl, 50 mM NaCl, 1.0 mM ATP and membrane vesicles (0.07–0.15 mg protein ml $^{-1}$); when required 100 μ M ouabain was added. The mixtures were incubated at 37 °C for 20 min and the reaction was stopped by the addition of ice-cold 10% trichloroacetic acid. After 10 min in a freezer (–10 °C), the samples were centrifuged at 11 600g and the supernatant used for phosphate assay according to Fiske and Subbarow (1925). The Na $^+$ /K $^+$ -adenosine triphosphatase activity was calculated by subtracting the values obtained with the incubation medium A (K $^+$ alone; Mg $^{2+}$ -ATPase activity) from those ones obtained with incubation medium B (Na $^+$ + K $^+$).

Data analysis and calculations

Metabolic rates were calculated from input–output differences and the total flow and were referred to the wet weight of the liver. Statistical analysis (Student's *t*-test) was done by means of the Primer program; *p* < 0.05 was adopted as a criterion of significance.

The outflow profiles of [5 - ^{3} H]glucose were analysed according to the model of Goresky *et al.* (1970). This model produces the following equation for a substance ($Q(t)$) that is injected as a bolus and which during its passage through the liver microcirculation has reversible access to the cellular space into which it can be irreversibly transformed (Goresky *et al.* 1970, Bracht *et al.* 1980):

$$Q(t) = Q_{\text{suc}}(t)e^{-k_{\text{in}}\theta(t-t_o)} + \int_0^{t-t_o} e^{-k_{\text{in}}\theta\tau} e^{-(k_{\text{ef}}+k_m)(t-t_o-\tau)} Q_{\text{suc}}(\tau+t_o) \sum_{n=1}^{\infty} \frac{(k_{\text{in}}k_{\text{ef}}\theta\tau)^n (t-t_o-\tau)^{n-1}}{n!(n-1)!} d\tau, \quad (1)$$

where t is time, τ the variable sinusoidal transit times, t_o the uniform transit time of the large non-exchanging vessels and θ the ratio of cellular to extracellular water spaces. The parameters k_{in} and k_{ef} are, respectively, the transfer coefficients for influx into the cell space and for efflux from the cellular space; and k_m is the irreversible sequestration coefficient. The dimensions of the rate coefficients are inverse time (e.g. s^{-1}) and they are referred to the intracellular distribution space (e.g. $\text{ml s}^{-1} \text{ ml cellular distribution space}^{-1}$). $Q_{\text{suc}}(t)$ is the $[\text{U-}^{14}\text{C}]$ sucrose outflow profile, the extracellular reference for $[\text{5-}^3\text{H}]$ glucose. Equation (1) was fitted to the experimental outflow profiles of $[\text{5-}^3\text{H}]$ glucose. A non-linear iterative least-squares procedure was used (Björck and Dahlquist 1972). Preliminary estimates of k_{in} , k_{ef} and k_m were introduced as well as values for t_o and θ . Values for these parameters were obtained from the optimized linear superimposition of the $[\text{3H}]$ water and $[\text{14C}]$ sucrose curves, according to Goresky (1963):

$$Q_{\text{water}}(t) = \left[\frac{1}{1+\theta} \right] Q_{\text{suc}} \left(\frac{t-t_o}{1+\theta} + t_o \right), \quad (2)$$

where $Q_{\text{water}}(t)$ is the impulse response of the liver to $[\text{3H}]$ water, $Q_{\text{suc}}([t-t_o]/[1+\theta] + t_o)$ is the $[\text{14C}]$ sucrose curve at time $([t-t_o]/[1+\theta] + t_o)$. Optimization of the superimposition was accomplished by means of a non-linear iterative least-squares procedure.

Integrals and derivatives of the experimental $[\text{14C}]$ sucrose and $[\text{3H}]$ water curves were calculated analytically after approximating the curves by cubic splines, with monoexponential extrapolation to infinite time from the last experimental point (90 s). Interpolation between experimental points was accomplished by means of a spline-function (Björck and Dahlquist 1972).

The cellular aqueous space of the liver per unit wet weight (V_i) was calculated from the mean transit times of $[\text{14C}]$ sucrose ($\bar{t}_{\text{suc}}$) and $[\text{3H}]$ water ($\bar{t}_{\text{water}}$), the flow (F) through the liver (Bracht *et al.* 1980) and the wet liver weight (W):

$$V_i = \frac{F(\bar{t}_{\text{water}} - \bar{t}_{\text{suc}})}{W}. \quad (3)$$

The mean transit times of tracers was calculated according to the well known formula (Meier and Zierler 1954):

$$\bar{t} = \int_0^{\infty} Q(t) \cdot t dt, \quad (4)$$

where $Q(t)$ is the normalized outflow profile of the tracer and t represents time.

Results

In the first experiments, the action of quercetin on mitochondrial respiration was investigated. This was justified because there are reports in the literature showing that this compound influences mitochondrial functions (Santos *et al.* 1997, Takahashi *et al.* 1998). The most important results are summarized in figure 1. The action of quercetin on mitochondrial respiration driven by succinate was characterized by a reduction in energy transduction. This is revealed by the fact that state III respiration, which designates the activated respiration which follows the addition of exogenous ADP, was clearly decreased in a concentration-dependent manner by quercetin. Conversely, state IV respiration, which is the respiration that follows the activated state, was stimulated. Both events evidently produced a progressive decrease of the respiratory control ratio (RC). At $300 \mu\text{M}$ quercetin, the respiratory control ratio was reduced to unity, meaning that a clear transition between state III and state IV was no longer detectable.

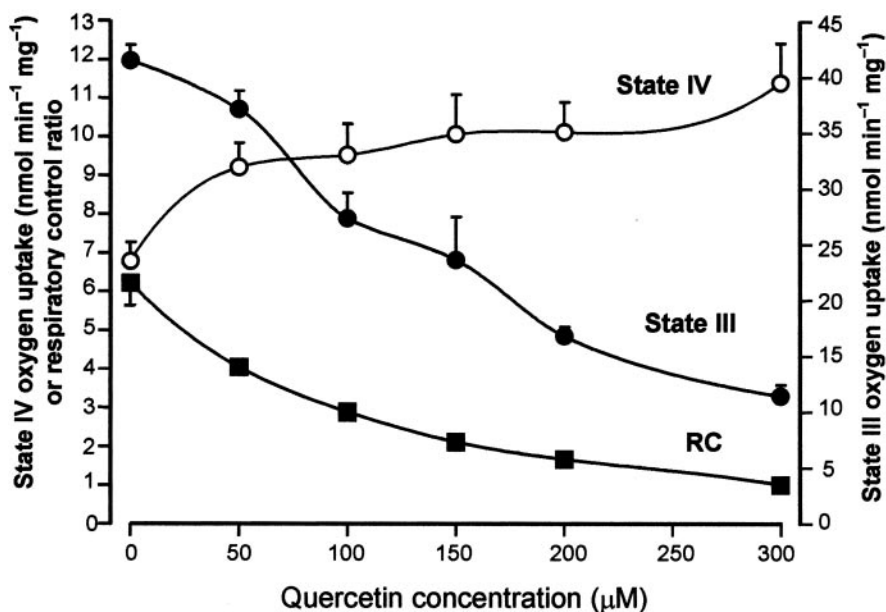


Figure 1. Action of quercetin on state III and IV respiration and on the respiratory control ratio (state III/IV). Rat liver mitochondria were isolated as described in the Materials and methods. Mitochondrial suspensions ($2 \text{ mg protein ml}^{-1}$) were incubated in a closed vessel (oxygraph) and the oxygen concentration was measured polarographically. Rates were computed from the slopes of the traces; state III respiration refers to the activated respiration after addition of ADP (250 nmol); state IV respiration to the respiration rate observed after the activated phase (Chance and Williams 1955). Data are the mean of $\pm \text{SEM}$ from four determinations.

From the data in figure 1, it is difficult to predict the action of quercetin on oxygen uptake in intact liver cells. Either stimulation or reduction of oxygen uptake can occur, depending on the conditions. On the other hand, since glycogen catabolism is generally strongly affected by changes in mitochondrial energy metabolism, one should expect some influence of quercetin on parameters such as glucose release and glycolysis. To investigate this possibility, several experiments were undertaken in which quercetin in the range $50\text{--}500 \mu\text{M}$ was infused over 30 min in livers from fed rats. Such livers respire at the expense of endogenous fatty acids and glycogen catabolism manifests itself by means of glucose release and lactate plus pyruvate production. Figures 2 and 3 show the time courses of the mean results obtained with 200 and $500 \mu\text{M}$ quercetin, respectively. Figure 2 reveals that quercetin-stimulated oxygen uptake and glucose release, and also that it tended to decrease lactate production. The stimulative effects on glucose release and oxygen uptake were clearly reversible, i.e. cessation of quercetin infusion led to a return to the basal levels. The same occurred with the reduction in lactate production, which increased again when quercetin was removed from the perfusion system. Pyruvate production, which was not affected during quercetin infusion, decreased upon cessation of the infusion.

The pattern shown by figure 2 was similar for all concentrations in the range $50\text{--}300 \mu\text{M}$, although to different extents. When the concentration was raised to 400 and $500 \mu\text{M}$, however, a different pattern emerged (figure 3). Glucose release and oxygen uptake only transiently increased and returned gradually to values slightly below the basal values. Cessation of quercetin infusion, however, was

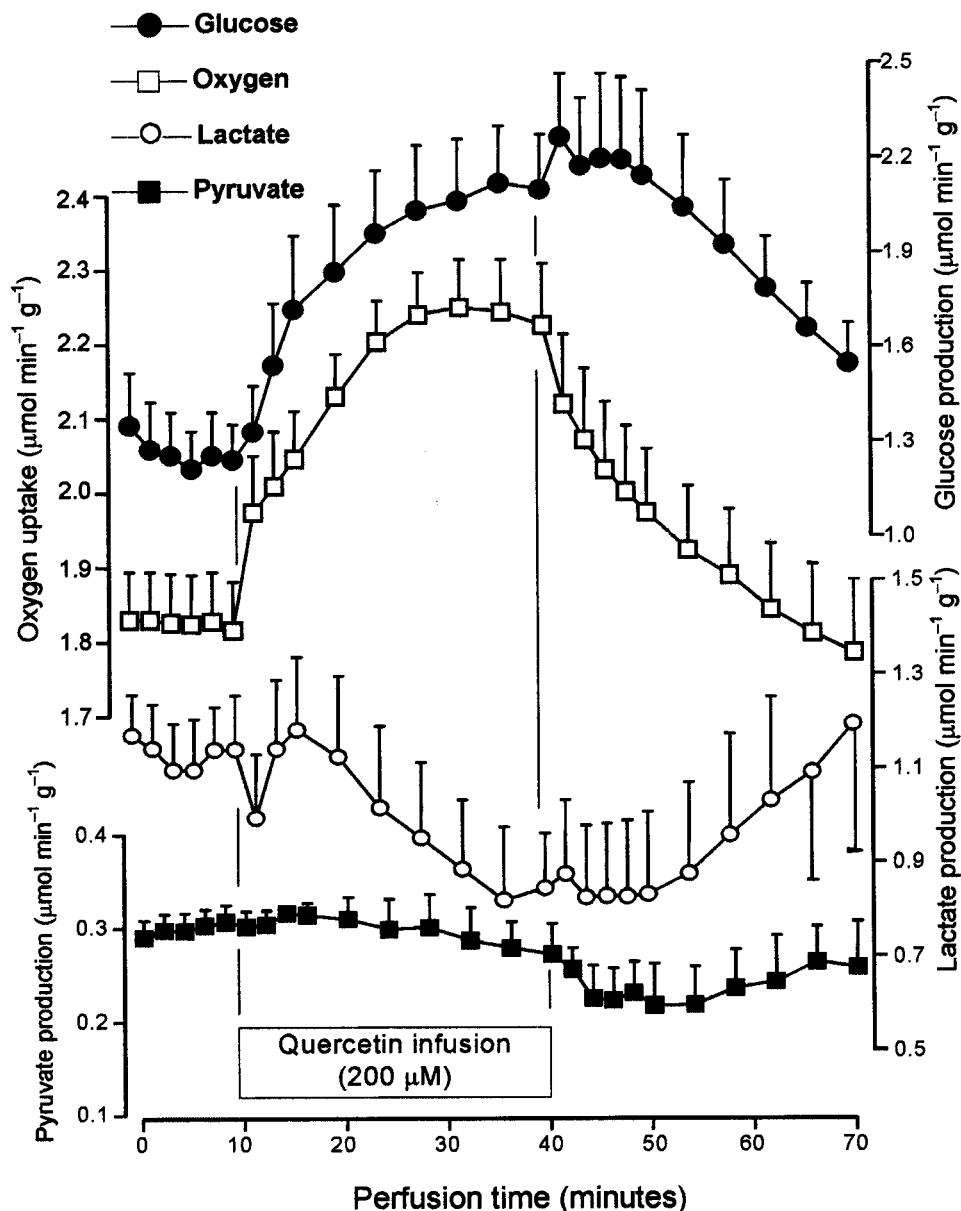


Figure 2. Time course of the changes caused by quercetin in glycogen catabolism and oxygen uptake in the perfused rat liver. Livers from fed rats were perfused as described in the Materials and methods. Quercetin (200 μM) was infused during 30 (10–40) min. Samples were taken for the measurement of glucose, lactate and pyruvate. Oxygen was determined polarographically. The data are the means of five liver perfusion experiments. Vertical bars are the mean standard errors.

followed by a substantial increase in both oxygen uptake and glucose release. Lactate production, on the hand, was decreased by quercetin and remained so during the whole infusion period, with some recovery after cessation of the infusion. Pyruvate production behaved similarly to the way already described for lower quercetin concentrations.

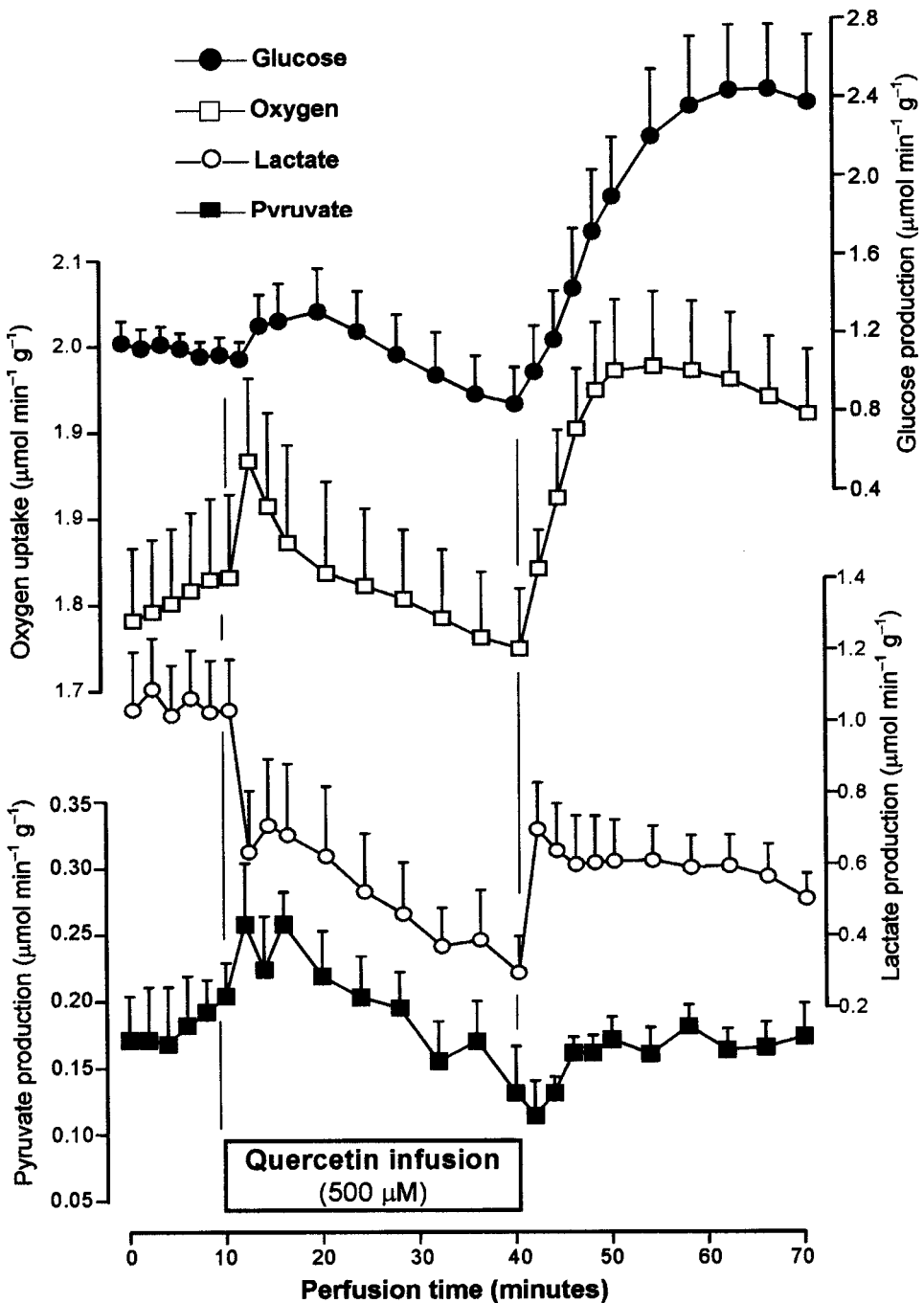


Figure 3. Time course of the changes caused by quercetin in glycogen catabolism and oxygen uptake in the perfused rat liver. Livers from fed rats were perfused as described in the Materials and methods. Quercetin (500 μM) was infused during 30 (10–40 min). Samples were taken for the measurement of glucose, lactate and pyruvate. Oxygen was determined polarographically. The data are the means of five liver perfusion experiments. Vertical bars are the mean standard errors.

Figure 4 shows the concentration dependence of the effects of quercetin on glucose release and oxygen uptake. Differences between the basal values and the values at the end of the infusion period of quercetin were evaluated. Glucose release was maximal at 300 μM , but was already evident at 50 μM . It declined with 400 μM and was slightly below the basal rates at 500 μM . Maximal oxygen uptake was found at 200 μM , but at 50 μM the effect was already around two-thirds of the maximum. Figure 5 shows the concentration dependence of the effects on lactate and pyruvate production. Pyruvate production was essentially unchanged, although a tendency toward higher values was apparent at 500 μM quercetin. Lactate production, however, was decreased, even though the concentration-dependence curve exhibited several irregularities. The decrease in lactate production without significant changes in pyruvate production also means that the lactate to pyruvate ratio was decreased. In this respect it should be noted that it is generally accepted that the lactate-to-pyruvate ratio in the liver reflects the cytosolic NAD^+/NADH redox potential (Sies 1982). This means, consequently, that quercetin increased the cytosolic NAD^+/NADH redox potential.

Several additional experiments were done with the aim of obtaining information that would allow an interpretation of the results of the perfusion experiments in terms, at least, of a working hypothesis. Inhibition of ATPase activities have been reported by several authors in several mammalian cell types, the Na^+/K^+ -adenosine triphosphatase being particularly sensitive (Suolinna *et al.* 1975, Mur-

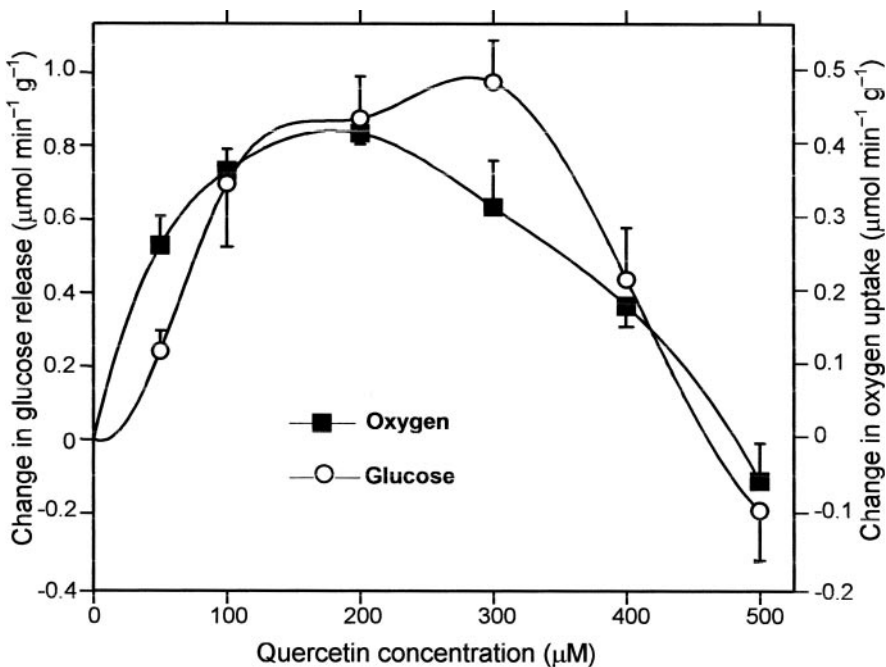


Figure 4. Action of quercetin on oxygen uptake and glucose release: dose-effect relationships. Changes in glucose release and oxygen uptake were computed from experiments of the kind shown by figures 2 and 3. The basal rates of glucose release or oxygen uptake were subtracted from the corresponding rates at the end of the quercetin infusion period in each perfusion experiment. Each computed difference is the mean of five to eight liver perfusion experiments. Error bars are standard errors of the mean.

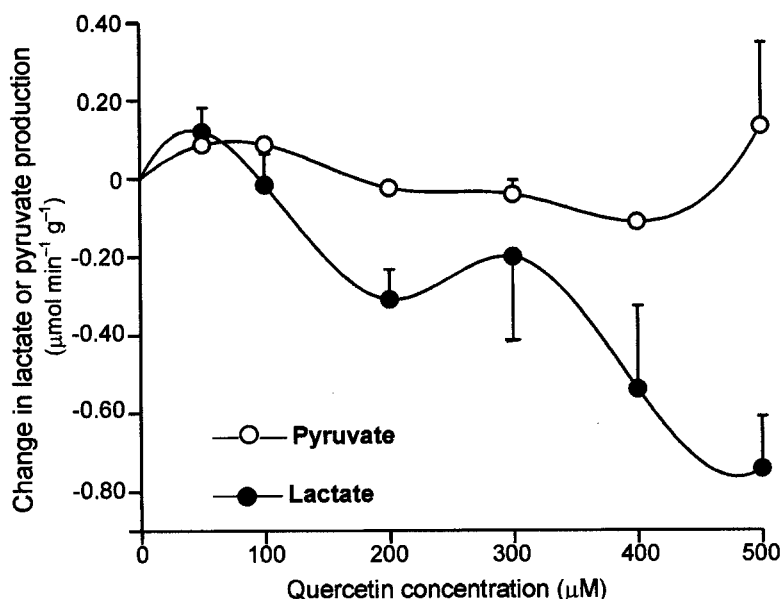


Figure 5. Action of quercetin on lactate and pyruvate production: dose-effect relationships. Changes in lactate and pyruvate production were computed from experiments of the kind shown by figures 2 and 3. The basal rates of lactate or pyruvate production were subtracted from the corresponding rates at the end of the quercetin infusion period in each perfusion experiment. Each computed difference is the mean of five to eight liver perfusion experiments. Error bars are standard errors of the mean.

akami *et al.* 1992, Gu *et al.* 1994). Table 1 shows the action of quercetin on several hepatic ATPase activities: the mitochondrial ATPase in intact and freeze-thawing disrupted mitochondria, the Na^+/K^+ -adenosine triphosphatase (ouabain sensitive) of plasma membrane vesicles and the Mg^{2+} -adenosine triphosphatase of plasma membrane vesicles. At least for the quercetin concentration of 300 μM , and with individual variations, inhibition was found for all four activities listed in table 1, but the Na^+/K^+ -adenosine triphosphatase was by far the most sensitive.

Lactate dehydrogenase, the enzyme that converts pyruvate to lactate, was not affected by quercetin up to 500 μM (data not shown). This would have indeed affected the assay of both metabolites, lactate and pyruvate, since this enzyme provides the basis for their determination. Glucose 6-phosphatase, however, was inhibited by quercetin (figure 6). The effect was almost maximal at 300 μM quercetin. Consistently, the glucose 6-phosphate levels were increased several fold by quercetin 500 μM (table 2). The levels of AMP, ADP and ATP, however, were not significantly affected by quercetin (table 2). Only some tendency toward lower levels of ATP and higher levels of ADP was found. However, these were not statistically significant.

The substantial stimulation of glucose release after cessation of 400 or 500 μM quercetin infusion raises the question whether glucose transport is affected. This question was approached by multiple-indicator dilution experiments (figure 7 and table 3). [$5\text{-}^3\text{H}$]glucose was rapidly injected together with indicators for the extracellular space ([^{14}C]sucrose) and for the total aqueous space ([^3H]water). The inflowing perfusate contained 1 μM non-labelled glucose. The outflow profiles

Table 1. Actions of quercetin on several ATPase activities.

ATPase activity	Control nmol min ⁻¹ (mg protein) ⁻¹	Quercetin (300 μM) nmol min ⁻¹ (mg protein) ⁻¹
Intact mitochondria (<i>n</i> = 11)	98.3 ± 4.4 ^a	57.7 ± 4.7 ^a
Freeze-thawing disrupted mitochondria (<i>n</i> = 11)	237.0 ± 10.1 ^b	185.0 ± 11.6 ^b
Na ⁺ /K ⁺ -dependent of plasma membrane vesicles (<i>n</i> = 3)	65.8 ± 5.1	≈ 0.0
Mg ⁺ -dependent of plasma membrana vesicles (<i>n</i> = 6)	314.4 ± 7.7 ^c	245.8 ± 22.3 ^c

Assays were undertaken as described in the Materials and methods. *p* refers to the Student's *t*-test. Error parameters are standard errors of the mean.

^a *p* < 0.001, ^b *p* = 0.016, ^c *p* = 0.003.

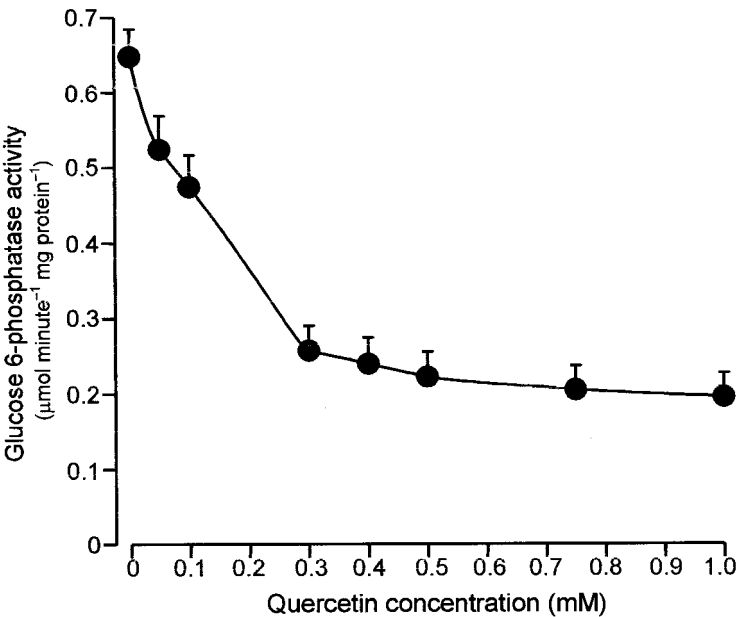


Figure 6. Action of quercetin on the activity of glucose 6-phosphatase. Livers from fed rats were homogenized and subjected to differential centrifugation as described in the Materials and methods. The microsomal fraction was used for glucose 6-phosphatase assay. Initial rates were measured at various quercetin concentrations. The data points are the means of 10 determinations. Bars are standard errors of the mean.

were normalized in such a way that the area under the normalized curves reflects the fractional recovery, which was unity in the case of [¹⁴C]sucrose and [³H]water. In the case of labelled glucose, however, the area was less than unity, indicating that some of the [5-³H]glucose was sequestered within the liver. As shown in figure 7, in the presence of quercetin, the outflow profile of [5-³H]glucose was closer to the outflow profile of the extracellular reference ([¹⁴C]sucrose). This could be due to inhibition of [5-³H]glucose removal, inhibition of glucose transport or to both phenomena. The removal rate of [5-³H]glucose can be determined from the recovered fractional [5-³H]glucose radioactivity (*R_c*), the flow rate (*F*), the wet liver weight (*W*) and the extracellular glucose concentration (*G*), i.e. removal

Table 2. Adenine nucleotide and glucose 6-phosphate contents of livers from fed rats in the presence and absence of quercetin.

Compound	Control ($n = 4$) $\mu\text{mol (g liver wet weight)}^{-1}$	Quercetin ($500 \mu\text{M}$)($n = 4$) $\mu\text{mol (g liver wet weight)}^{-1}$
ATP	1.908 ± 0.157	1.748 ± 0.070
ADP	0.624 ± 0.047	0.703 ± 0.066
AMP	0.206 ± 0.024	0.251 ± 0.028
Glucose 6-phosphate	0.191 ± 0.032^a	0.596 ± 0.048^a

Isolated perfused livers from fed rats were freeze-clamped and extracted as described in the Materials and methods. Assays were undertaken by standard enzymatic techniques. p refers to the Student's t -test. Error parameters are standard errors of the mean.

^a $p < 0.001$.

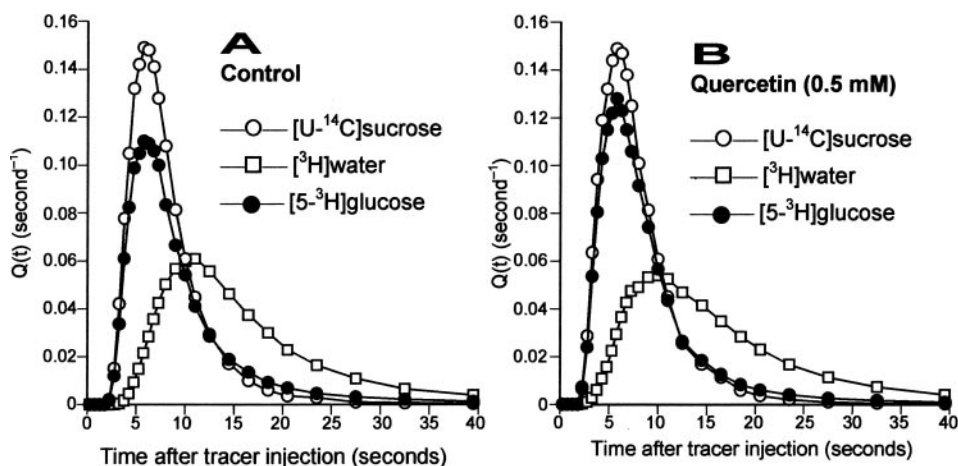


Figure 7. Outflow profiles of $[5\text{-}^3\text{H}]\text{glucose}$ and indicators. Livers were perfused as described in the Materials and methods. Mixtures containing $[5\text{-}^3\text{H}]\text{glucose}$, $[U\text{-}^{14}\text{C}]\text{sucrose}$ and $[^3\text{H}]\text{water}$ were injected twice, before and during $0.5 \mu\text{M}$ quercetin infusion. The outflowing perfusate was fractionated by means of a rapid fraction collector (0.5 s). $[5\text{-}^3\text{H}]\text{glucose}$ and $[^3\text{H}]\text{water}$ were separated by lyophilization and ^3H and ^{14}C were measured by isotope discrimination. The radioactivity in each fraction was divided by the total injected radioactivity and the time interval (fraction per second).

rate = $(1 - R_c) \cdot F \cdot G/W$. The rates calculated in this way are shown in table 3 and demonstrate that quercetin decreases $[5\text{-}^3\text{H}]\text{glucose}$ removal by approximately 33%. The rates of $[5\text{-}^3\text{H}]\text{glucose}$ removal do not mean net glucose uptake. Actually, the liver from fed rats shows net glucose release. $[5\text{-}^3\text{H}]\text{glucose}$ removal reflects most probably the unidirectional flow of free glucose into glycolysis (Malaisse *et al.* 1988). The rate of glucose transport, on the other hand, can be calculated from the transfer coefficient for influx (k_{in} ; see equation 1), the extracellular glucose concentration (G) and the cellular space per gram liver weight (V_i), i.e. influx = $k_{in} \cdot G \cdot V_i$. The unidirectional transfer coefficient was obtained by fitting equation (1) to the normalized outflow profiles of $[5\text{-}^3\text{H}]\text{glucose}$. As shown in table 3, glucose transport was not significantly affected by quercetin. It is, thus, unlikely that inhibition of transport could be causing a reduction in $[5\text{-}^3\text{H}]\text{glucose}$ removal or glucose accumulation within the cytosol in the presence of high quercetin concentrations.

Table 3. Rates of unidirectional influx and sequestration rate of 1 mM [5-³H]glucose in the perfused rat liver and the influence of quercetin.

Parameter	Control (<i>n</i> = 5)	Quercetin (500 µM) (<i>n</i> = 5)
Rate of [5- ³ H]glucose sequestration (µmol min ⁻¹ g ⁻¹)	0.393 ± 0.026 ^a	0.263 ± 0.047 ^a
Glucose unidirectional influx rate (µmol min ⁻¹ g ⁻¹)	3.21 ± 0.34 ^b	2.76 ± 0.51 ^b

Data were obtained from indicator dilution experiments like those shown in figure 1. Sequestration rates were estimated from the recovered fractional [5-³H]glucose radioactivity (R_c), the flow rate (F), the wet liver weight (W) and the extracellular glucose concentration (G), i.e. net uptake = $(1 - R_c) \cdot F \cdot G / W$. The unidirectional influx rates were calculated from the computed unidirectional transfer coefficient (k_{in} ; see equation 1), the extracellular glucose concentration (G) and the cellular space per gram liver weight (V_i), i.e. influx = $k_{in} \cdot G \cdot V_i$. The unidirectional transfer coefficient was obtained by fitting equation (1) to the normalized outflow profiles of [5-³H]glucose. p refer to the Student's t -test.

^a $p = 0.042$, ^b $p = 0.48$.

Discussion

In general terms, it is apparent from the results obtained here that quercetin can stimulate glycogenolysis in the liver and can also affect several related parameters as, for example, oxygen uptake and glycolysis. Additionally, quercetin affects several enzymatic systems, including ATPases and energy transduction in mitochondria. All these effects when taken together comprise a complex picture and present several apparent contradictions that make it difficult to derive precise cause-effect relationships.

Oxygen uptake stimulation is most probably the consequence of an uncoupling action, as evidenced by state IV respiration stimulation in isolated mitochondria as well as by the decrease in the respiratory control ratio (RC), both indicators of a decrease in the efficiency of energy transduction. Decreases in the efficiency of energy transduction in isolated mitochondria have also been suggested by Santos *et al.* (1997). It is generally accepted that the metabolic state of the mitochondria within the hepatic cells in the substrate-free perfused liver resembles closely that of state IV of isolated mitochondria (Scholz and Bücher 1965). On the other hand, diminution of energy transduction efficiency usually generates glycogenolysis stimulation, a phenomenon that was effectively observed (Constantin *et al.* 1995, Lopez *et al.* 1998).

Besides those phenomena that can be, partly at least, considered as a consequence of a deleterious action on energy transduction in the mitochondria, there are other observations that require alternative interpretations. Decreases in the efficiency of energy transduction in the mitochondria should equally lead to increases in glycolysis as a compensatory phenomenon (Constantin *et al.* 1995, Lopez *et al.* 1998). The opposite, however, was found: glycolysis dependent on endogenous glycogen was diminished by quercetin. On the other hand, ATP levels, which should have been diminished, were not affected to any significant extent. This phenomenon can be interpreted as a consequence of a significant drop in the ATP demand, caused mainly by inhibition of the Na⁺/K⁺-ATPase, whose activity under normal conditions is quite significant. In non-hepatic cells, diminution of the ATP demand due to Na⁺/K⁺-ATPase inhibition leads to a substantial diminution of the ADP concentration, a phenomenon that is likely to cause inhibition of glycolysis (Suolinna *et al.* 1973, 1975, Trejo *et al.* 1995). In liver cells, this phenomenon cannot be the cause for glycolysis inhibition, because the

ADP content was not changed by quercetin. Inhibition of the Na^+/K^+ -ATPase and other ATPases, however, can lead to substantial alterations in the concentration of several substances and ions, as K^+ , for example. For the latter, a diminution of its cytosolic concentration can be expected, a phenomenon that should lead to a diminution in the pyruvate kinase activity which is strictly dependent on K^+ (Ramirez-Silva *et al.* 2001). Furthermore, a direct inhibitory effect on pyruvate kinase seems equally possible (Grisolia *et al.* 1975). A diminution of the pyruvate kinase activity, in turn, should also lead to a diminution of glycolysis. It should be stressed that besides decreasing glycolysis from endogenous glycogen, as revealed by the decreased lactate production, quercetin also decreased $[5\text{-}^3\text{H}]\text{glucose}$ uptake even under conditions of net glucose release. Therefore, it seems that inhibition of glycolysis is not simply a feature of tumour or testis cells (Suolinna *et al.* 1973, 1975, Trejo *et al.* 1995) but more likely a property of quercetin that can be extended to mammal cells in general.

A finding of the present work that can be very important for the metabolic effects of quercetin is the increased concentration of glucose 6-phosphate and is most probably caused by the diminished glucose 6-phosphatase activity. It could be very important for three reasons: (1) glucose 6-phosphate is an important intermediate for several metabolic pathways: glycogen synthesis, the pentose monophosphate shunt, glycolysis and gluconeogenesis; (2) the compound is also an important signalling molecule whose effects can be very complex, especially within a context of additional complexities already generated by the action of quercetin on other parameters (Dijk *et al.* 2001); and (3) increased concentrations of glucose 6-phosphate could appear in combination with increases in other phosphorylated compounds due, especially, to enzyme equilibria within the cytosol. As a signalling compound, glucose 6-phosphate, for example, could have contributed to a diminution of net $[5\text{-}^3\text{H}]\text{glucose}$ uptake, which depends on the activity of glucokinase. Inhibition of this enzyme in the intact cell is one of the metabolic effects of glucose 6-phosphate (Dijk *et al.* 2001). Glucose 6-phosphate could equally contribute to the characteristic concentration dependence of glucose release: maximal stimulation at $300\text{ }\mu\text{M}$, less stimulation at 400 and no stimulation at $500\text{ }\mu\text{M}$. It is possible that at the highest quercetin concentrations the glucose 6-phosphate concentration within the cell is high enough so as to cause a decrease in glycogen degradation. In fact, as a signalling compound glucose 6-phosphate stimulates the activity of glycogen synthase b and of glycogen synthase phosphatase, thus leading to an increase in glycogen synthesis and consequently to a decrease in glycogen degradation (Bollen *et al.* 1998). A repartitioning of glucose 6-phosphate into glycogen synthesis at high cellular glucose 6-phosphate concentrations has indeed been reported by Dijk *et al.* (2001). The accumulation of glucose 6-phosphate caused by quercetin cannot explain all the excess of glucose release that occurred when the 400 and $500\text{ }\mu\text{M}$ infusions of the drug were interrupted (figure 3). This is probably an indication that high quercetin concentrations can influence the cell economy to such an extent that considerable time and many compensatory phenomena are necessary for restoration of the normal conditions when the chemical is removed.

An observation of the present work is particularly difficult to interpret: quercetin reduced lactate production but had no effect on pyruvate production. This means, obviously, a reduction in the lactate-to-pyruvate ratio. In addition, since lactate dehydrogenase in the liver is a near-equilibrium enzyme, this also

means a shift of the cytosolic NAD^+ -NADH potential in the direction of more positive values (Sies 1982). If extensively inhibited, the enzyme would no longer be able to operate under near-equilibrium conditions and the rate of pyruvate transformation into lactate would be decreased. However, in contrast to Grisolia *et al.* (1975), we found no inhibition of lactate dehydrogenase by quercetin, a phenomenon that would have actually influenced our lactate and pyruvate assays. It is thus highly probable that the cytosolic NAD^+ /NADH ratio was increased by quercetin. With the available data, it is impossible to arrive at any conclusion with respect to the mechanism of this phenomenon because there are so many possibilities. An autocatalytic oxidative action of quercetin on NADH can be excluded because no such phenomenon was observed during lactate dehydrogenase measurements and lactate or pyruvate assays. However, it could be that the introduction of quercetin stimulates reactions that require reducing equivalents in the cytosol. In addition, or alternatively, it could equally be that quercetin inhibits the transfer of reducing equivalents from the mitochondria to the cytosol. Irrespective of the mechanism that underlies this phenomenon, it is quite certain that a significant change in the cytosolic NAD^+ /NADH potential must have a significant influence on the metabolic state of the liver cells.

In conclusion, the results of the present work demonstrate that quercetin is a compound that can change liver metabolism amply and intensively. In this sense, the study fully corroborates previous investigations with other experimental systems (Suolinna *et al.* 1973, 1975, Murakami *et al.* 1992, Trejo *et al.* 1995, Santos *et al.* 1997). The quercetin concentrations employed here were those used in most studies. Such concentrations, however, are impossible to reach when quercetin is ingested in the normal diet (Peterson and Dwyer 1998). Consequently, effects like those reported here and by many other studies can only be expected under in vivo conditions if very high doses of quercetin are ingested or injected.

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Diterpene quinone tanshinone IIA selectively inhibits mouse and human cytochrome P4501A2

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Received 5 December 2002

1. Tanshinone IIA is the main active diterpene quinone in the herbal medicine *Salvia miltiorrhiza*. In untreated mouse liver microsomes, tanshinone IIA selectively inhibited 7-ethoxyresorufin *O*-deethylation (EROD) and 7-methoxyresorufin *O*-demethylation (MROD) activities without affecting the oxidation of benzo(a)pyrene, tolbutamide, *N*-nitrosodimethylamine and nifedipine. Tanshinone IIA was a competitive inhibitor of MROD activity with a K_i of 7.2 ± 0.7 nM.

2. In 3-methylcholanthrene-treated mouse liver microsomes, tanshinone IIA and two minor tanshinones, tanshinone I and cryptotanshinone, inhibited liver microsomal MROD activity without affecting EROD and benzo(a)pyrene hydroxylation activities at the concentrations up to 1 μ M. Tanshinone IIA induced a type I binding spectrum with a spectral dissociation constant K_s of 2.3 ± 0.8 μ M without cooperativity.

3. In human liver microsomes, tanshinone IIA decreased EROD and MROD activities without affecting the oxidation of benzo(a)pyrene, tolbutamide, chlorzoxazone and nifedipine.

4. In *Escherichia coli* membranes expressing bicistronic human CYP1A enzymes, tanshinone IIA inhibited EROD activity of CYP1A1 with an IC_{50} 48 times higher than that for CYP1A2. Tanshinone I and cryptotanshinone had the same IC_{50} ratio (1A1/1A2) of 4.

5. The results indicate that tanshinone represents a new group of CYP1A inhibitors, and tanshinone IIA had the highest selectivity in inhibition of CYP1A2.

Introduction

The root of *Salvia miltiorrhiza* has been used as a traditional Chinese medicine for the treatment of coronary heart diseases, cerebrovascular diseases and neuroathenic insomnia. Tanshinone IIA (figure 1), a diterpene quinone, is the main active constituent in *S. miltiorrhiza*. Two structurally related compounds, tanshinone I and cryptotanshinone, have been identified in minor quantities in chloroform extracts of *S. miltiorrhiza*. The estimated amounts of tanshinone IIA, tanshinone I and cryptotanshinone in chloroform extracts of *S. miltiorrhiza* were 1.11, 0.06 and 0.34 g kg⁻¹ dried root, respectively (Wu *et al.* 1991). However, the contents and relative abundance of tanshinones in *S. miltiorrhiza* vary between preparations using different fractionation solvents (Wu *et al.* 1991, Sato *et al.*

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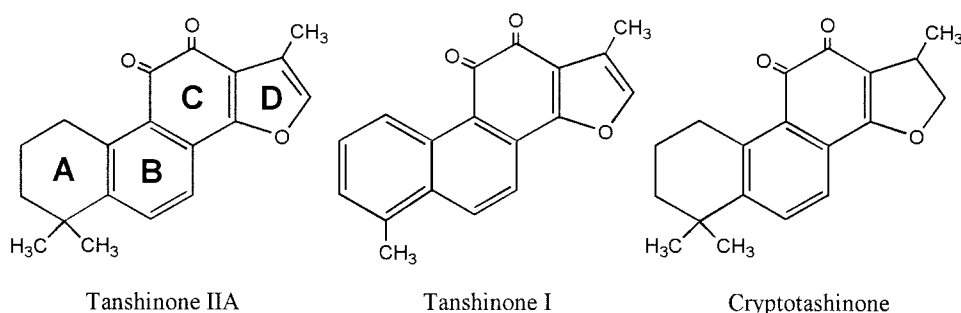


Figure 1. Structures of tanshinone IIA, tanshinone I and cryptotanshinone.

1992). Tanshinones possess a variety of pharmacological activities including antioxidative effects (Cao *et al.* 1996), diminution of cancer cell proliferation (Yoon *et al.* 1999), reduction of myocardial infarct size (Wu *et al.* 1999) and modulation of immunological responses (Kang *et al.* 2000).

Microsomal cytochrome P450 (CYP)-dependent monooxygenases play a key role in the detoxication and bioactivation of xenobiotics including drugs and chemical carcinogens. P450-catalysed oxidations can be modulated by natural products including medicinal herbs, fruits and vegetables (Ioannides 2002). The resulting induction and inhibition of P450 may change the pharmacological activities and toxicities of drugs (Guengerich 1997, Lin and Lu 1998). Tanshinones at low concentration enhance the mutagenicity of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) in *Salmonella typhimurium* TA98, but this enhancement was reduced at higher concentrations (Sato *et al.* 1992). Tanshinones are not mutagenic and CYP1A1/CYP1A2 are the main enzymes involved in the oxidative activation of Trp-P-1, but the effects of tanshinones on P450s have not been reported. The elevated incidence of concurrent use of herbal medicine and drugs increases the possibility of drug interactions. To assess the possible adverse effects resulting from the interaction of medicinal herbs and drugs, it is important to determine the effects of natural products and their active components on P450 enzymes. Here, the *in vitro* effects of tanshinones on P450-catalysed oxidations have been studied using mouse and human liver microsomes and bicistronic human CYP1A1 and CYP1A2.

Materials and methods

Chemicals

Benzo(a)pyrene, chlorzoxazone, cytochrome *c*, 7-ethoxyresorufin, 7-methoxyresorufin, NADH, NADPH and nifedipine were from Sigma-Aldrich (St Louis, MO, USA). Tolbutamide, 4-hydroxytolbutamide and 6-hydroxychlorzoxazone were from Sigma/RBI (Natick, MA, USA).

Isolation of tanshinones

Tanshinone IIA, tanshinone I and cryptotanshinone were isolated from *S. miltiorrhiza*. The root of *S. miltiorrhiza* (5 kg) was extracted with ethyl acetate and then concentrated *in vacuo* to prepare an ethyl acetate extract (about 10 g). This extract was subjected to silica gel column chromatography using an *n*-hexane and ethyl acetate gradient solvent system. The subfractions of 15% ethyl acetate eluate were recrystallized in *n*-hexane to obtain tanshinones I and IIA. The 20–25% ethyl acetate eluate was further purified using a silica gel column (20% ethyl acetate/*n*-hexane) and recrystallized in ethanol to obtain cryptotanshinone. Tanshinones were identified by comparison of their spectroscopic data including ^1H , ^{13}C -NMR and electron-impact mass spectra with literature values (Onitsuka *et al.* 1983, Honda *et al.* 1988, Ryu *et al.* 1997). The purity of tanshinones were 98–99% as analysed by NMR and HPLC.

Microsomal preparation and bicistronic human P450 expression

Male C57BL/6J mice (5 weeks, 13–15 g) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan, ROC). Mice were allowed a 1-week acclimation in the animal centre with air-conditioning and an automatically controlled photoperiod of 12 h light daily. Mice were treated with a single injection of 80 mg 3-methylcholanthrene (3-MC) kg⁻¹ intraperitoneally. After 48 h, mouse liver microsomes were prepared by differential centrifugation (Alvares and Mannerling 1970). Caucasian liver samples (CaL) were obtained through Tennessee Donor Services (Nashville, TN, USA). Chinese liver samples (ChL) were obtained from patients undergoing liver resection in the National Taiwan University Hospital (Taipei, Taiwan, ROC). Human liver microsomes were prepared following the method of Guengerich (1994). Bicistronic human CYP1A1 and CYP1A2 constructs consisting of the coding sequence of a P450 followed by that of NADPH-P450 reductase were constructed in a pCW vector and transformed to *Escherichia coli* DH5 α by electroporation. Bacterial membrane fractions were prepared as described by Parikh *et al.* (1997). Microsomes and bacterial membrane fractions were stored at -75 °C until use.

Enzyme assays

Microsomal protein concentration was determined by the method of Lowry *et al.* (1951). P450 content was determined by the spectrophotometric method of Omura and Sato (1964). NADPH-P450 reductase activity was determined following the method of Phillips and Langdon (1962) using cytochrome *c* as a substrate. Benzo(a)pyrene hydroxylation (aryl hydrocarbon hydroxylation, AHH) was determined by measuring the fluorescence of phenolic metabolites (Nebert and Gelboin 1968). *O*-dealkylation of 7-ethoxyresorufin (EROD) and 7-methoxyresorufin (MROD) were determined by measuring the fluorescence of resorufin (Pohl and Fouts 1980). Tolbutamide hydroxylation (TOH) was determined by HPLC analysis (Yamazaki *et al.* 1998). Chlorzoxazone hydroxylation was assayed following the method of Peter *et al.* (1990). *N*-nitrosodimethylamine demethylation (NDM) was determined by measuring the formation of formaldehyde using the Nash (1953) reagent. Nifedipine oxidation (NFO) was determined following the method of Guengerich *et al.* (1986). Concentrations of substrates used in assays were as described by Ueng *et al.* (2002). In the EROD assays of bacterial membrane expressing bicistronic P450s, the 1-ml reaction mixture contained 20 pmol P450, an NADPH-generating system (5 mM MgCl₂, 0.5 mM NADP⁺, 5 mM glucose 6-phosphate, and 0.35 μ M glucose 6-phosphate dehydrogenase), and 2 μ M 7-ethoxyresorufin in 0.1 M potassium phosphate buffer, pH 7.4. Tanshinones were dissolved in DMSO and stored in dark brown glass vials. The final concentration of DMSO in microsomal and membrane reaction mixture was less than 1%. The same volume of DMSO was added to the control.

Data and statistical analysis

The concentrations of tanshinones required for 50% inhibition of catalytic activities (IC₅₀) were calculated by curve fitting (Grafit, Erithacus Software Ltd, Staines, UK). Kinetic analysis of MROD activity was done by fitting the Michaelis–Menten equation. Velocities (*v*) at various substrate concentrations (*S*) were fitted by nonlinear least-squares regression without weight due to the equation, consistent with competitive inhibition according to the Michaelis–Menten equation:

$$v = V_m^* S / (K_m^* [1 + (I/K_i)] + S),$$

where *V_m*, *S* and *I* are the maximal velocity, substrate concentration and tanshinone IIA concentration, respectively (Sigma Plot, Jandel Scientific, San Rafael, CA, USA). For the spectral binding of tanshinone IIA with P450, absorbance differences ($\Delta A_{389-420\text{ nm}}$) at various concentrations of tanshinone IIA were fitted using nonlinear least-squares regression without weight due to the equation:

$$\Delta A = (\Delta A_{\text{max}}^* T^n) / (K_s^n + T^n),$$

where *T* is the tanshinone IIA concentration and *n* is a measure of the cooperativity, respectively. The statistical significance of differences between control and treated animals was evaluated by the Student's *t*-test. *p* < 0.05 was considered as statistically significant.

Results

Effects of tanshinones on mouse liver microsomal monooxygenase activities in vitro

In liver microsomes of untreated mice, pre-incubation of tanshinone IIA and microsomes in the presence and absence of 0.5 mM NADPH at 37 °C for 10 min had no effects on spectrally detectable P450 and tanshinone IIA at the concentra-

Table 1. IC_{50} values of tanshinone IIA for 7-ethoxyresorufin *O*-deethylation and 7-methoxyresorufin *O*-demethylation activities in mouse and human liver microsomes.

	7-Ethoxyresorufin <i>O</i> -deethylation		7-Methoxyresorufin <i>O</i> -demethylation	
	nmol min ⁻¹ mg ⁻¹	IC_{50} (μ M)	nmol min ⁻¹ mg ⁻¹	IC_{50} (μ M)
Mouse liver				
Control	0.34 ± 0.02	0.25 ± 0.06	0.59 ± 0.02	0.18 ± 0.02
3-Methylcholanthrene	$5.55 \pm 0.38^*$	5.53 ± 0.63	$4.49 \pm 1.80^*$	0.40 ± 0.04
Human Ch5L	0.30	0.20 ± 0.03	0.27	0.38 ± 0.04

Microsomal protein used in the activity determination was 0.1 mg ml^{-1} in the reaction mixture.

O-dealkylation of 7-ethoxyresorufin and 7-methoxyresorufin were determined using $2 \mu\text{M}$ 7-ethoxyresorufin and $20 \mu\text{M}$ 7-methoxyresorufin, respectively.

Human liver microsomes were prepared from a Chinese liver sample, Ch5L.

Results are the mean $\pm$ SE of three mice.

* Significantly different from the control values, $p < 0.05$.

For IC_{50} values, estimates of variance (denoted by $\pm$) are presented from analysis of the means of three mouse and individual human liver microsomes with duplicate determinations.

tions of $0.05\text{--}1 \mu\text{M}$ had no effects on the cytochrome *c* reduction activity of NADPH-P450 reductase (data not shown). In untreated mouse liver microsomes, tanshinone IIA inhibited EROD and MROD activities with $IC_{50} = 0.25 \pm 0.06$ and $0.18 \pm 0.01 \mu\text{M}$, respectively (table 1). However, microsomal AHH, TOH, NDM and NFO activities were not changed by tanshinone IIA at concentrations up to $1 \mu\text{M}$ (figure 2A). Addition of tanshinone I and cryptotanshinone also inhibited EROD and MROD activities without affecting AHH, NDM and NFO activities (figure 2B, C). Tanshinone I inhibited EROD and MROD activities with $IC_{50} = 0.60 \pm 0.08$ and $0.12 \pm 0.01 \mu\text{M}$, respectively. Cryptotanshinone inhibited EROD and MROD activities with $IC_{50} = 0.85 \pm 0.12$ and $0.21 \pm 0.02 \mu\text{M}$, respectively. At $1 \mu\text{M}$, tanshinone I and cryptotanshinone caused 22 and 18% decreases of TOH activities, respectively.

To elucidate the selectivity of inhibition on CYP1A1- and CYP1A2-catalysed oxidations, mice were treated with 3-MC to induce CYP1A1 and CYP1A2. Treatment of mice with 3-MC resulted in 16-, 8- and 12-fold increases of EROD, MROD and AHH activities, respectively (table 1). In liver microsomes of 3-MC-treated mice, tanshinone IIA, tanshinone I and cryptotanshinone inhibited MROD activity with $IC_{50} = 0.40 \pm 0.04$, 0.21 ± 0.01 and $0.44 \pm 0.04 \mu\text{M}$, respectively (table 1 and figure 2D–F). In contrast, microsomal EROD and AHH activities were not affected by tanshinone IIA, tanshinone I or cryptotanshinone at concentrations up to $1 \mu\text{M}$ (figure 2D–F).

Kinetics of inhibition of 7-methoxyresorufin O-demethylation activity by tanshinone IIA in mouse liver microsomes

The velocity (*v*) versus substrate concentration (*S*) plot is shown in figure 3. Analysis of Lineweaver–Burk and Dixon plots indicated that tanshinone IIA was a competitive inhibitor of MROD activity with a $K_i = 7.2 \pm 0.7 \text{ nM}$ as analysed by nonlinear regression with the initial estimate from Dixon plot (13.8 nM). Pre-incubation of mouse liver microsomes with tanshinone IIA in the presence of an

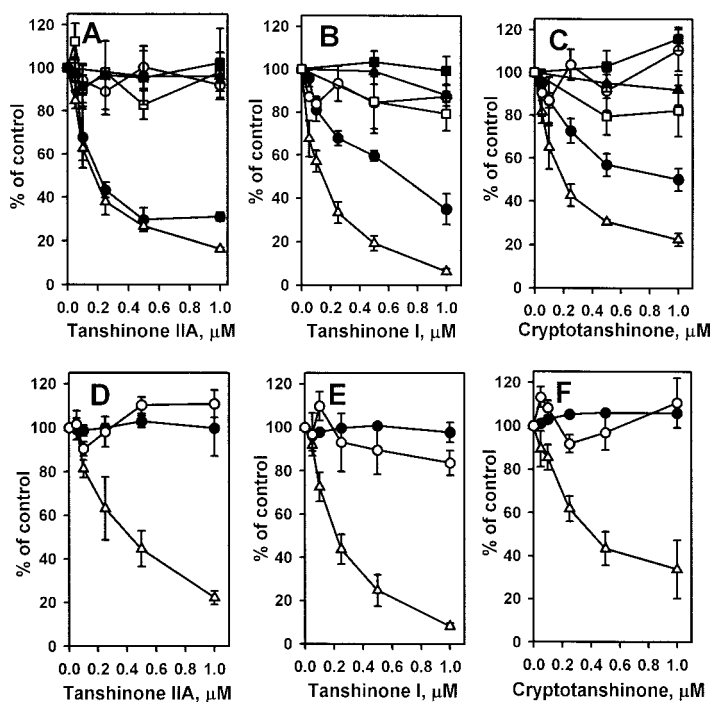


Figure 2. Effects of tanshinone IIA, tanshinone I and cryptotanshinone on monoxygenase activities of liver microsomes in untreated mice (A–C) and 3-methylcholanthrene-treated mice (D–F). Microsomal 7-ethoxyresorufin *O*-deethylation (●), 7-methoxyresorufin *O*-demethylation (Δ), benzo(a)pyrene hydroxylation ($\circ$), tolbutamide hydroxylation ($\square$), *N*-nitrosodimethylamine demethylation ($\blacktriangle$) and nifedipine oxidation ($\blacksquare$) activities were determined as described in the Materials and methods and are given as the mean $\pm$ SE of three mice.

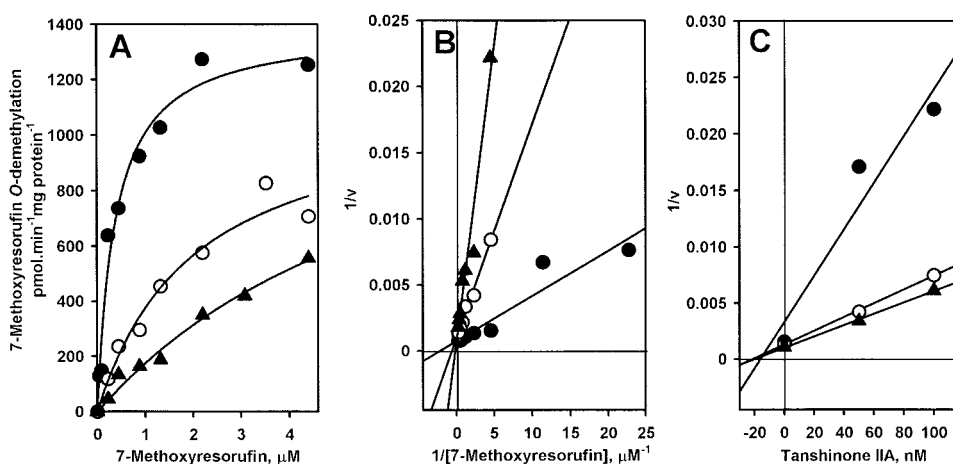


Figure 3. Kinetic properties of the inhibitory effect of tanshinone IIA on 7-methoxyresorufin *O*-demethylation activity. (A) velocity (v) versus substrate concentration (S) plot. Tanshinone IIA concentrations were: 0 (●), 50 ($\circ$) and 100 ($\blacktriangle$) nM. (B) Lineweaver–Burk plot. Tanshinone IIA concentrations employed were: 0 (●), 50 ($\circ$) and 100 (Δ) nM. (C) Dixon plot of 7-methoxyresorufin *O*-demethylation at 0.22 (●), 0.44 ($\circ$) and 0.88 ($\blacktriangle$) μM 7-methoxyresorufin. Solid lines represent the lines of best fit as determined by linear regression analysis. Data are the means of duplicate determinations.

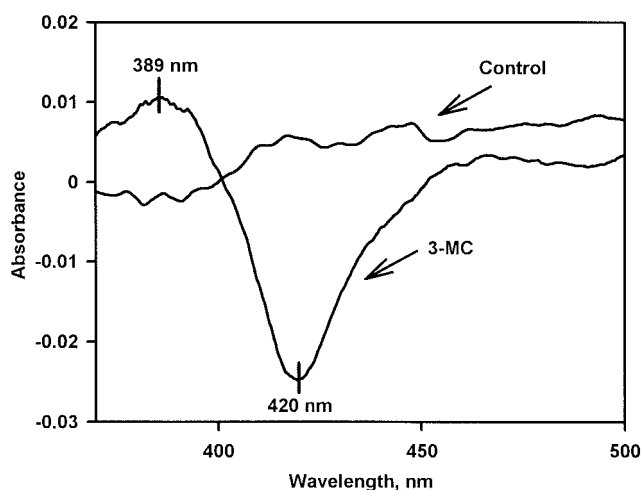


Figure 4. Spectral difference due to interactions of tanshinone IIA with liver microsomal cytochrome P450. Spectra were recorded at 23 °C after addition of 1.4 μ M tanshinone IIA to liver microsomes from control (2.2 mg protein ml^{-1}) and 3-MC-treated (2.6 mg protein ml^{-1}) mice.

NADPH-generating system at 37 °C for 10 min had no effect on the inhibition of MROD activity by tanshinone IIA (data not shown).

To examine the binding of tanshinone IIA to microsomal P450, difference spectra for the interaction of tanshinone IIA and microsomal P450 were analysed. Tanshinone IIA yielded a typical type I binding spectrum in 3-MC-treated mouse liver microsomes (figure 4). The binding yielded a hyperbolic ΔA versus tanshinone IIA concentration plot. The apparent spectral dissociation constant K_s analysed from the binding spectra was $2.3 \pm 0.8 \mu\text{M}$. A ΔA_{max} was 0.04 ± 0.01 (mg protein) $^{-1}$ (figure 5A). The spectrum differences induced by tanshinone IIA were fitted to both hyperbolic (ΔA versus tanshinone IIA concentration) and Scatchard plots using nonlinear regression. The cooperativity parameters (n) determined from the hyperbolic and Scatchard plots were 1.0 ± 0.0 ($r^2 = 0.99$) and 1.2 ± 0.2 (0.99), respectively (figure 5A,C), and the n calculated from the linear regression of the Hill plot was 0.91 ($r^2 = 0.97$) (figure 5B).

Effects of tanshinone IIA on monooxygenase activities in human liver microsomes and bicistronic human CYP1A1 and CYP1A2

Tanshinone IIA inhibited human liver (Ch5L) microsomal EROD and MROD activities with $\text{IC}_{50} = 0.20 \pm 0.03$ and $0.38 \pm 0.04 \mu\text{M}$, respectively. Tanshinone IIA (0.5 μM) resulted in 52 and 47% reduction of EROD and MROD activities in six human liver samples, respectively (table 2). In contrast, microsomal oxidation of benzo(a)pyrene, tolbutamide, chlorzoxazone and nifedipine were not inhibited by tanshinone IIA. To elucidate the selectivity of tanshinone inhibition on CYP1A1 and CYP1A2, the EROD activity of bacterial membranes expressing human P450s was determined in the presence of increasing concentrations of tanshinones. EROD activities of CYP1A1 and CYP1A2 were 67 ± 3 and $5.3 \pm 1.0 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ P450}$, respectively. The IC_{50} of tanshinone IIA for CYP1A1-catalysed EROD activity was 48 times higher than that for CYP1A2

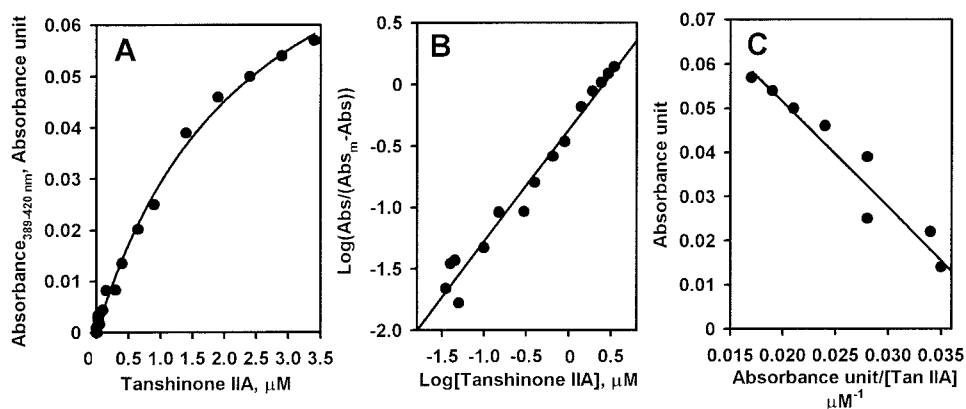


Figure 5. Kinetic properties of spectral difference due to interactions of tanshinone IIA with liver microsomal cytochrome P450. (A) Absorbance versus tanshinone IIA concentration plot. (B) Hill plot. (C) Absorbance versus absorbance/tanshinone IIA concentration plot.

Table 2. Effects of tanshinone IIA on cytochrome P450-catalysed oxidations in human liver microsomes.

Oxidations	Percentage of control
7-Ethoxyresorufin <i>O</i> -deethylation	48 $\pm$ 5
7-Methoxyresorufin <i>O</i> -demethylation	53 $\pm$ 7
Benzo(a)pyrene hydroxylation	125 $\pm$ 14
Tolbutamide hydroxylation	109 $\pm$ 15
Chlorzoxazone hydroxylation	126 $\pm$ 10
Nifedipine oxidation	116 $\pm$ 16

Results are the per cent of activity in the presence of 0.5 μM tanshinone IIA to that in the absence of tanshinone IIA.

Results are the mean $\pm$ SE of liver microsomes of three Chinese and three Caucasians.

The representative control activities of 7-ethoxyresorufin *O*-deethylation, 7-methoxyresorufin *O*-demethylation, benzo(a)pyrene hydroxylation, tolbutamide hydroxylation, chlorzoxazone hydroxylation and nifedipine oxidation were in the ranges (mean $\pm$ SE) of 0.08–0.30 (0.17 $\pm$ 0.05), 0.03–0.24 (0.12 $\pm$ 0.03), 0.10–0.29 (0.18 $\pm$ 0.03), 0.29–0.43 (0.35 $\pm$ 0.02), 0.55–1.34 (0.87 $\pm$ 0.11) and 0.09–1.11 (0.41 $\pm$ 0.15) nmol min⁻¹ mg⁻¹ protein, respectively.

(table 3). The IC₅₀'s of tanshinone I and cryptotanshinone for CYP1A1 were four and four times higher than those for CYP1A2, respectively.

Discussion

Microsomal P450 comprises a family of enzymes that show relatively broad substrate specificities. EROD and MROD activities are mainly catalysed by CYP1A including CYP1A1 and CYP1A2. In untreated mice and most human tissue samples, CYP1A2 is the major hepatic CYP1A member and CYP1A1 is essentially an extrahepatic enzyme (Dey *et al.* 1999). *N*-nitrosodimethylamine and chlorzoxazone were mainly oxidized by CYP2E1 and tolbutamide and nifedipine were mainly oxidized by CYP2C and CYP3A in mammals, respectively (Guengerich 1995). The tanshinones isolated from *S. miltiorrhiza* have a common structural feature of a planar phenanthrene quinone. Sato *et al.* (1992) reported

Table 3. IC_{50} values of tanshinone IIA, tanshinone I and cryptotanshinone on 7-ethoxyresorufin *O*-deethylation activity catalysed by bicistronic human CYP1A1 and CYP1A2.

Tanshinone	IC_{50} (μ M)		IC_{50} ratio (1A1/1A2)
	CYP1A1	CYP1A2	
Tanshinone IIA	4.30 ± 0.72	0.09 ± 0.03	48
Tanshinone I	0.75 ± 0.02	0.19 ± 0.03	4
Cryptotanshinone	2.20 ± 0.21	0.56 ± 0.02	4

Membranes expressing human CYPs used in the activity determination were added at the level of $20 \text{ pmol P450 ml}^{-1}$ in the reaction mixture.

O-dealkylation of 7-ethoxyresorufin was determined using $2 \mu\text{M}$ 7-ethoxyresorufin.

Bacterial membrane 7-ethoxyresorufin *O*-deethylation activities of bicistronic human CYP1A1 and CYP1A2 were 67.3 ± 3.1 and $5.3 \pm 1.0 \text{ nmol min}^{-1} \text{ nmol P450}^{-1}$, respectively.

Estimates of variance (denoted by $\pm$) are presented from analysis of the means of duplicate determinations.

that $20 \mu\text{g/plate}$ tanshinones enhanced the mutagenicity of Trp-P-1 activated by Aroclor 1254-treated rat liver microsomes. This concentration was in the range used in our study, but tanshinones had no stimulatory effects on mouse and human liver microsomal P450 activities. To address the mechanism of mutagenicity modulation, the possible causes such as effects of tanshinones on rat P450 and the bacterial sensitivity need further studies. Our results have shown that tanshinones extensively inhibit EROD and MROD activities in liver microsomes of untreated mice (figure 2A–C). In contrast, the oxidations of benzo(a)pyrene, tolbutamide, *N*-nitrosodimethylamine and nifedipine are not affected or only slightly inhibited. These results indicate that tanshinones are selective CYP1A inhibitors without affecting CYP2C, CYP2E1 and CYP3A. Tanshinone IIA inhibited EROD and MROD activities with similar IC_{50} values. Tanshinone I and cryptotanshinone inhibited EROD activity with IC_{50} values five times and four times higher than that of MROD activity, respectively. 7-Methoxyresorufin was preferentially *O*-demethylated by CYP1A2 in 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin-treated mice and 7-ethoxyresorufin is preferentially *O*-deethylated by CYP1A1 in 3-MC-treated mice (Gradelet *et al.* 1997). CYP1A1 has the highest AHH activity and was induced by 3-MC. In liver microsomes of 3-MC-treated mice, tanshinone IIA, tanshinone I and cryptotanshinone inhibited MROD activity without affecting EROD and AHH activities at concentrations up to $1 \mu\text{M}$ (figure 2D–F). These results revealed that tanshinone IIA, tanshinone I and cryptotanshinone were mouse CYP1A2 inhibitors. The presence of unsaturated bonds in the A and D moieties of tanshinones (figure 1) ad no obvious influence on CYP1A2 selectivity.

Our results with liver microsomes of 3-MC-treated mice showed that tanshinone IIA yielded a typical type I difference spectrum (figure 4), corresponding to a shift from low- to high-spin iron (Schenkman *et al.* 1967). The Hill coefficient obtained from the linear regression of Hill plot and nonlinear regression of Scatchard plot were 0.98 and 1.01, respectively (figure 5C, D), revealing no cooperativity. 3-MC-treatment caused substantial induction of CYP1A1 and CYP1A2. In liver microsomes of 3-MC-treated mice, the K_s value of tanshinone

IIA was relatively high compared with the IC_{50} and K_i values of MROD activity. The high K_s might attribute to the less inhibition of EROD activity, which is mainly catalysed by CYP1A1 in 3-MC-treated mice. Tanshinone IIA was a competitive inhibitor of MROD activity of mouse liver microsomes (figure 3). This inhibition was not enhanced by the pre-incubation of microsomes and tanshinone IIA in the presence of a NADPH-generating system (data not shown). This result indicates that NADPH-dependent microsomal metabolism is not a major requirement for the inhibitory effect. The pre-incubation of microsomes with benzoquinone in the presence of NADPH could cause protein destruction (Souček 1999). Thus, the furano-*o*-quinone moiety of tanshinones could be responsible for the production of reactive free radicals to cause enzyme destruction. However, our results showed that the microsomal CO-difference spectra were not affected by the pre-incubation of microsomes with tanshinones in the absence and presence of an NADPH-generating system. The cytochrome *c* reduction activity of NADPH-P450 reductase was not affected by the addition of tanshinone IIA. Souček (1999) reported that CYP3A and CYP1A enzymes are sensitive to destruction by hydroquinone and benzoquinone and that CYP3A was the P450 enzyme most sensitive to this destruction. However, our results showed that the quinone tanshinones had no effect on mouse and human CYP3A-catalysed nifedipine oxidation. The mechanisms of inhibition caused by tanshinones are therefore likely different from that of benzoquinone.

In human liver microsomes, CYP1A2 is the only CYP1A member in the majority of human liver samples and CYP1A1 is expressed at very low levels in some human liver samples (Guengerich 1995). Tanshinone IIA inhibited human hepatic CYP1A2-catalysed EROD and MROD activities without affecting the oxidation of benzo(a)pyrene, tolbutamide, chlorzoxazone and nifedipine (table 2). Tanshinone IIA had the smallest IC_{50} for EROD activity of bacterial membranes expressing bicistronic human CYP1A2 and NADPH-P450 reductase. The IC_{50} of tanshinone IIA for CYP1A1-catalysed EROD activity was 48 times higher than that for CYP1A2 (table 3). The IC_{50} 's of tanshinone I and cryptotanshinone for CYP1A1 were also higher than those for CYP1A2, but with a lesser IC_{50} ratio. This is the first paper to show the selective CYP1A inhibition by the diterpene quinone tanshinone and that tanshinone IIA is a selective mouse and human CYP1A2 inhibitor.

Acknowledgements

This paper was supported by National Research Institute of Chinese Medicine, Taipei.

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***In vitro* inhibition of human small intestinal and liver microsomal astemizole *O*-demethylation: different contribution of CYP2J2 in the small intestine and liver**

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Received 5 December 2002

1. The effects of chemical agents on the metabolism of the antihistamine drug astemizole were investigated to evaluate drug–drug interactions.

2. Chemical inhibitors of astemizole *O*-demethylation were screened using the small intestinal and liver microsomes from rabbit as an animal model for the first-pass metabolism of humans. In the rabbit small intestine, astemizole *O*-demethylation was clearly inhibited by ebastine, arachidonic acid, α -naphthoflavone, ketoconazole, tranylcypromine, troglitazone and terfenadine.

3. In humans, these inhibitors also reduced microsomal astemizole *O*-demethylation in both the small intestine and liver. However, the inhibition rate of almost all these chemicals were clearly greater in the small intestine than in the liver. Thus, a different contribution of cytochrome P450 in each tissue is suggested.

4. All the chemicals inhibited astemizole *O*-demethylation in recombinant CYP2J2 microsomes. The results suggest that CYP2J2 is involved in astemizole *O*-demethylation in both the human small intestine and liver; however, the contribution in the liver is lower than in the small intestine. The effects of the CYP2J2 inhibitors during first-pass metabolism may be more important in the small intestine than in the liver. Since all the inhibition profiles of astemizole *O*-demethylation were different in the liver and small intestine, involvement of another P450 in astemizole *O*-demethylation in human liver may be speculated.

5. In the rabbit microsomal systems, the same metabolites found in humans were qualitatively detected and the inhibition profiles of the chemical agents in the microsomes resembled that of humans.

Introduction

Astemizole {1-(4-fluorophenylmethyl)-*N*-1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl-1*H*-benzimidazole-2-amine} is a potent non-sedative H₁-receptor antagonist effective for the treatment of allergic rhinitis or chronic urticaria (Richards *et al.* 1984). This drug is rapidly absorbed from the gastrointestinal tract and undergoes extensive first-pass metabolism to the pharmacologically active metabolite desmethylastemizole and other metabolites (figure 1) (Heykants *et al.* 1986, Meuldermans *et al.* 1986). Consequently, the AUC of astemizole is approximately 20 times less than that of desmethylastemizole. Recently, the involvement of CYP2J2 in the intestinal first-pass metabolism of astemizole to desmethylastemi-

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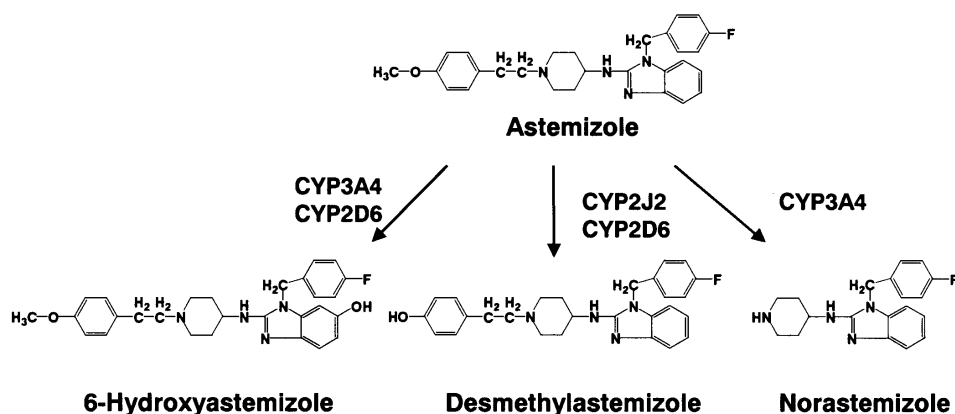


Figure 1. Proposed metabolic pathways of astemizole.

zole was reported (Matsumoto *et al.* 2002). Since higher concentrations of astemizole compared with desmethylastemizole were detected in the plasma during the first few hours after the oral administration of astemizole to humans (Heykants *et al.* 1986), the elimination of astemizole may have occurred in both the small intestine and the liver. However, the contribution of CYP2J2 in the metabolism of astemizole in the liver has not yet been clarified.

CYP2J2 is expressed in several tissues such as the small intestine, liver, heart, kidney and lung and may play a role in the oxidative bioactivation of arachidonic acid (Wu *et al.* 1996, Zeldin *et al.* 1996, 1997). However, the enzymatic properties of CYP2J2 with respect to drug–drug interactions have not been well characterized (Scarborough *et al.* 1999), nor has chemical inhibition of CYP2J2. In addition, substrate specificities or inhibition profiles of the CYP2J form have also not been studied between species. The high expression of CYP2J2 immunoreactive protein as well as the high activity of astemizole *O*-demethylation in the rabbit small intestinal microsomes may indicate the rabbit as a possible animal model for the CYP2J2-mediated drug interactions (Matsumoto *et al.* 2002). Thus, in the current study, the effect of several chemical agents on astemizole *O*-demethylation have been investigated using small intestinal and liver microsomes from both humans and rabbits.

Materials and methods

Materials

Individual human liver and intestinal microsomes were purchased from Xenotech LLC (Cambridge, KS, USA), Gentest Corp. (Woburn, MA, USA) and Tissue Transformation Technologies (Edison, NJ, USA). The rabbit liver and intestinal microsomes were prepared from male New Zealand white rabbits (2.0–2.4 kg) according to Nakamura *et al.* (2000). Recombinant CYP2J2 microsomes were prepared from COS-1 cells as described by Matsumoto *et al.* (2002). Sulfaphenazole, quinidine, tranilcyproline, terfenadine, α -naphthoflavone, arachidonic acid (Sigma Chemical Co., St Louis, MO, USA), coumarin (Wako Pure Chemical Industries, Osaka, Japan), astemizole and R55248 (1-[(4-fluorophenyl)methyl]-2-[[1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl]methyl]-1H-benzimidazole; Research Diagnostic, Inc., Flanders, NJ, USA), and ketoconazole (Research Biochemicals International, Natick, MA, USA) were purchased from the indicated sources. Troglitazone and ebastine were gifts from Sankyo Co., Ltd and Dainippon Pharmaceutical Co., Ltd, respectively. All other chemical used were of the highest grade available. The experiments with the human livers and intestines

were approved by the Tohoku University Ethics Committee. The animal experiments were undertaken under the instructions of the Tohoku University Animal Care and Use Committee.

Astemizole metabolism by human, recombinant CYP2J2 and rabbit microsomal systems

The incubation mixture for astemizole metabolism consisted of 10 μ M astemizole, 0.2 mg ml⁻¹ microsomes, an NADPH-generating system (6.0 mM magnesium chloride, 3.3 mM glucose 6-phosphate, 1.3 mM NADP, 1.0 U ml⁻¹ glucose 6-phosphate dehydrogenase) and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.2 ml. The reaction was initiated by the addition of the NADPH-generating system after 5 min of pre-incubation at 37°C and continued for 20–30 min. The reaction was terminated by the addition of 0.2 ml acetonitrile. After the addition of R55248 as the internal standard (2 μ M), the samples were centrifuged at 1800g for 5 min. Aliquots of the supernatant were analysed by liquid chromatography/tandem mass spectrometry (LC/MS). Astemizole was added as a methanolic solution where the final methanol concentration was less than 1% in the reaction mixtures.

Inhibition

α -Naphthoflavone (5–50 μ M, Newton *et al.* 1995), coumarin (50 μ M, Torchin *et al.* 1996), sulfaphenazole (50 μ M), quinidine (50 μ M), ketoconazole (1–10 μ M, all Newton *et al.* 1995), terfenadine (5–50 μ M, Jones *et al.* 1998), tranilcypromine (5–100 μ M, Taavitsainen *et al.* 2001, Zhang *et al.* 2001), troglitazone (5–50 μ M, Yamazaki *et al.* 2000), arachidonic acid (10–100 μ M) and ebastine (5–100 μ M, both Matsumoto *et al.* 2002) were used as chemical inhibitors. Arachidonic acid and ebastine, substrates of CYP2J2, were used as competitive inhibitors. The other inhibitors used are known inhibitors of human P450 activities or substrates of human P450s. The incubation mixture consisted of astemizole (10 μ M), an inhibitor, 0.2 mg ml⁻¹ human, rabbit or recombinant 2J2 microsomes, the NADPH-generating system and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.2 ml. The reaction was initiated by the addition of the NADPH-generating system after 5 min of pre-incubation at 37°C and continued for 20–30 min. The samples were processed and analysed as described above. Astemizole and the inhibitors were added as a methanolic or dimethylsulfoxide solution whose final methanol or dimethylsulfoxide concentration was less than 1% in the reaction mixtures.

LC/MS/MS apparatus

For the LC/MS/MS analysis of the astemizole metabolism, a Hewlett-Packard 1050 HPLC system (Palo Alto, CA) and Finnigan mat TSQ 700 (San Jose, CA) were used. Separation of the metabolites was accomplished at 40°C in a reversed-phase mode using Develosil ODS-HG-5 (2.0 $\times$ 150 mm, Nomura Chemical Co., Ltd, Tokyo, Japan). The mobile phase consisted of acetonitrile–20 mM ammonium acetate in the proportions of 54:46. The flow rate was set at 0.2 ml min⁻¹. The metabolite and internal standard were detected in the positive-ion electrospray ionization mode at the ion spray interface temperature of 200°C and identified using the transitions m/z 445 $\rightarrow$ 204 (desmethyl-astemizole) and 458 $\rightarrow$ 307 (R55248), or identified the m/z 325 (norastemizole) and m/z 475 (6-hydroxyastemizole). The offset voltage was –30 to –10 V, and nitrogen was used as the sheath gas. The rate of astemizole metabolism was determined according to a concurrently generated standard curve.

Results

Oxidative metabolism of astemizole in microsomes from human and rabbit

The oxidative metabolism of astemizole was examined in systems containing microsomes from human livers and small intestines, and rabbit livers and small intestines in the presence of 10 μ M astemizole (table 1). Astemizole was mainly converted to *O*-desmethylastemizole at 171 ± 57 pmol mg protein⁻¹ min⁻¹, norastemizole at 30 ± 13 pmol mg protein⁻¹ min⁻¹ and 6-hydroxyastemizole at 13 ± 7 pmol mg protein⁻¹ min⁻¹ in the human small intestine. Astemizole was also mainly converted to desmethylastemizole at 207 ± 82 pmol mg protein⁻¹ min⁻¹, norastemizole at 85 ± 47 pmol mg protein⁻¹ min⁻¹ and 6-hydroxyastemizole at 21 ± 5 pmol mg protein⁻¹ min⁻¹ in the human liver (mean $\pm$ SD of three samples). In the rabbit small intestine, astemizole was mainly converted to desmethylastemizole at 1017 pmol mg protein⁻¹ min⁻¹, norastemizole at 3 pmol mg

Table 1. Oxidative metabolism of astemizole in microsomes from human and rabbit.

Species	Microsome	Metabolite formation (pmol mg protein ⁻¹ min ⁻¹)†		
		Desmethylastemizole	Norastemizole	6-Hydroxyastemizole
Human‡	small intestine	171 ± 57	30 ± 13	13 ± 7
	liver	207 ± 82	85 ± 47	21 ± 5
Rabbit§	small intestine	1017	3	15
	liver	544	26	55

† Astemizole (10 μm) was incubated with microsomes from human and rabbit at 37 °C for 20–30 min in the presence of an NADPH-generating system.
‡ Mean ± SD of three samples.
§ Average of duplicate assays.

protein⁻¹ min⁻¹ and 6-hydroxyastemizole at 15 pmol mg protein⁻¹ min⁻¹. In rabbit liver prepared from the same animal, astemizole was also mainly converted to desmethylastemizole at 544 pmol mg protein⁻¹ min⁻¹, norastemizole at 26 pmol mg protein⁻¹ min⁻¹ and 6-hydroxyastemizole at 55 pmol mg protein⁻¹ min⁻¹ (average of duplicate assays).

Effects of the chemical agents on astemizole O-demethylation in microsomes from the rabbit small intestine and liver

The effects of chemical agents on astemizole O-demethylation using microsomes from the rabbit small intestine and liver were evaluated in order to screen the inhibition of CYP2J2 activities (figure 2). Among the tested agents, arachidonic acid and ebastine, CYP2J2 substrates, clearly decreased the rate of microsomal astemizole O-demethylation in a concentration-dependent manner in both organs. The inhibitory potencies of both agents were rather weak in the liver. The

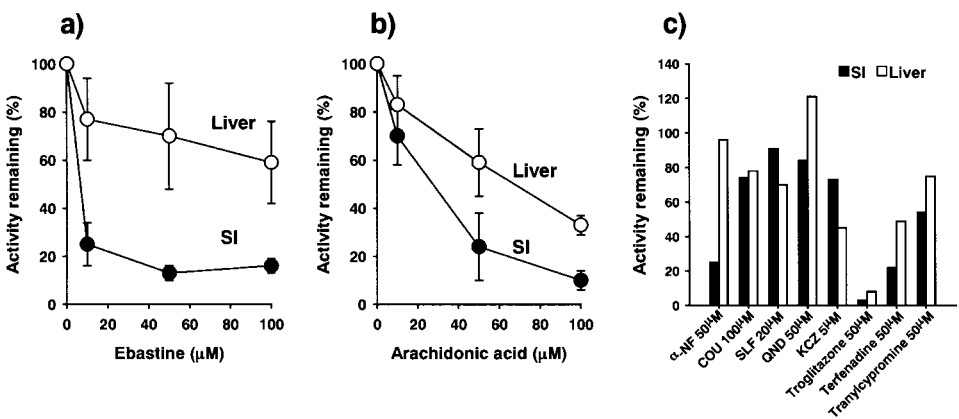


Figure 2. Effects of chemical agents on astemizole O-demethylation by rabbit small intestinal and liver microsomes. (a,b) Data are the per cent activity remaining relative to a control (methanol alone) incubation and are the mean ± SD of three samples. SI, small intestine. Control activities: SI 238, 679, 690 pmol mg protein⁻¹ min⁻¹; liver 127, 206, 223 pmol mg protein⁻¹ min⁻¹. (c) α-NF: α-naphthoflavone (50 μM); COU, coumarin (100 μM); SLF, sulfaphenazole (20 μM); QND, quinidine (50 μM); KCZ, ketoconazole (5 μM); troglitazone (50 μM); terfenadine (50 μM); tranilcypropine (50 μM). Data are the per cent activity remaining relative to control (methanol or dimethylsulfoxide alone) incubation and are the average of two samples. SI, small intestine. Control activities: SI 115 pmol mg protein⁻¹ min⁻¹; liver 93 pmol mg protein⁻¹ min⁻¹.

activity decreased to 22 and 49% (terfenadine 50 μM), 25 and 96% (α -naphthoflavone 50 μM), 3 and 8% (troglitazone 50 μM), 54 and 75% (tranilcypromine 50 μM), and 73 and 45% (ketoconazole 5 μM) of the control by the addition of the indicated inhibitors in the small intestine and the liver, respectively. The inhibitory potencies of terfenadine, tranilcypromine and α -naphthoflavone were rather strong in the small intestine and vice versa for ketoconazole. Other inhibitors (coumarin 100 μM , sulfaphenazole 100 μM , quinidine 100 μM) showed little (< 30%) or no inhibitory effect in both the small intestine and the liver.

Effects of the chemical agents on astemizole O-demethylation in human intestinal and liver microsomes

Microsomal astemizole O-demethylation in the small intestine and liver from humans was examined in the presence of α -naphthoflavone, ketoconazole, troglitazone, ebastine, tranilcypromine and terfenadine (figure 3). All the tested chemical agents decreased the rate of microsomal astemizole O-demethylation in a concentration-dependent manner, especially in the small intestine. The activity was decreased to 60% (α -naphthoflavone 50 μM), 48% (ketoconazole 5 μM), 28% (troglitazone 50 μM), 31% (ebastine 100 μM), 65% (tranilcypromine 100 μM) and 25% (terfenadine 50 μM) of the control value by the addition of the indicated

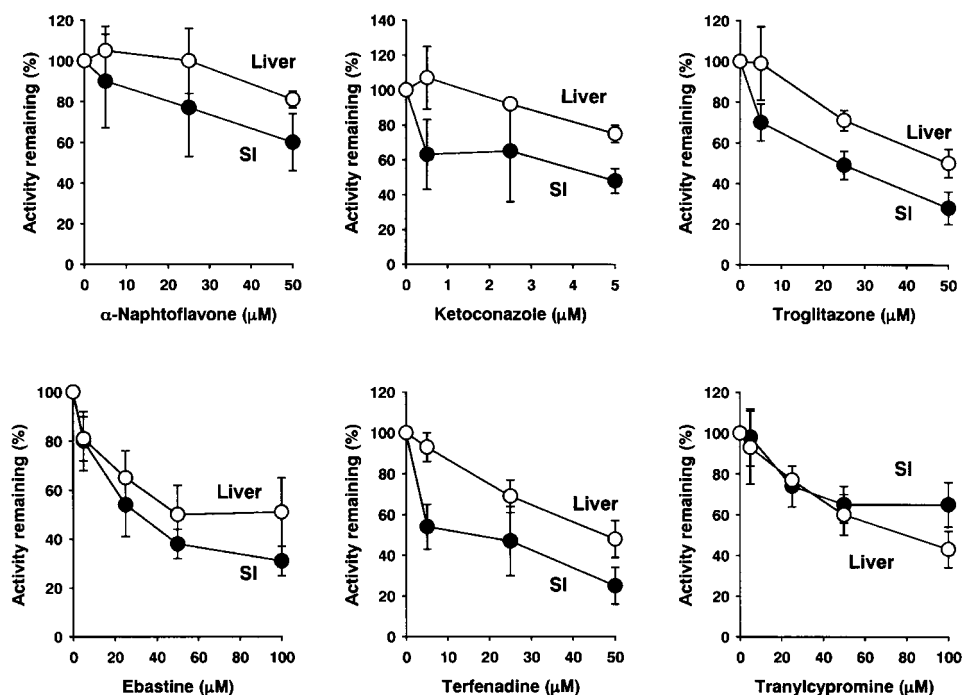


Figure 3. Effects of chemical agents on astemizole O-demethylation by human small intestine and liver microsomes. Small intestine samples HJM0058, HJM0061, HJM0065 (Tissue Transformation Technologies), and liver sample pooled microsomes (Xenotech), H042, H112 (Gentest) were used. Data are the per cent activity remaining relative to a control (methanol or demethylsulfoxide alone) incubation and are the mean $\pm$ SD of three samples. SI, small intestine. Control activities: 64 (HJM0058), 165 (HJM0061), 178 (HJM0065), 310 (pooled), 150 (H042), 116 (H112) pmol mg protein⁻¹ min⁻¹.

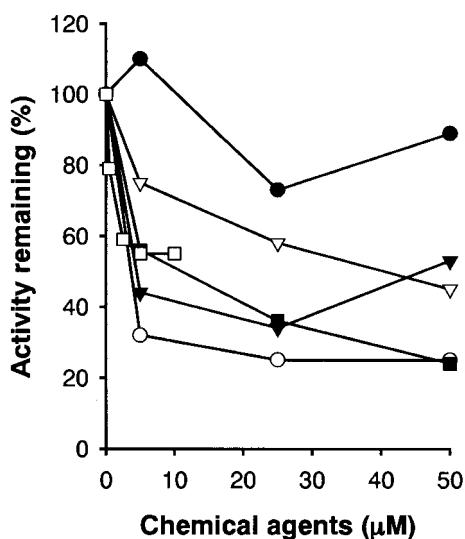


Figure 4. Effects of chemical agents on astemizole *O*-demethylation by recombinant CYP2J2 microsomes. Closed triangle, α -naphthoflavone (5–50 μ M); open square, ketoconazole (0.5–10 μ M); open triangle, troglitazone (5–50 μ M); open circle, ebastine (5–50 μ M); open triangle, terfenadine (5–50 μ M); closed circle, tranlycypromine (5–50 μ M). Data are the per cent activity remaining relative to control (methanol or demethylsulfoxide alone) incubation and are single samples. Control activities: 4.4 pmol mg protein⁻¹ min⁻¹.

chemical agents in the small intestine. These agents also inhibited the activity in the liver, although the inhibitory potencies were relatively weak except for tranlycypromine.

Effects of chemical agents on astemizole O-demethylation in recombinant CYP2J2 microsomes

The effects of the chemical agents on astemizole *O*-demethylation using recombinant CYP2J2 microsomes were determined in order to evaluate the inhibitory potencies of these agents on CYP2J2 activities (figure 4). All the tested chemical agents decreased the rate of microsomal astemizole *O*-demethylation in a concentration-dependent manner. The activity was decreased to 44% (α -naphthoflavone 50 μ M), 55% (ketoconazole 10 μ M), 45% (troglitazone 50 μ M), 25% (ebastine 50 μ M), 89% (tranlycypromine 50 μ M) and 24% (terfenadine 50 μ M) of the control value by the addition of the indicated chemical agents.

Discussion

The oxidative metabolism of orally administered drugs during the first pass is often critical for the pharmacological efficacy of drugs. Recent clinical investigations have demonstrated that the small intestine is involved in the first-pass metabolism of orally administered drugs including cyclosporine, midazolam, terfenadine, saquinavir and tacrolimus (Thummel *et al.* 1997, Lin *et al.* 1999). All these drugs are metabolized by CYP3A4, thus most studies of intestinal metabolism have focused on this enzyme. Antihistamine drugs such as astemizole

(Heykants *et al.* 1986) and ebastine (Yamaguchi *et al.* 1994) are extensively biotransformed in the small intestine and liver, although the contribution of CYP3A4 to the metabolism of these drugs is minor (Hashizume *et al.* 1998, Matsumoto and Yamazoe 2001). Recently, the involvement of CYP2J2 in the intestinal first-pass metabolism of these drugs has been reported (Hashizume *et al.* 2002, Matsumoto *et al.* 2002).

Chemical agents that inhibit astemizole O-demethylation were screened using rabbit small intestinal/liver microsomes and then the effects of the inhibitors on astemizole O-demethylation in human small intestinal and liver microsomes were investigated. In our previous study, astemizole O-demethylation in human liver microsomes was not inhibited by several P450 form-specific inhibitors (furafylline, CYP1A2; coumarin, CYP2A6; sulfaphenazole, CYP2C9; omeprazole, CYP2C19; quinidine, CYP2D6; diethyldithiocarbamate, CYP2E1; troleandomycin, CYP3A4; Matsumoto and Yamazoe 2001). In the current study, clear inhibition of α -naphthoflavone, ketoconazole, troglitazone, tranilcypromine, ebastine and terfenadine on the rate of astemizole O-demethylation in human small intestine were observed. All these chemical agents also inhibited the rate of astemizole O-demethylation in recombinant CYP2J2 microsomes. Although these chemicals are inhibitors or substrates of several P450s (α -naphthoflavone: CYP1A2, 2C9 inhibitor, ketoconazole: CYP1A2, 2C9, 2D6, 3A4 inhibitor, tranilcypromine: CYP2A6, 2C inhibitor, troglitazone: CYP2C8, 9, 3A4 inhibitor, ebastine: CYP2J2, CYP3A4, CYP4F12 substrate, terfenadine: CYP3A4, CYP2D6 substrate, Newton *et al.* 1995, Ono *et al.* 1996, Jones *et al.* 1998, Yamazaki *et al.* 2000, Taavitsainen *et al.* 2001, Zhang *et al.* 2001, Hashizume *et al.* 2002), these chemicals may also inhibit CYP2J2 activity in human liver and small intestinal microsomes.

The rates of inhibition by these chemical agents were relatively weak in human liver except for tranilcypromine. These results suggest that the contribution of hepatic CYP2J2 is lower than that of the small intestine. Although an immunoreactive CYP2J2 protein band was detected in human liver microsomes by Western blot analysis (Matsumoto *et al.* 2002), the difference in the expression level of CYP2J2 may influence the contribution of CYP2J2 in the small intestine and liver. In our previous report, recombinant CYP2D6 microsomes catalyse astemizole O-demethylation, whereas an inhibitory antibody to CYP2D6 and a CYP2D6 inhibitor, quinidine, did not inhibit human liver microsomal astemizole O-demethylation (Matsumoto and Yamazoe 2001). Therefore, the contribution of CYP2D6 seemed to be low, whereas astemizole O-demethylation in liver microsomes were relatively higher than that in the small intestinal microsomes in this study. Among the tested chemical agents, tranilcypromine strongly inhibited liver astemizole O-demethylation, although the inhibition potency of the recombinant CYP2J2 microsomes was relatively weak. These results may suggest the possible involvement of another P450 in the liver. In addition, the higher contribution of CYP2J2 in the small intestine than in the liver may suggest that the effect of the CYP2J2 inhibition on the first-pass metabolism would be higher in the small intestine than in the liver.

Astemizole is metabolized in both human and rabbit intestinal/liver microsomes to the same three metabolites, desmethylastemizole, norastemizole and 6-hydroxyastemizole. Astemizole O-demethylation in the rabbit small intestine was higher than that in the liver and correlated with the content of the immunoreactive

CYP2J2 protein in the small intestine and liver (Matsumoto *et al.* 2002). In addition, CYP2J2 substrates, arachidonic acid and ebastine clearly inhibited this activity in the rabbit small intestine. The CYP2J may also be involved in intestinal astemizole *O*-demethylation in the rabbit. These results may indicate that the metabolism of astemizole in the rabbit at least qualitatively resembles that in humans. In addition, the potent inhibitors of astemizole *O*-demethylation in the rabbit small intestine also inhibited this activity in recombinant CYP2J2 microsomes and in human small intestinal microsomes. Nakamura *et al.* (2000) investigated the immunoreactive CYP3A contents, catalytic activities and response of a human CYP3A inducer in the rabbit small intestine and suggested it to be useful as an *in vivo* model for studies of CYP3A-related drug interactions. Since the results of the present study may suggest the importance of the CYP2J2-related drug interaction in the human small intestine rather than in the liver, the rabbit would be a beneficial *in vivo* model for the assessment of drug interactions involving CYP2J2 during the first-pass of ingested drugs.

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Pharmacokinetics and metabolism of the cholecystokinin antagonist dexloxiglumide in male human subjects

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Received 31 October 2002

1. Mean concentrations of total ¹⁴C and of dexloxiglumide at the end of single 20-min infusion doses of ¹⁴C-dexloxiglumide (200 mg) to four healthy male subjects were 18.5 µg eq. ml⁻¹ and 19.5 µg ml⁻¹, respectively. The mean plasma clearance (0.221 h⁻¹ kg⁻¹) and mean volume of distribution (*V*_{ss} = 0.181 kg⁻¹) were low.

2. Single oral doses of a solid formulation of ¹⁴C-dexloxiglumide (200 mg) to the same subjects appeared to be rapidly and well absorbed. Mean peak plasma concentrations (*C*_{max}) of total ¹⁴C (2.8 µg eq. ml⁻¹) and of dexloxiglumide (2.2 µg ml⁻¹) occurred at about 1.5 h. Systemic availabilities of the oral dose based on total ¹⁴C and dexloxiglumide were 70 and 48%, respectively. Thus, a proportion of an oral dose was subjected to presystemic elimination and the absorbed dose mainly eliminated by metabolism. Binding of dexloxiglumide to plasma proteins was extensive (96.6–99.2%).

3. Total ¹⁴C was excreted mainly in the faeces. Mass balance of ¹⁴C excretion was almost complete within 7 days when a mean of > 93% of the dose had been recovered. After the intravenous (i.v.) dose, mean totals of 23.7 and 69.8% of the dose were excreted in urine and faeces, respectively, during 7 days, and 19.5 and 73.7% of the dose, respectively, after the oral dose. The data were consistent with biliary excretion and perhaps some enterohepatic circulation of conjugates of dexloxiglumide and at least one of its metabolites.

4. LC-MS/MS of urine extracts showed that dexloxiglumide was metabolized by oxidation and conjugation. The former included at least two metabolites formed by monohydroxylation in the *N*-(3-methoxypropyl) pentyl side chain, and *O*-demethylation of this side chain followed by subsequent oxidation of the resultant alcohol to the dicarboxylic acid. At least one glucuronide was also present in urine. The main components in faeces appeared to be dexloxiglumide and a dicarboxylic metabolite formed by *O*-demethylation followed by oxidation of the *N*-(3-methoxypropyl) side chain. Both compounds were identified as their corresponding methyl esters formed because acid and methanol were used in the extraction procedure. Dexloxiglumide and the dicarboxylic acid were presumably excreted in bile as the glucuronic acid conjugates.

Introduction

The peptide hormone cholecystokinin (CCK) is a major regulator of gall bladder contraction and pancreatic enzyme secretion, and is secreted in response to food intake (Woodruff and Hughes 1991, D'Amato *et al.* 1999b). It also regulates motor and sensory functions in the gut (Grider 1994) and CCK receptors are present in the intestines and brain.

Dexloxiglumide, (*R*)-4-(3,4-dichlorobenzamido)-5-[*N*-(3-methoxypropyl)-*N*-pentylamino]-5-oxopentanoic acid, is a selective and potent CCK Type 1 receptor (CCK₁) antagonist being developed for the treatment of constipation-prone irritable bowel syndrome (Revel *et al.* 1999). By contrast, the (*S*)-enantiomer is virtually inactive (Scarpignato *et al.* 1996). Basic pharmacological (Fioramonti *et al.* 1996, Rouzade *et al.* 1996) and clinical (Meier *et al.* 1997, D'Amato *et al.* 1999a, 2001, Feinle *et al.* 1999) studies of dexloxiglumide have been reported. As a chemical class of CCK receptor antagonists, dexloxiglumide is a glutamic acid derivative (D'Amato *et al.* 1999b).

The present study was designed to evaluate the pharmacokinetics and metabolism of dexloxiglumide in humans using the ¹⁴C-labelled compound. The studies used the clinical route of administration, which is oral, whereas the i.v. route was included so that basic pharmacokinetic parameters could be obtained.

Materials and methods

Materials

Dexloxiglumide (batch no. D/4740, chemical purity 99.8%, enantiomeric purity 99.5%), lorglumide (sodium salt, batch no. D/867, chemical purity 99.8%), the corresponding racemate of dexloxiglumide as the methyl ester and *O*-desmethyldexloxiglumide were provided by Rotta Research Laboratorium (Monza, Italy). ¹⁴C-dexloxiglumide (32.83 mCi mmole⁻¹) was synthesized at Huntingdon Life Sciences. Its radiochemical purity was > 99% (tlc in three different solvent systems) as was its enantiomeric purity (hplc of the L-phenylalaninamide derivative). Chemical identity was verified chromatographically (tlc, hplc) and by mass spectrometry.

Reagents were analytical or hplc grade as appropriate and obtained commercially.

Dose formulations

Non-radioactive and ¹⁴C-dexloxiglumide were dissolved in ethyl acetate: the solvent was removed by evaporation under N₂ at room temperature and the solid residue dried by vacuum desiccation. The radiochemical purity of the resultant ¹⁴C-dexloxiglumide was > 99% (tlc in three solvent systems), its enantiomeric purity was 98% (hplc of the corresponding L-phenylalaninamide derivative) and its chemical identity was verified by mass spectrometry and chromatographic properties compared with an authentic standard of dexloxiglumide.

The oral dose formulation was ¹⁴C-dexloxiglumide solid (200 mg) in a gelatin capsule.

A bulk i.v. dose (5 mg ml⁻¹) was prepared by dissolving ¹⁴C-dexloxiglumide in 1 M NaOH to which aq. 0.84% (w/v) NaCl, 0.11% (w/v) glycine and ultrapure water were added; the pH of the resultant solution was 8.5. This was dispensed aseptically into individual vials (10 ml), which were autoclaved. The contents of randomly selected vials were shown to be sterile and pyrogen-free. ¹⁴C-dexloxiglumide in the vials was stable for at least 30 days.

Clinical phase

The clinical phase was conducted at Leicester Clinical Research Centre (Leicester, UK) under the supervision of the Medical Director (Dr J. Harry). The study was approved by the local ethics committee and by the UK Administration of Radioactive Substances Advisory Committee.

Four male subjects (three smokers, one non-smoker; age mean 43 years, range 35–55 years; bodyweight mean 71 kg, range 58–82 kg) volunteered for this study after its purpose, procedures and possible adverse events were explained. They were enrolled following acceptable medical histories and results in physical and laboratory screening, and after they provided informed consent. The subjects were resident in the clinical unit from the evening preceding dose administration through to sample collection during 7 days. They were rescreened on completion of the study to ensure that the administered drug had no detectable effect on their health.

Following an overnight fast of at least 8 h, individual doses of 200 mg ¹⁴C-dexloxiglumide were administered to each subject orally or by i.v. infusion over 20 min. At each of two dosing sessions, two subjects received either single oral doses (one capsule) or single i.v. infusions (the contents of four vials per subject) on a crossover basis. Ten days elapsed between each of the two dosing sessions. The infusion device was analysed for residual radioactivity so that the exact dose administered to each subject could be accurately determined. Orally administered doses were within a mean of 97 ± 4% of target and i.v. infusions were also within a mean of 97 ± 3% of target.

Sample collection

Blood samples were withdrawn from an indwelling cannula in an arm vein (contralateral to that used for infusion) at predose and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144 and 168 h post-dose. Additional blood samples were collected at mid-infusion, end-infusion and 5 min (infusion dose only) and at 5 and 10 h (oral dose only). Urine samples were collected at predose and during 0–3, 3–6, 6–9, 9–12, 12–24 and then at 24-h intervals up to 168 h post-dose. Faeces were similarly collected, but during 24-h intervals.

All samples were stored at about -20°C until taken for analysis except whole blood which was stored at about 4°C .

Preparation of samples for measurement of radioactivity

Plasma (0.5 ml) and urine (0.5 ml) were mixed with MI-31 scintillator (Canberra Packard Ltd, Pangbourne, UK). Whole blood (0.5 ml) and aq. (1:1 w/v) faecal homogenates (about 0.2 g) were combusted in an automatic sample oxidizer (Model 307, Canberra Packard). The combustion products were absorbed in Optisorb I to which Optisorb S scintillator (Fisher Scientific UK Ltd, Loughborough, UK) was added before liquid scintillation analysis in a Wallac 1409 automatic scintillation counter (Wallac Oy, Turku, Finland). Combustion recoveries using ^{14}C standards exceeded 95% for which the results were corrected. The concentrations of radioactivity in blood cells were calculated from those in whole blood and plasma using measured haematocrits at each time point. These ranged between 48 and 39 and tended to diminish during the course of blood withdrawals, as would be expected.

Plasma protein binding

The binding of dexloxiglumide to human plasma proteins was measured *in vitro* in fresh control human plasma to which ^{14}C -dexloxiglumide was added over a concentration range similar to that measured *in vivo* by ultrafiltration (Centrifree Micropartition System, Amicon Ltd, Stonehouse, UK).

Determination of dexloxiglumide in plasma

Plasma (100 μl), mixed with aq. 50% (v/v) acetonitrile (25 μl), internal standard (lorglumide, 20 $\mu\text{g ml}^{-1}$) dissolved in the same solvent (25 μl), and 0.5 M phosphate buffer (500 μl , pH 2), was extracted with ethyl acetate (6 ml). After centrifugation (about 1000 g for 5 min), the organic phase was evaporated to dryness (under nitrogen at 40°C). The resultant residue was reconstituted in acetonitrile (200 μl), and after centrifugation (about 20 000 g), the solvent phase was evaporated to dryness (under nitrogen at 40°C). The resultant residue was reconstituted in mobile phase (200 μl), and after centrifugation (about 20 000 g), an aliquot (50 μl) was analysed by hplc with UV detection at 244 nm. Both the analytical (150 $\times$ 4.6 mm i.d., at 35°C) and guard (10 $\times$ 3.2 mm i.d.) columns were YMC-Pack ODS-AM-302 (5 μm particle size) supplied by Capital HPLC (London, UK) and Hichrom Ltd (Reading, UK), respectively, and the mobile phase was acetonitrile: 50 mM phosphate buffer (pH 6) (50:50 v/v) at a flow rate of 1 ml min^{-1} . Data capture (peak heights) was with a TurboChrom data acquisition system (PE Nelson Ltd, Beaconsfield, UK). The internal standard was lorglumide, which only differs from dexloxiglumide in that the 3-methoxypropyl side chain is replaced by a pentyl side chain (figure 3). Their retention times during hplc were typically 8.5 (lorglumide) and 3.4 (dexloxiglumide) min, respectively.

The method was validated before use (Brodie *et al.* 2002). The calibration line was linear ($r = 0.9998$) over the concentration range 0.2–20 $\mu\text{g ml}^{-1}$ when intra- and interday precision (as coefficient of variation) and accuracy (as relative error) ranged between 1.7 and 6.3% and -3.2 to $+7.6\%$, respectively. Recoveries of dexloxiglumide and internal standard from plasma were 79.8–83.0 and 61.3%, respectively. Dexloxiglumide was stable in plasma during analysis and during storage before analysis.

Thin-layer chromatography

Tlc was carried out on prelayered Merck silica gel 60F254 plates (E. Merck, Darmstadt, Germany) of layer thickness 0.25 mm in a solvent system of chloroform:methanol (80:20 v/v). Radioactive components present on tlc plates were detected and quantified using a Fujix BAS2000 Bioimage Analyser (Lablogic Ltd, Sheffield, UK). During this procedure, developed tlc plates were superimposed on bioimage analyser plates impregnated with a photostimulable phosphor (BaFBr:Eu^{2+}), which is a store for emitted radioactive energy. These latter plates were then evaluated by exposure to a laser when luminescence emitted in proportion to the amount of radioactivity present was measured.

Preparation of samples for tlc

Plasma. Equal volumes (0.5 ml) of plasma obtained for up to six different but adjacent time intervals (two to three plasma samples per subject per time interval) up to 16 h (i.v., i.e. 0.08–0.25, 0.5–1, 1.5–2.5,

3–6 and 8–16 h) or 24 h (p.o., i.e. 0.5–1, 1.5–2.5, 3–4, 5–6, 8–10 and 12–24 h) were combined and, without adjusting the pH, were extracted with ethyl acetate. After centrifugation, the organic phase was evaporated to dryness and the residue reconstituted in methanol. The methanol solution was subjected to solid-phase extraction (Oasis cartridge, Waters HLB 30 ml/60 mg, Waters UK Ltd, Watford, UK), and radioactive components eluted with methanol after non-specific material had first been eluted with water. The eluate was evaporated to dryness and the residue reconstituted in methanol (about 50 μ l) followed by tlc (up to six samples per subject per dose route). Recovery of ^{14}C through this procedure deteriorated from about 85% at early time intervals to about 45% later (8 h onwards), presumably reflecting failure of the solid-phase column to retain more polar metabolites during the water elution stage.

Urine. Urine samples (1 ml) from each subject for five different time intervals for each dose route were acidified (1 ml HCl) and extracted with ethyl acetate (10 ml). The organic phase was evaporated to dryness, and the residue reconstituted in methanol (400 μ l), of which a portion (50 μ l) was subjected to tlc (five samples per subject per dose route). Recovery of ^{14}C through this procedure exceeded 90%.

Faeces. Aqueous (1:1 w/v) faecal homogenates (about 1 g) from each subject for three different time intervals for each dose route were acidified (1 ml HCl) and extracted with acetonitrile (2 $\times$ 10 ml). After centrifugation, the combined organic phases were evaporated to dryness and the residue reconstituted in methanol (800 μ l), which was then filtered through a DynaGard syringe filter (0.2 μ m, Microgon, Inc., Laguna Hills, CA, USA). The resultant solution was processed by solid-phase extraction as described for plasma. The residue thus obtained was reconstituted in methanol (about 40 μ l) followed by tlc (three samples per subject per dose route). Recovery of ^{14}C through this procedure was about 65% with losses almost entirely associated with the acetonitrile extraction of faeces homogenates.

In all sample preparation, the organic phase was evaporated to dryness under nitrogen at about 40 $^{\circ}\text{C}$, and centrifugation was at about 2000 g for 10 min.

Preparation of samples for hplc

Hplc was used to assess whether inversion of dexloxiglumide to its opposite antipode occurred *in vivo* and to check enantiomeric purity of dexloxiglumide in dose formulations. This involved preparation of the corresponding diastereoisomers as follows: 0.005 M triethylamine in acetonitrile (0.5 ml) was added to the biological extract or dose formulation. After mixing for 1 min, 0.06 M ethylchloroformate in acetonitrile (0.25 ml) and 0.2 M L-phenylalaninamide in acetonitrile (0.25 ml) were successively added and mixed. After 20 min at room temperature, water (0.5 ml) was added. After mixing, the resultant solution was subjected to hplc with on-line radioactivity detection (β -Ram, Lablogic). The hplc column was Econosphere C18 (particle size 5 μ m, 250 $\times$ 4.6 mm i.d., HiChrom Ltd, Reading, UK), and the gradient mobile phase consisted of 0.0125 M phosphate buffer, pH 6.7, acetonitrile and water at a flow rate of 1 ml min $^{-1}$. The 30-min gradient commenced and ended with 50:50 (v/v) buffer and acetonitrile but consisted entirely of water during 17–25 min. Detection was by UV absorbance at 244 nm and radioactivity. Under these conditions, the corresponding diastereoisomers were separated by about 1 min with that of dexloxiglumide eluting latest at about 13.8 min.

Mass spectrometry

Before LC-MS/MS, urine extracts (0.5 ml) were evaporated to dryness under nitrogen at about 40 $^{\circ}\text{C}$, and reconstituted in aq. 0.05% ammonium acetate:acetonitrile (0.25 ml, 50:50 v/v). Faeces extracts were injected without further processing.

A Finnigan TSQ 7000 LC-MS/MS system (Finnigan MAT, San Jose, CA, USA) coupled to on-line UV (Shimadzu SPD-10A, Dysons Instruments Ltd, Tyne & Wear, UK) and radioactivity (β -RAM, Lablogic) detectors were used. The hplc column was Phenomenex Luna C $_5$ (150 $\times$ 4.6 mm i.d., Phenomenex, Macclesfield, UK) and the gradient mobile phase was aq. 0.05% (v/v) ammonium acetate and acetonitrile at a flow rate of 1 ml min $^{-1}$. For separation of ^{14}C components in faeces extracts, the gradient was 50:50 (v/v) for 15 min, then 10:90 (v/v) for 2 min and back to 50:50 (v/v) before injection of the next sample. For separation of ^{14}C components in urine extracts, the gradient was 95:5 (v/v) for 15 min, then 5:95 (v/v) for 0.5 min and back to 95:5 (v/v) before injection of the next sample. The eluate from the hplc flowed through the UV detector and then a stream splitter such that 90% of the eluate flowed through the radioactivity detector and the remainder was introduced into the source of the mass spectrometer for electrospray ionization. The spray voltage and capillary temperature of the ionization source were set at 4.5 kV and 250 $^{\circ}\text{C}$, respectively. Nitrogen was the sheath gas at a pressure of about 65 psi and also the auxiliary gas at about 10 units. Mass spectrometric data were acquired over a designated scan range at a rate of 1 scan s $^{-1}$.

Possible dexloxiglumide-related components were located from the chromatograms of both the UV and the radioactivity detectors so that their mass spectra could be obtained by averaging several scans across the analogous region of the mass chromatogram after background subtraction. Ions of interest

(e.g. $[M + H]^+$) were selected for MS/MS analysis in product ion scan mode where the precursor ion was fragmented by collision-induced dissociation (argon, about 2 m torr) in the Q2 region at collision energy empirically optimized so as to provide maximum structural information. A mass spectrum of the fragments produced was obtained by scanning Q3 across the appropriate mass range at a rate of 1 scans⁻¹. Several scans were averaged to produce the product ion mass spectrum of the putative metabolite.

Pharmacokinetic data processing

Concentrations of radioactivity in plasma and whole blood are expressed in terms of $\mu\text{g eq. dexloxiglumide ml}^{-1}$. Maximum plasma concentrations of radioactivity and of dexloxiglumide (C_{max}) and their times of occurrence (T_{max}) were the observed values. The respective areas under the plasma analyte concentration versus time curves were estimated by the linear trapezoidal rule to the last sampling time at which concentrations were at or above the limit of accurate detection (AUC_t). Except for dexloxiglumide i.v. route data, areas were extrapolated to infinite time (AUC_{∞}) by the addition of C/λ_z , where λ_z is the terminal rate constant calculated by linear regression of $\ln$ concentration on time using at least three data points which appeared to be randomly distributed about a single straight line, and C is the predicted plasma analyte concentration at the last quantifiable sampling time given by the regression. For the regression analysis to be accepted, the following criteria had to be satisfied: (1) regression coefficient ≥ 0.95 , (2) the amount of variance accounted for by the regression ≥ 0.9 and (3) the ratio of the measurement period for the data points used in the regression analysis to the estimated half-life ≥ 2 . Terminal half-lives were calculated as $\ln 2/\lambda_z$. Systemic clearance (Cl) was calculated as dose/ AUC_{∞} . Apparent volumes of distribution during the post-distribution (terminal) phase (V_z) and at steady-state (V_{ss}) were calculated as dose/ $\text{AUC}_{\infty}\lambda_z$ and dose. $\text{AUMC}_{\infty}/(\text{AUC}_{\infty})^2$, respectively, where AUMC_{∞} is the area under the first moment dexloxiglumide concentration-time curve. Systemic availability (F) was calculated as $F = 100 \times (\text{AUC}_{\infty \text{ p.o.}}/\text{AUC}_{\infty \text{ i.v.}}) \times (\text{dose}_{\text{i.v.}}/\text{dose}_{\text{p.o.}})$. If AUC_{∞} was not adequately determined, AUC_t was used to calculate F . Mean residence times (MRT) were calculated as $\text{AUMC}_{\infty}/\text{AUC}_{\infty}$. A two-compartment model with constant i.v. input (i.e. infusion) and first-order output was used to fit the plasma dexloxiglumide concentrations obtained after i.v. dosing. The equation of the model was $C_t = A.e^{-\alpha t} + B.e^{-\beta t}$, where C_t is the plasma dexloxiglumide concentration at time t , and A , B , α and β are constants. The area under the plasma dexloxiglumide concentration-time curve was calculated as $(A/\alpha) + (B/\beta)$, the volume of distribution (V_c) of the central compartment as dose/ $(A + B)$ and K_{10} as $A + B/(A/\alpha + B/\beta)$. These and other parameters, calculated as described above, were generated using WinNonlin (version 1.1, Scientific Consulting, Inc., NC, CA, USA).

Results

Plasma concentrations

Mean concentrations of total ^{14}C and of dexloxiglumide are shown in figure 1 and pharmacokinetic parameters in table 1. The half-lives shown in table 1 should be considered to be approximate because all the acceptance criteria were not satisfied, particularly that the period during which the half-life was determined did not exceed the measured half-life by at least twofold owing to the lower limit of quantification, $\text{LLOQ} = 0.2 \mu\text{g ml}^{-1}$.

After single i.v. infusion doses, the peak mean concentration of total ^{14}C was $18.5 \mu\text{g eq. ml}^{-1}$ at 20 min, the end of the infusion, similar to that of dexloxiglumide of $19.5 \mu\text{g ml}^{-1}$ (figure 1a). Concentrations of total ^{14}C and of dexloxiglumide apparently declined bi-exponentially with harmonic means for the terminal half-lives of approximately 7.3 and 1.2 h, respectively (table 1). The proportion of total ^{14}C associated with dexloxiglumide progressively declined from 90% at 15 min post-infusion to 55% at 1 h and to 20% at 4 h, the last time point at which dexloxiglumide could be quantified in three of the four subjects owing to the $\text{LLOQ} = 0.2 \mu\text{g ml}^{-1}$. Comparison of mean areas under the respective plasma concentration-time curves (AUC_t), indicated that the mean AUC_t of dexloxiglumide was 50% of that for total ^{14}C implying that dexloxiglumide had been metabolized and that one or more of its metabolites was cleared more slowly.

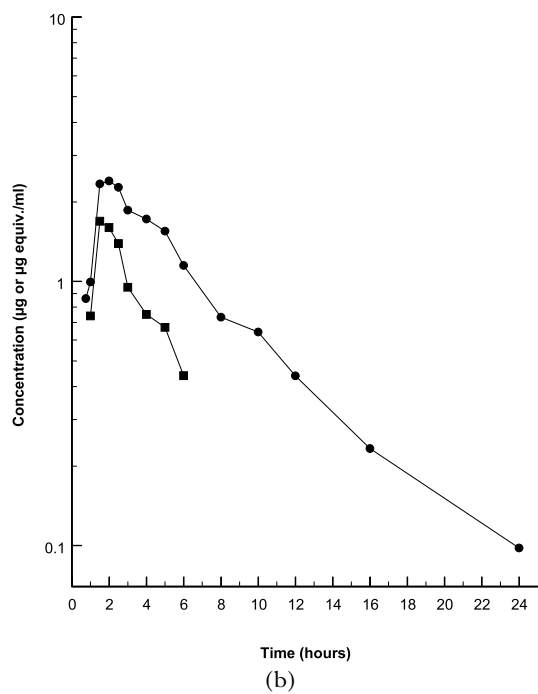
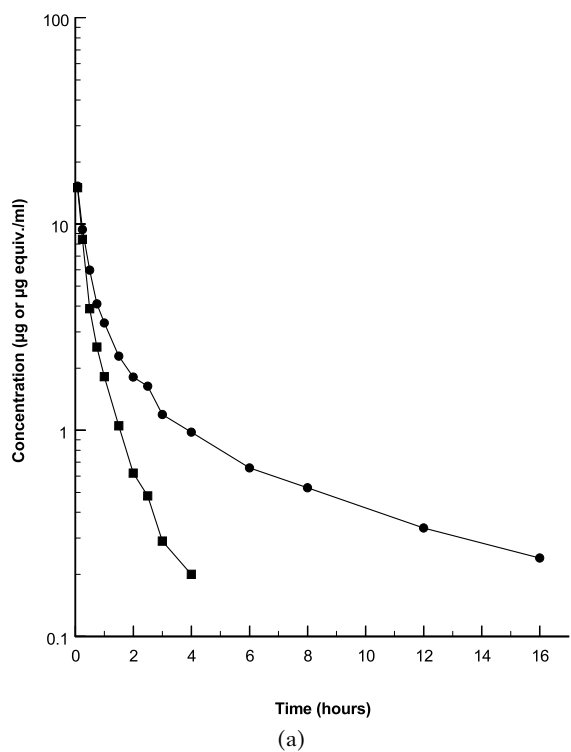


Figure 1. Mean plasma concentration-time profiles of total ^{14}C (●) and dexloxiglumide (■) after (a) intravenous infusion during 20 min (post-infusion concentrations are shown) or (b) oral administration of 200 mg ^{14}C -dexloxiglumide to four healthy male subjects.

Table 1. Pharmacokinetic parameters of total ^{14}C and dexloxiglumide after intravenous or oral administration of ^{14}C -dexloxiglumide (200 mg) to four healthy male subjects.

Parameter*	Intravenous		Oral	
	^{14}C	Dexloxiglumide	^{14}C	Dexloxiglumide
$C_{\max}$ ($\mu\text{g ml}^{-1}$)	18.5 ± 2.3	19.5 ± 2.9	2.8 ± 0.3	2.2 ± 0.3
$T_{\max}$ (h)	0.33 $\dagger$ ± 0.00	0.33 $\dagger$ ± 0.00	1.63 ± 0.63	1.44 ± 0.52
AUC_t ($\mu\text{g h ml}^{-1}$)	21.6 ± 2.4	11.6 ± 1.3	16.0 ± 4.1	5.6 ± 1.3
AUC_{∞} ($\mu\text{g h ml}^{-1}$)	24.2 ± 2.6	12.0 ± 1.1	18.0 ± 4.2	6.9 ± 1.5
$t_{1/2\ddagger}$ (h)	7.3 —	1.2 —	5.8 —	2.3 —
F (%)	—	—	70 ± 11	48 ± 10

* ^{14}C data are $\mu\text{g eq.}$ to dexloxiglumide $\pm\text{SD}$.

$\dagger T_{\max}$ was at the end of infusion.

$\ddagger$ Terminal phase half-life expressed as a harmonic mean and values should be regarded as approximate as acceptance criteria were not satisfied.

The interindividual variability in plasma dexloxiglumide concentrations was moderate with coefficients of variation generally less than 25% (range 5–69%).

After single oral doses, the peak mean concentration of total ^{14}C was $2.4 \mu\text{g eq. ml}^{-1}$ at 2 h, greater than that of dexloxiglumide of $1.69 \mu\text{g ml}^{-1}$ at 1.5 h (figure 1b). Concentrations of both analytes apparently declined bi-exponentially with apparent mean terminal half-lives of approximately 5.8 and 2.3 h, respectively (table 1). The proportion of total ^{14}C associated with dexloxiglumide progressively declined from 72% at 1.5 h to 51% at 3 h and to 38% at 6 h, the last time point at which dexloxiglumide was quantifiable. These data indicated that dexloxiglumide was absorbed at a moderate rate and that it was subjected to appreciable presystemic first-pass metabolism during its absorption from the gut into the systemic circulation. Comparison of the respective AUC_{∞} 's indicated that the mean AUC_{∞} of dexloxiglumide was 38% of that for total ^{14}C , lower than that (50%) after i.v. infusion, confirming that some first-pass metabolism of dexloxiglumide had occurred. The systemic availability of an oral dose of dexloxiglumide based on total radioactivity and unchanged drug were 70 and 48%, respectively (table 1). The interindividual variability in plasma dexloxiglumide concentrations was greater after oral than after i.v. doses with coefficients of variation generally less than 35% (range 15–153%).

After i.v. doses, the plasma dexloxiglumide concentration–time profile was adequately described by a two-compartment model with elimination from the central compartment. The resultant pharmacokinetic parameters are shown in table 2. The systemic clearance of dexloxiglumide was $0.221 \text{ h}^{-1} \text{ kg}^{-1}$ and its volumes of distribution were 0.41 kg^{-1} (V_z) and 0.181 kg^{-1} (V_{ss}) (table 2). The difference in these values is expected for a bi-exponential decline in plasma drug concentrations.

The whole-blood concentration–time profile of total ^{14}C paralleled that for plasma (data not shown), and whole blood to plasma ratios were about 0.50

Table 2. Pharmacokinetic parameters obtained by fitting a two-compartment model to the plasma dexloxiglumide concentration-time data obtained after intravenous administration of ^{14}C -dexloxiglumide (200 mg) to four healthy male subjects.

Parameter	Mean $\pm$ SD
A ($\mu\text{g ml}^{-1}$)	28.18 ± 3.11
B ($\mu\text{g ml}^{-1}$)	2.34 ± 0.44
α (h^{-1})	3.5499 ± 0.4345
β (h^{-1})	0.5813 ± 0.1085
k_{10} (h^{-1})	2.5557 ± 0.3811
$t_{1/2}$ (h)	0.3
Cl ($\text{l h}^{-1} \text{ kg}^{-1}$)	0.22 ± 0.03
V_c (l kg^{-1})	0.09 ± 0.01
V_{ss} (l kg^{-1})	0.18 ± 0.04
V_z (l kg^{-1})	0.40 ± 0.09
MRT (h)	0.79 ± 0.17

suggesting that uptake of ^{14}C into blood cells was minimal. This conclusion was confirmed when concentrations in blood cells were calculated from whole-blood and plasma concentrations and the respective haematocrits.

Plasma protein binding

Over the concentration range $0.1\text{--}20 \mu\text{g ml}^{-1}$, ^{14}C -dexloxiglumide was extensively bound to plasma proteins *in vitro*. There was an apparent trend for a decrease in binding as the concentration increased from 0.1 (99.2%) to 1 (97.6%) to $10 \mu\text{g ml}^{-1}$ (96.6%).

Excretion

^{14}C from i.v. and oral doses of ^{14}C -dexloxiglumide was excreted fairly slowly, mostly in faeces. In some subjects at least, excretion of traces of ^{14}C in faeces probably continued beyond the last sampling interval (table 3) as implied by the excretion of about 1% dose during the last (144–168 h) sampling interval.

During the first 24 h after i.v. infusion, means of 22.5% of the dose were excreted in urine and 9.5% of the dose in faeces increasing to 23.7 and 58.7% of the dose, respectively, during 72 h, and 23.7 and 69.8% of the dose, respectively, during the 7-day collection period.

During the first 24 h after oral administration, means of 17.8% of the dose were excreted in urine and 8.1% of the dose in faeces increasing to 19.5 and 57.1% of the dose, respectively, during 72 h, and 19.5 and 73.7% of the dose, respectively, during the 7-day collection period.

The proportions of the dose excreted during the respective time intervals by the individual subjects in faeces differed, perhaps reflecting output of faeces. Urinary excretion of ^{14}C was not detected beyond 72 h. Coefficients of variation for urinary and faecal excretion were about 2.4 and 10% or less, respectively (table 3).

Table 3. Mean ($\pm$ SD) excretion of ^{14}C in the urine and faeces after intravenous or oral administration of ^{14}C -dexloxiglumide (200 mg) to four healthy male subjects.

Time interval (h)	Intravenous	Oral
Urine		
0-3	11.9 $\pm$ 2.8	4.2 $\pm$ 0.9
3-6	4.4 $\pm$ 2.0	5.4 $\pm$ 1.8
6-9	2.7 $\pm$ 0.5	3.8 $\pm$ 1.0
9-12	1.7 $\pm$ 0.3	2.3 $\pm$ 0.8
12-24	1.9 $\pm$ 0.5	2.2 $\pm$ 0.4
24-48	1.0 $\pm$ 0.2	1.4 $\pm$ 0.6
48-72	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2
72-168	ND	-†
Total (0-168)	23.7 $\pm$ 5.8*	19.5 $\pm$ 3.5*
Faeces		
0-24	9.5 $\pm$ 11.1	8.1 $\pm$ 14.0
24-48	18.4 $\pm$ 28.1	28.4 $\pm$ 26.2
48-72	30.8 $\pm$ 24.3	20.6 $\pm$ 20.7
72-96	14.3 $\pm$ 16.4	13.4 $\pm$ 20.0
96-120	0.2‡	15.7 $\pm$ 15.9
120-144	3.2 $\pm$ 5.2	1.4‡
144-168	1.2 $\pm$ 1.8	0.7 $\pm$ 1.0
Total (0-168)	69.8 $\pm$ 7.1*	73.7 $\pm$ 3.6
Total excretion (0-168)	93.5 $\pm$ 3.11*	93.2 $\pm$ 3.6*

Results are the percentage administered dose.

* Means were calculated from the total for each subject.

† Only one subject excreted ^{14}C (0.2%) in urine after 72 h.

‡ Only one subject excreted ^{14}C in this period.

Metabolite profiles

Plasma. With the procedure used, the proportion of ^{14}C extracted from plasma progressively decreased with time, presumably due to the corresponding increase in the proportions of polar non-extractable components. These polar metabolites were probably glucuronides as the extractability of ^{14}C was approximately doubled after incubation of selected pooled plasma samples (2 h i.v. and 4 h p.o.) with β -glucuronidase (C. Webber, unpublished data). In profiles of radioactive components in plasma (i.v. up to 2.5 h, p.o. up to 4 h), only one component corresponding chromatographically to unchanged dexloxiglumide was detected. This result is in agreement with comparisons of plasma concentrations of ^{14}C and of dexloxiglumide measured by hplc.

Chiral hplc showed that the ^{14}C in a plasma sample collected 4 h after oral dosing was associated only with dexloxiglumide and not with its opposite enantiomer, indicating that dexloxiglumide did not invert to any detectable extent *in vivo*.

Urine. Extracts of the five samples of urine obtained from each subject during 0-24 h after single i.v. doses contained up to seven ^{14}C components, the most abundant of which corresponded chromatographically to dexloxiglumide (32-60% of the sample ^{14}C , about 11% of the dose) (e.g. Subject 3M, figure 2). Of the other ^{14}C components detected, five were more polar and one (HM6) less polar than dexloxiglumide: three of these were prominent accounting for means of about 13% (4-25%, HM4), 20% (7-39%, HM5) and 18% (1-33%, HM6) of the sample ^{14}C , respectively, and for about 3.1, 3.4 and 3.3% dose, respectively (figure 2). The

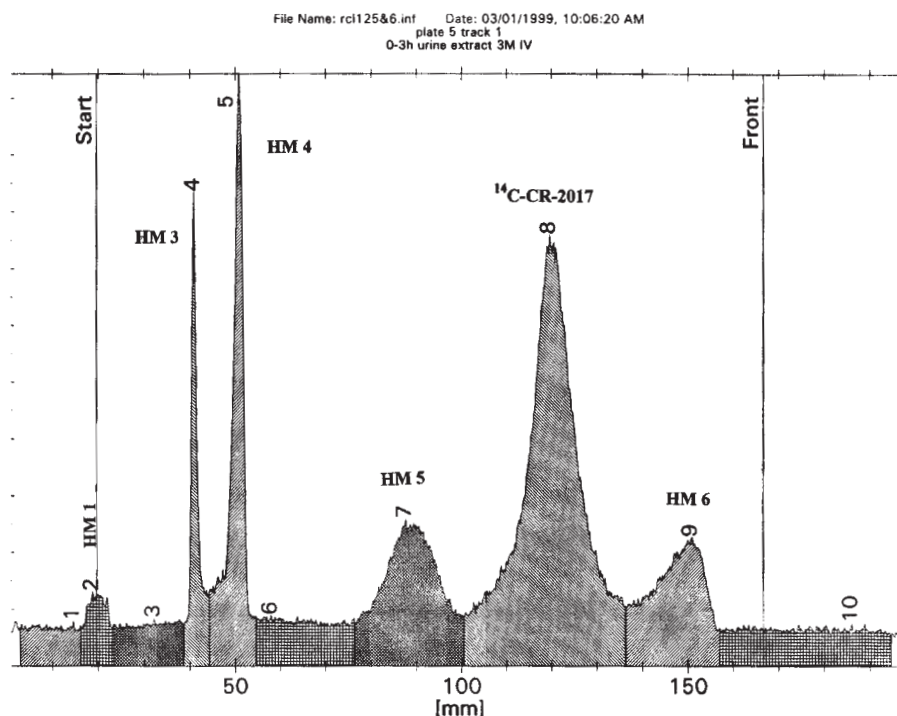


Figure 2. Thin-layer radiochromatogram of a 0–3-h extract of urine obtained after intravenous administration of 200 mg ^{14}C -dexloxiglumide (CR-2017) to a healthy male subject (#3).

other trace components, such as HM3, were only detected in some samples. Proportions of dexloxiglumide and of HM4, HM5 and HM6 did not appear to vary systematically with respect to the period during which the urine was excreted.

Results from urine samples collected at intervals during 0–12 h after single oral doses were similar to those obtained after single i.v. doses with dexloxiglumide of similar importance (23–62% of the sample ^{14}C , about 6% of the dose) whereas HM3 (mean about 10%, 5–18%) was more prominent than HM4 (mean about 6%, 2–11%). HM5 and HM6 accounted for means of about 17 (8–32) and 25% (10–38%) of the sample ^{14}C , respectively. These four components accounted for about 1.6, 0.9, 2.6 and 3.5% of the dose, respectively (table 4).

Faeces. Almost all the ^{14}C recovered from faeces was associated chromatographically with a single component HM6 (mean $\geq 93\%$ of the sample ^{14}C), which was less polar than dexloxiglumide. Only traces of the latter were present in some samples, particularly after the oral dose.

Identification of excreted components

Urine. HM5 corresponded chromatographically to a metabolite previously identified in dog urine and rat bile as the product of O-demethylation of dexloxiglumide (figure 3).

Table 4. LC-MS data generated from pooled urine extracts (0–3 h) obtained after intravenous administration of ^{14}C -dexloxiglumide (200 mg) to four healthy male subjects. The proportion of dose data are those obtained after oral administration (see text).

Retention time (min)	Molecular ion (M-H) ⁻	Proposed structure*	Proportion (% dose)
5.96	459	dexloxiglumide carboxylic acid (HM1)	0.8
6.73/7.37†	475	hydroxylated dexloxiglumide (HM4)	0.9
7.67	445	O-demethylated dexloxiglumide (HM5)	2.6
7.98	635	dexloxiglumide glucuronide (HM3)	1.6
8.43	459	dexloxiglumide (CR-2017)	6.2
8.62	487	ethyl ester of dexloxiglumide carboxylic acid (HM6)‡	3.5
12.02	473	methyl ester of dexloxiglumide (HM6)‡	
13.23	487	ethyl ester of dexloxiglumide (HM6)‡	

* Structures are shown in figure 3.

† A third peak, retention time 7.05 min, also possibly had this structure.

‡ Components represented by HM6 are considered to be artefacts of the extraction procedure.

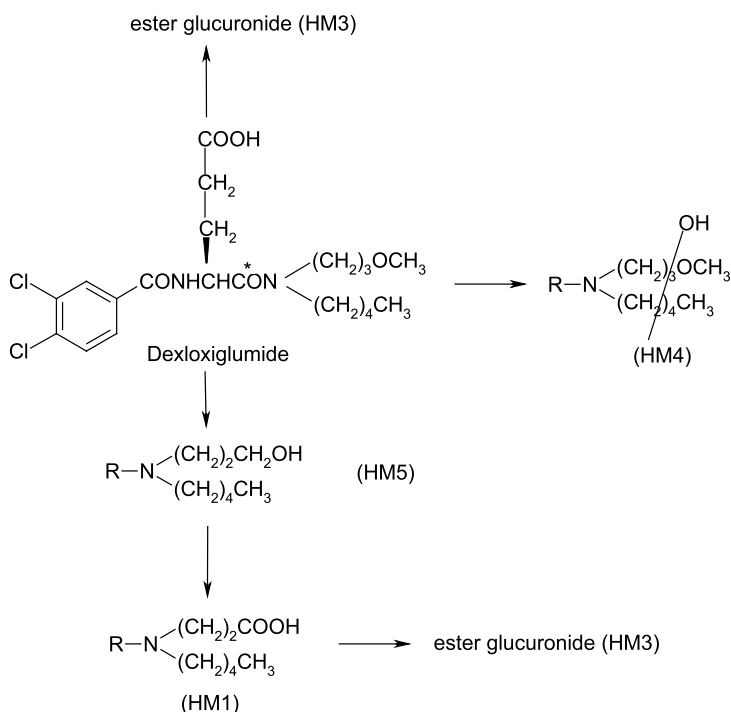


Figure 3. Proposed metabolism pathway of dexloxiglumide in humans. *Position of radiolabel.

Table 4 summarizes the data generated from LC-MS of a 0–3-h pooled urine extract obtained after the i.v. dose. Besides unchanged dexloxiglumide, at least eight other components were detected that had originated from dexloxiglumide because their Q1-MS contained M, M + 2 and M + 4 molecular ions in the expected ratio of 9:6:1 characteristic of the two chlorine atoms present in dexloxiglumide. In almost all cases, the molecular ion (M) was the base peak: the exception was the Q1-MS of the component with a retention time of 13.23 min where the molecular ion's relative abundance was about 92%. Table 5 summarizes

Table 5. LC-MS/MS data generated from pooled urine extracts (0–3 h) obtained after intravenous administration of ¹⁴C-dexloxiglumide (200 mg) to four healthy male subjects.

Retention time (min)	Precursor ion (<i>m/z</i>)*	Product ions (<i>m/z</i>)†	Assignment
5.82	459	459 (2)	(M–H) [–]
		387 (68)	<i>m/z</i> 459–CH ₂ CH ₂ CO ₂ [–]
		369 (100)	<i>m/z</i> 387–H ₂ O
		300 (19)	<i>m/z</i> 459–HNCH ₂ CH ₂ CO ₂ H(pentyl)
		256 (4)	<i>m/z</i> 300–CO ₂
		238 (8)	<i>m/z</i> 256–H ₂ O
		189 (5)	dichlorobenzamide
6.63/7.09	475	475 (100)	(M–H) [–]
		457 (86)	<i>m/z</i> 475–H ₂ O
		403 (3/38)	<i>m/z</i> 475–CH ₂ CH ₂ CO ₂ [–]
		300 (32/75)	<i>m/z</i> 475–N(CH ₂) ₃ OCH ₃ (pentyl),–OH
		256 (8/20)	<i>m/z</i> 300–CO ₂
		238 (7/23)	<i>m/z</i> 256–H ₂ O
		228 (21/12)	<i>m/z</i> 256–CO
		189 (12/17)	dichlorobenzamide
7.59	445	445 (100)	(M–H) [–]
		401 (4)	<i>m/z</i> 445–CO ₂
		373 (39)	<i>m/z</i> 445–CH ₂ CH ₂ CO ₂
		300 (96)	<i>m/z</i> 445–HN(CH ₂) ₂ CH ₂ OH(pentyl)
		256 (35)	<i>m/z</i> 300–CO ₂
		238 (50)	<i>m/z</i> 256–H ₂ O
		228 (35)	<i>m/z</i> 256–CO
		189 (85)	dichlorobenzamide
		145 (3)	dichlorophenyl
7.99	635	635 (17)	(M–H) [–]
		459 (100)	<i>m/z</i> 635–glucuronic acid moiety (C ₆ H ₈ O ₆)
		175 (11)	glucuronic acid moiety (C ₆ H ₇ O ₆)
8.32	459	459 (32)	(M–H) [–]
		441 (4)	<i>m/z</i> 459–H ₂ O
		415 (14)	<i>m/z</i> 459–CO ₂
		387 (28)	<i>m/z</i> 459–CH ₂ CH ₂ CO ₂ [–]
		300 (73)	<i>m/z</i> 459–HN(CH ₂) ₃ OCH ₃ (pentyl)
		256 (66)	<i>m/z</i> 300–CO ₂
		228 (30)	<i>m/z</i> 256–CO
		189 (82)	dichlorobenzamide
		145 (5)	dichlorophenyl
8.83	487	487 (30)	(M–H) [–]
		444 (25)	<i>m/z</i> 487–OC ₂ H ₅ , + 2H
		427 (43)	<i>m/z</i> 444–O, –OH
		300 (8)	<i>m/z</i> 487–HNCH ₂ CH ₂ CO ₂ C ₂ H ₅ (pentyl)
		256 (28)	<i>m/z</i> 300–CO ₂
		238 (20)	<i>m/z</i> 256–H ₂ O
		228 (14)	<i>m/z</i> 256–CO
		189 (100)	dichlorobenzamide
12.01	473	473 (19)	(M–H) [–]
		228 (100)	<i>m/z</i> 473–HN(CH ₂) ₃ OCH ₃ (pentyl), –(CH ₂) ₂ CO ₂ CH ₂
		189 (34)	dichlorobenzamide
13.24	487	441 (23)	<i>m/z</i> 487–OC ₂ H ₅ , –H
		387 (100)	<i>m/z</i> 487–CH ₂ CH ₂ CO ₂ C ₂ H ₅ , + H
		369 (8)	<i>m/z</i> 387–H ₂ O
		228 (51)	<i>m/z</i> 387–HN(CH ₂) ₃ OCH ₃ (pentyl)

* See table.
† Approximate relative abundance (%) is shown in parentheses.

the data generated from LC-MS/MS of the pooled urine extract. The product ion mass spectra support the structural assignments made.

There were at least two peaks in the mass chromatogram for m/z 475 corresponding to monohydroxylated dexloxiglumide suggesting that at least two such metabolites were present. The Q1 mass spectrum of a component with a retention time of 7.05 min also contained the M, M + 2 and M + 4 ions, but not in the ratio characteristic of the presence of two chlorines in the molecule. However, its Q3 mass spectrum was similar to those of the components at 6.73 and 7.37 min suggesting that it was probably also a monohydroxylated dexloxiglumide. Each of these metabolites presumably arose from monohydroxylation at a different position in either the *N*-pentyl or the *N*-3-methoxypropyl side chains. However, hydroxylation in the side chain creates a second asymmetric centre and consequently, the separate metabolites could also be diastereoisomers formed by oxidation at the same carbon atom.

There were at least two peaks in the mass chromatogram for m/z 487. From their respective polarities, the first was probably an ethyl ester of the dicarboxylic acid that arose after *O*-demethylation of dexloxiglumide and subsequent oxidation of the resultant alcohol: another possibility would be that of the dimethyl ester of the dicarboxylic acid. The second, with the longest retention time (13.23 min), is likely to correspond to the ethyl ester of dexloxiglumide since the retention time (12.02 min) of the corresponding methyl ester (m/z 473) was slightly shorter, as would be expected.

LC-MS/MS data generated from pooled urine extracts (0–9 h) obtained after oral administration of ^{14}C -dexloxiglumide showed that the same components were present as those characterized after i.v. administration.

Comparison of profiles obtained by tlc with those by LC-MS suggested that HM6 probably corresponded to methyl and ethyl esters of dexloxiglumide, HM5 to *O*-desmethyldexloxiglumide, HM4 to monohydroxylated dexloxiglumide and HM1/HM3 either to the dicarboxylic acid formed after *O*-demethylation of dexloxiglumide and subsequent oxidation of the resultant alcohol, or to glucuronic acid conjugates. Although dexloxiglumide and its corresponding glucuronide have similar retention times during LC-MS/MS, they were likely to have been well resolved during tlc such that HM1 or HM3 probably corresponded to the glucuronide. The latter supposition was indicated by the absence of HM3 in a urine sample incubated with β -glucuronidase (C. Webber, unpublished data).

Faeces. LC-MS/MS (electrospray positive-ion mode) analysis of the major and almost only ^{14}C component detected in faeces by tlc after single i.v. and oral doses showed that it contained mainly two compounds. One was identified as the methyl ester of dexloxiglumide and the second as a dimethyl ester of *O*-demethylated and further oxidized (in the *N*-3-methoxypropyl side chain) dexloxiglumide (figure 3), i.e. of 'dexloxiglumide carboxylic acid' (table 4).

The methyl ester of dexloxiglumide was identified by its identical chromatographic and mass spectral properties to an available reference compound, the methyl ester of *R,S*-4-(3,4-dichlorobenzamido)-5-[*N*-(3-methoxypropyl)-*N*-pentylamino]-5-oxopentanoic acid: this racemate is a 50:50 mixture of methylated dexloxiglumide and its opposite antipode. Under the conditions used, this methyl ester had a retention time of 8.27 min and its mass spectrum contained protonated

molecular ions at m/z 475/477/479. The Q1 mass spectrum also contained m/z 497/499/501 corresponding to sodium adducts and fragment ions at m/z 316/318/320 and 160, the latter corresponding to loss of the *N*-(3-methoxypropyl)-*N*-pentylamino side chain and the former to the remainder. The product ion mass spectrum in Q3 for m/z 475 contained the latter and prominent fragment ions m/z 160 and 316. Their relative intensities were about 100, 75 and 33%, respectively.

The dimethyl ester (of 'dexloxiglumide carboxylic acid') had a retention time of 8.0 min and protonated molecular ions at m/z 489/491/493. The spectrum also contained the ions corresponding to the sodium adducts (m/z 511/513/575) and the fragment ions at m/z 316/318/320 and 174. The product ion mass spectrum in Q3 for m/z 489 contained the latter and the fragment ions m/z 174 and 457, the latter corresponding to loss of methanol. Their relative intensities were about 100, 3 and 55%, respectively.

The M , $M+2$ and $M+4$ ions in the mass spectra of both of the above compounds were in the expected ratio of 9:6:1 for the two chlorine atoms present.

Discussion

Because of its physicochemical properties (e.g. $\log D$ 1.56 at pH 7.4), dexloxiglumide would be expected to be well absorbed from the gastrointestinal tract. Indeed, an oral dose of ^{14}C -dexloxiglumide was well absorbed but was also partly subjected to presystemic elimination as indicated by absolute bioavailabilities of 70 and 48% for total ^{14}C and unchanged drug, respectively (table 1). Receptors for the action of dexloxiglumide are located in the intestines (D'Amato *et al.* 1999b). The absorption rate of the oral dose appeared to be moderate as T_{max} occurred at about 1.5 h. Extensive absorption of the oral dose was also indicated by the excretion of less than 10% of the dose in the faeces during 0–24 h.

The mean total body clearance of dexloxiglumide of $0.22 \text{ l h}^{-1} \text{ kg}^{-1}$ (table 2) was low in relation to the average cardiac plasma output of about $2.1 \text{ l h}^{-1} \text{ kg}^{-1}$ and less than half of hepatic plasma flow of about $0.54 \text{ l h}^{-1} \text{ kg}^{-1}$ (Davies and Morris 1993). The mean volume of distribution of dexloxiglumide at steady-state (V_{ss} , table 2) was 0.18 l kg^{-1} compared with an average body water volume of about 0.6 l kg^{-1} (Davies and Morris 1993), suggesting that binding of dexloxiglumide in plasma exceeded its binding in tissues. Indeed, studies in rats have shown that after single oral doses of ^{14}C -dexloxiglumide, ^{14}C concentrations in most tissues, are less than those in plasma (C. Webber, unpublished data).

The mean terminal half-life of dexloxiglumide observed here was about 2.3 h after an oral dose. However, the calculated half-lives (table 1) did not satisfy acceptance criteria and were probably underestimates. Use of more sensitive bioanalytical methodology (i.e. an LC-MS method with an LLOQ of 50 ng ml^{-1}), which would enable acceptance criteria to be satisfied, yielded a half-life of about 5–10 h (Forest Laboratories, unpublished data). Compartmental modelling of i.v. route data showed that the elimination half-life ($t_{1/2}$) was 0.3 h (table 2), whereas the half-life associated with the second (β) phase was longer, 1.2 h (table 1). In addition, the volumes of distribution V_{ss} and V_z differed suggesting that elimination mainly occurred during the initial (α) phase and that the second (β) phase represented distribution of dexloxiglumide from the tissues back to the plasma. This latter phase probably accounts for the terminal half-life measured after oral doses.

Intersubject variability in the pharmacokinetics of dexloxiglumide was relatively low as indicated by coefficients of variation of about 20% or less for $C_{\max}$ and AUC (table 1).

In another pharmacokinetic study of dexloxiglumide in 12 healthy male volunteers also involving an hplc bioanalytical method, $C_{\max}$ and AUC were $5.4 \mu\text{g ml}^{-1}$ and $8.6 \mu\text{g h ml}^{-1}$, respectively, which are greater than the values observed in the present study (table 1). Intersubject variability was also greater (coefficient of variation of about 30–40%). AUCs were dose proportional over the dose range 100–400 mg. The half-life was about 3 h (Persiani *et al.* 2002), similar to the approximate value (2.3 h) in the present study.

Most of the ^{14}C in plasma was associated with the unchanged drug soon after dosing. However, the proportion of ^{14}C associated with unchanged drug progressively diminished such that, after the oral dose, it was less than 50% of plasma ^{14}C after 3 h. The identity of the circulating metabolites was not determined, but it is likely that these included at least one glucuronic acid conjugate and a dicarboxylic acid formed by O-demethylation of dexloxiglumide and subsequent oxidation of the resultant alcohol to a carboxylic acid. None of these metabolites would be expected to have been extractable from plasma under the conditions used for metabolite profiling which only detected dexloxiglumide in the plasma samples profiled. Further work is ongoing to determine the identity of circulating metabolites.

After i.v. or oral doses the majority of the ^{14}C dose was excreted in the faeces. The prolonged excretion of ^{14}C in faeces after i.v. or oral administration of ^{14}C -dexloxiglumide can be explained by biliary excretion and perhaps also some enterohepatic circulation of dexloxiglumide and/or its metabolites. Biliary excretion and some enterohepatic circulation of dexloxiglumide occurs in rats (C. Webber, unpublished data).

Likely metabolites include glucuronic acid conjugates of dexloxiglumide and of the corresponding dicarboxylic acid as only such conjugates would have the requisite physicochemical properties for biliary excretion, particularly in respect of molecular weight. The molecular weight of either glucuronide would be 637 and in excess of the threshold for biliary excretion in humans (Hirom *et al.* 1977). Urinary excretion of ^{14}C was not detected after about 3 days whereas faecal excretion was not quite complete after 7 days (table 3). Dexloxiglumide glucuronide has been detected in the bile of a dog dosed orally with ^{14}C -dexloxiglumide (C. Webber, unpublished data).

This study has demonstrated that dexloxiglumide is metabolized by oxidation and conjugation (table 4). The former is responsible for monohydroxylation in more than one position in the *N*-(3-methoxypropyl) pentylamino side chain as well as O-demethylation of the 3-methoxy group followed by subsequent oxidation of the resultant alcohol to the corresponding carboxylic acid. *In vitro* metabolism studies have shown that cytochromes P450 3A4, 2B6 and 2C9 are implicated in the metabolism of dexloxiglumide (Hall *et al.* 2001). The oxidation products are expected metabolites of a molecule with the structure of dexloxiglumide (Gibson and Skett 1986). Based on the large proportion of the i.v. or oral dose excreted in the faeces, it is probable that the major metabolites were conjugates of dexloxiglumide and the corresponding dicarboxylic acid. Such metabolites would be excreted in the bile and hydrolysed by the gut flora (Scheline 1973) to yield the corresponding aglycones which were identified as the corresponding methyl esters

owing to the extraction conditions used. The proportion of an oral dose of ^{14}C -dexloxiglumide excreted in urine was low, less than 2% of the dose in the study of Persiani *et al.* (2002), indicating that renal excretion was unimportant in the elimination of unchanged dexloxiglumide.

The methyl and ethyl esters of dexloxiglumide and the corresponding dicarboxylic acid, identified by LC-MS/MS, are unlikely to be metabolites but were more probably artefacts of the extraction conditions used before metabolite profiling of urine and faeces and subsequent LC-MS/MS identification. Urine was extracted with ethyl acetate under acidic conditions, and it is probable that the large excess of ethyl acetate was partly hydrolysed, under the acidic conditions used to ethyl alcohol and acetic acid. Again, owing to the acidic conditions, the former was involved in the partial esterification of dexloxiglumide and its carboxylic acid metabolite to produce the corresponding ethyl esters. Unesterified carboxylic acids would have then been partially esterified to methyl esters when evaporated extracts were dissolved in methanol. Faeces were extracted with methanol under acidic conditions that would have facilitated the formation of methyl esters. This was confirmed by spiking control faeces with ^{14}C -dexloxiglumide and employing the same extraction conditions (data not shown). Carboxylic acids are known to be esterified if mixed with an excess of an alcohol such as methanol in the presence of an acid such as hydrochloric acid (Fieser and Fieser 1956).

Dexloxiglumide is a single isomer drug that did not appear to racemise *in vivo*. Conversion of dexloxiglumide to its opposite enantiomer would not be expected because the chiral centre is not located at a probable site of metabolism.

Acknowledgements

The authors thank Leicester Clinical Research Centre for its care of the subjects who participated in the study and R. R. Brodie, C. Jelley, C. Miller and A. Poole for respective contributions.

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Interaction with indinavir to enhance systemic exposure of an investigational HIV protease inhibitor in rats, dogs and monkeys

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Received 28 October 2002

1. The use of a beneficial interaction between indinavir and compound A, a potent investigational HIV protease inhibitor to enhance systemic exposure of compound A, was investigated.

2. When administrated alone, compound A underwent extensive hepatic first-pass metabolism in rats and monkeys, resulting in low oral bioavailability.

3. *In vitro* studies with liver microsomes revealed that compound A metabolism was mediated exclusively by CYP3A enzymes in rats, dogs and monkeys. Indinavir, which also was metabolized predominantly by CYP3A enzymes, extensively inhibited compound A metabolism in microsomes, whereas compound A showed weak inhibitory potency on indinavir metabolism.

4. Consistent with *in vitro* observations, co-administration of the two compounds resulted in a 17-fold increase in oral AUC of compound A in rats owing to the inhibition of metabolism of compound A by indinavir, whereas compound A did not affect indinavir metabolism as indicated by the unchanged indinavir AUC. Similarly, the systemic exposure of compound A in dogs and monkeys was increased substantially following oral co-administration with indinavir by 7- and > 50-fold, respectively.

5. Enhancement in compound A systemic exposure by indinavir in humans, as predicted based on the *in vivo* animal and *in vitro* human liver microsomal data, was confirmed in subsequent clinical studies.

Introduction

Metabolic drug interactions are normally considered undesirable because they can cause potentially dangerous clinical consequences. However, there are times when these interactions can be advantageous. Cyclosporin–ketoconazole combination therapy is an example of the successful use of a beneficial interaction between the drugs, which are both metabolized by CYP3A4. Ketoconazole prolongs the elimination of cyclosporin via competitive inhibition of its metabolism (Yee *et al.* 1990). With this approach, the effective dose of cyclosporin in cardiac transplantation patients is reduced by 80% by addition of ketoconazole (Keogh *et al.* 1995). This results in a cost saving of approximately US\$5200 per patient every year. Use of other drugs to reduce the cost of cyclosporin also has been reported (Jones 1997).

Human immunodeficiency virus (HIV) protease inhibitors are highly effective in the treatment of acquired immune deficiency syndrome (AIDS) (Vacca and Condra 1997). However, clinical use of these drugs has experienced difficulties in

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compliance with the treatment protocols. Most of the current HIV protease inhibitors have limited oral bioavailability owing to extensive first-pass metabolism (Lin 1997). Consequently, these drugs must be taken in relatively large quantities two or three times a day. For example, the recommended dosage is 1200 mg three times daily for saquinavir, 800 mg three times daily for indinavir and 600 mg twice a day for zidovudine (package inserts for Fortovase (saquinavir mesylate), Crixivan (indinavir sulfate), and Norvir (zidovudine)).

Recently, beneficial drug–drug interactions have been exploited to overcome the pharmacokinetic limitations of HIV protease inhibitors. One example is the lopinavir–ritonavir combination that increases the oral bioavailability of lopinavir through inhibition of its metabolism by ritonavir (Sham *et al.* 1998, Kumar *et al.* 1999). Lopinavir plasma concentrations are increased significantly by concomitant administration of ritonavir. However, ritonavir is known to be a suicide inhibitor of the drug-metabolizing enzyme CYP3A4 (Koudriakova *et al.* 1998) and the long-term consequence of enzyme destruction remains largely unknown. For this reason, we investigated an alternative combination that uses a reversible inhibitor, indinavir, to enhance the systemic exposure of another HIV protease inhibitor candidate.

Compound A (*N*-tertiary-butyl 4-(benzofuran-2-ylmethyl)-1-[2(*S*)-hydroxy-4(*R*)-[2(*R*)-hydroxy-indan-1(*S*)-ylcarbamoyl]-5-phenyl-pentyl]-piperazine-2(*S*)-carbox amide) is a structural analogue of indinavir (Dorsey *et al.* 2000). While exhibiting better antiviral activity than indinavir, compound A is precluded from stand-alone antiviral therapy because extensive first-pass metabolism by CYP3A enzyme limits its oral bioavailability. The present study demonstrates the concept of beneficial interaction in which indinavir inhibits the metabolism of compound A, thereby enhancing its systemic exposure in animals.

Materials and methods

Materials

Indinavir, compound A, deuterated compound A (D4-compound A) and [^{14}C] compound A were synthesized at Merck Research Laboratories. The ^{14}C label was incorporated at the carbonyl carbon of the pentanamide with the specific activity of $38.13 \mu\text{Ci mg}^{-1}$ and radiochemical purity of $> 99\%$. Pooled male rat, dog and rhesus monkey hepatic microsomes were obtained from XenoTech LLC (Kansas City, KS, USA). Pooled human liver microsomes were purchased from Human Biologics International (Scottsdale, AZ, USA). Monoclonal anti-human CYP3A4, CYP2C and CYP2D6 antisera were provided by Dr Magang Shou (Merck Research Laboratories, West Point, PA, USA) (Mei *et al.* 1999, 2002). All three antibodies showed high specificity to the respective enzymes, which inhibited the metabolism of corresponding marker substrates by $> 98\%$, whereas having little activities on other P450s (Mei *et al.* 1999, 2002). Anti-rat CYP3A2 and CYP2C11 antisera, which are specific to rat CYP3A and 2C, were purchased from Gentest Corp. (Woburn, MA, USA). All other reagents were purchased from commercial sources and were of the highest purity available.

Animals

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA), weighing 250–350 g, male beagle dogs (Marshall Research, North Rose, NY, USA), 9–11 kg, and male rhesus monkeys (Charles River, Key Lois, FL, USA), 3–6 kg, were used. All procedures performed on animals were in accordance with established guidelines and were reviewed and approved by the Merck Research Laboratories Institutional Care and Use Committee.

In vivo studies

Compound A was suspended in 0.05 M citric acid for oral administration and dissolved in DMSO for dosing intravenously. Indinavir was dissolved in 0.05 M citric acid for oral administration.

Studies in rats. For kinetic studies, all rats had an indwelling cannula (silicone rubber/polyethylene) implanted in the right jugular vein for blood sampling and drug administration. Surgery was performed under pentobarbital anaesthesia (40 mg kg^{-1} i.p.) 1 day before the experiment. Animals were fasted overnight before oral administration.

In single drug administration studies, groups of rats ($n = 4-6$ per group) received compound A at 2 mg kg^{-1} i.v., 10 mg kg^{-1} p.o. or 40 mg kg^{-1} p.o. A separate group of animals received an oral dose of indinavir at 40 mg kg^{-1} . In co-administration studies, one group of rats received an oral dose of both indinavir and compound A at 40 mg kg^{-1} of each drug, and another group received an i.v. dose (10 mg kg^{-1}) of compound A immediately after an oral dose of indinavir (40 mg kg^{-1}). Blood samples were collected periodically at appropriate time points from the jugular vein. The plasma samples were obtained by centrifugation and stored frozen at -20°C .

The extent of hepatic first-pass effect of compound A was determined in rats by comparing the drug concentrations in the systemic circulation during portal and femoral vein infusion. Each animal in the group had an additional cannula implanted either in the portal ($n = 5$) or femoral vein ($n = 5$) 1 day before dosing. The drug was infused at a constant rate of $4 \mu\text{g min}^{-1}$ for 150 min. Blood samples were collected from right jugular vein at 0, 15, 30, 60, 90, 120, 150, 180, 240 and 300 min.

The *in situ* isolated rat intestinal loop method (Barr and Riegelman 1970) was used to determine the extent of intestinal first-pass metabolism of compound A in rats. Briefly, rats ($n = 4$) were anaesthetized with pentobarbital (40 mg kg^{-1} i.p.), and a cannula was inserted into the left jugular vein. Implantation of the mesenteric venous cannula and preparation of the isolated intestinal loop system *in situ* were then performed. A segment of proximal jejunum 10–15 cm in length was selected for use. All mesenteric venous blood from the loop was collected continuously for 60 min, and replacement rat blood (fresh) was infused simultaneously via jugular cannulation at approximately the same rate as blood was drained from the mesenteric venous cannula ($0.1-0.15 \text{ ml min}^{-1}$). Blood samples were collected every 5 or 10 min up to 60 min after intrajejunal injection of [^{14}C] compound A (10 mg kg^{-1} , 1.5 ml). The plasma samples were analysed for unchanged drug and total radioactivity.

Studies in dogs. In single drug administration studies, male beagle dogs ($n = 4$) received 10 mg kg^{-1} oral dose or 2 mg kg^{-1} i.v. dose of compound A. In co-administration studies, the same dogs received an oral dose of both indinavir (20 mg kg^{-1}) and compound A (5 mg kg^{-1}). On a separate occasion, these dogs received an i.v. dose of compound A (2 mg kg^{-1}) immediately after an oral dose of indinavir (20 mg kg^{-1}). Blood samples were collected periodically at appropriate time points and plasma was obtained by centrifugation and stored frozen at -20°C until assayed.

Studies in rhesus monkeys. In single drug administration studies, rhesus monkeys ($n = 3$) were given compound A at 10 mg kg^{-1} p.o. and 2 mg kg^{-1} i.v. In co-administration studies, these monkeys received an oral dose of both indinavir (20 mg kg^{-1}) and compound A (10 mg kg^{-1}), or an i.v. dose of compound A (2 mg kg^{-1}) immediately after an oral dose of indinavir (20 mg kg^{-1}).

The extent of hepatic first-pass effect of compound A in monkeys was determined by comparing the drug concentrations in the systemic circulation during portal and cephalic vein infusion. The drug was given by a constant infusion at $20 \mu\text{g min}^{-1}$ for 60 min. Blood samples were collected at 0, 15, 30, 60, 65, 75, 90, 120, 180, 240, 300, 360, 480 and 1440 min.

In vitro studies

Distribution of [^{14}C] compound A in blood. The partition of [^{14}C] compound A between red blood cells and plasma was assessed in rats, dogs and monkeys at final concentrations of 0.5, 1, 2, 5, 10 and $20 \mu\text{M}$. Blood was incubated at 37°C for 15 min; the plasma was separated immediately by centrifugation. A preliminary study indicated that the partitioning equilibrium was reached within 5 min. Blood and plasma were combusted to $^{14}\text{CO}_2$ with a Packard oxidizer and radioactivity was determined by liquid scintillation spectrometry.

Antibody and chemical inhibition study. Anti-human CYP3A4 and CYP2C antibodies were used in incubation with human and monkey liver microsomes, whereas anti-rat CYP3A2 and CYP2C11 were used in rat and dog liver microsomal incubations. Anti-human CYP2D6 was used in all species. The cross-reactivity of each antibody was verified by examining the inhibitory effect of the antiserum on CYP3A, CYP2C and CYP2D activities in each species. Anti-human CYP3A4 almost completely inhibited testosterone 6β -hydroxylase activity ($> 98\%$) in monkey liver microsomes, and anti-rat CYP3A2 inhibited 94% of testosterone 6β -hydroxylase activity in dog liver microsomes. Anti-human CYP2C antibody inhibited 72% of diclofenac 4'-hydroxylase activity in monkey liver microsomes, and anti-rat CYP2C11 inhibited 89% of diclofenac 4'-hydroxylase activity in dog liver microsomes. Anti-human CYP2D6 antibody inhibited bufuralol 1'-hydroxylase activity by approximately 88% in rats, 76% in dogs and 83% in monkeys, respectively.

For the immunochemical inhibition study, hepatic microsomes were mixed with various amounts ($0\text{--}40\text{ }\mu\text{l}$ (mg microsomal proteins) $^{-1}$) of antiserum, and incubated on ice for 20 min. An aliquot of the antibody-treated microsomes was added to a reaction mixture in 0.1 M phosphate buffer ($\text{pH } 7.4$) containing an NADPH-regenerating system (20 mM glucose 6-phosphate, 4 IU ml^{-1} glucose 6-phosphate dehydrogenase, 10 mM MgCl_2 and 1 mM NADPH) to yield a final microsomal protein concentration of $0.1\text{--}0.2\text{ mg ml}^{-1}$. After warming at 37°C for 5 min, the reaction was initiated by adding compound A ($1\text{ }\mu\text{M}$), and terminated by adding acetonitrile after a 5-min incubation. The extent of compound A metabolism was measured as the difference of compound A concentrations between a control sample (quenched at 0 min) and the test samples. Similar experimental conditions were used to determine the effect of ketoconazole on the metabolism of compound A. Compound A ($1\text{ }\mu\text{M}$) was incubated with liver microsomes in the presence of various concentrations of ketoconazole for 5 min.

Indinavir–compound A interactions in hepatic microsomes. For the determination of IC_{50} values of indinavir for inhibiting compound A metabolism, various concentrations of indinavir ($0.01\text{--}5\text{ }\mu\text{M}$) were added to microsomal incubation mixtures containing a fixed concentration of compound A ($1\text{ }\mu\text{M}$). The final concentration of microsomal proteins was $0.1\text{--}0.2\text{ mg ml}^{-1}$. Similar conditions were used to determine the IC_{50} values of compound A for indinavir metabolism. Hepatic microsomes (1 mg ml^{-1}) were incubated with indinavir ($1\text{ }\mu\text{M}$) in the presence of various concentrations of compound A ($0.1\text{--}100\text{ }\mu\text{M}$).

Analytical procedure

The concentrations of indinavir in plasma were determined by HPLC as reported by Chen *et al.* (1995). The concentrations of compound A in plasma and liver microsomal incubates were determined either by LC-UV or LC-MS/MS. Briefly, the internal standard (D_4 -compound A) was added to an aliquot of biological specimen ($0.05\text{--}0.5\text{ ml}$), and extracted with diethyl ether ($0.8\text{--}5\text{ ml}$). Following centrifuging, the organic layer was transferred to a clean tube where it was evaporated to dryness under nitrogen. The residue was constituted with 30% aqueous acetonitrile containing 0.1% trifluoroacetic acid ($250\text{ }\mu\text{l}$).

Samples analysed by the LC-UV method were injected onto a Vydac protein peptide C18 column ($4.6\text{ mm} \times 25\text{ cm}$). The mobile phase was 36% aqueous acetonitrile containing 0.1% trifluoroacetic acid, and the flow rate was 1 ml min^{-1} . The column effluent was monitored at 220 nm .

LC-MS/MS analysis of the samples was carried out on a Perkin-Elmer Sciex (Toronto, Canada) 3000 triple-quadrupole mass spectrometer equipped with a heated nebulizer interface. Separation was achieved by gradient elution reverse-phase chromatography using a Zorbax 300SB-C18 column ($4.6\text{ mm} \times 5\text{ cm}$). The mobile phases consisted of solvents A (0.1% aqueous formic acid) and B (acetonitrile containing 0.1% formic acid), which were delivered in linear gradient of 20 to 80% B in 2.5 min . Quantitation was carried out in the positive-ion detection mode by monitoring the transitions $m/z\ 653 \rightarrow 504$ for compound A and $657 \rightarrow 508$ for D_4 -compound A.

Pharmacokinetic analysis

Plasma clearance (CL_p), plasma volume distribution at steady state ($V_{d_{ss,p}}$) and apparent half-life ($t_{1/2}$) were calculated by standard non-compartmental methods (Gibaldi and Perrier 1982, Rocci *et al.* 1983, Wilkinson 1987). Plasma pharmacokinetic PK parameters were converted to corresponding blood values using the blood-to-plasma distribution ratios. Bioavailability (F) was calculated as the ratio between dose-normalized AUC_0^∞ values measured following oral and intravenous administration.

Results

Pharmacokinetics of compound A

Compound A had somewhat lower clearance than indinavir, but was also cleared rapidly in all species following intravenous administration (table 1). The terminal $t_{1/2}$ of compound A ranged from 54 min in rats to 70 min in monkeys. For comparison, the pharmacokinetic parameters of indinavir are also shown in table 2 (Lin 1996). Similar to indinavir, compound A exhibited marked species difference in elimination kinetics.

Following oral administration, significant species differences in the absorption kinetics were observed for compound A. The C_{max} and AUC values were high in

Table 1. Pharmacokinetic parameters of compound A in rats, dogs and monkeys following intravenous administration (mean $\pm$ SD, $n = 3-6$).

Species	Dose (mg kg ⁻¹) i.v./p.o.	Intravenous dose			Oral dose		
		CL _B (ml min ⁻¹ kg ⁻¹)	$Vd_{ss,B}$ (l kg ⁻¹)	$t_{1/2}$ (min)	C_{max} (μ M)	AUC (μ M*h)	F (%)
Rat	10/20	81.5 $\pm$ 23.5	3.57 $\pm$ 1.24	42 $\pm$ 17	0.32 $\pm$ 0.22	1.3 $\pm$ 0.9	13.0
Dog	2/9	8.2 $\pm$ 1.8	0.49 $\pm$ 0.05	54 $\pm$ 18	28.4 $\pm$ 17.5	41.0 $\pm$ 28.7	70.3 $\pm$ 30.9
Monkey	2/10	18.3 $\pm$ 8.7	0.89 $\pm$ 0.20	39 $\pm$ 1	< 0.03	ND ^a	ND ^a

^a ND, not determined due to insufficient data points.

Table 2. Pharmacokinetic parameters of indinavir rats, dogs and monkeys following intravenous administration (mean $\pm$ SD, $n = 3-6$).

Species	Dose (mg kg ⁻¹) i.v./p.o.	Intravenous dose			Oral dose		
		CL _B (ml min ⁻¹ kg ⁻¹)	$Vd_{ss,B}$ (l kg ⁻¹)	$t_{1/2}$ (min)	C_{max} (μ M)	AUC (μ M*h)	F (%)
Rat	10/10	107.0 $\pm$ 31.0	2.24 $\pm$ 0.46	25 $\pm$ 17	0.44 $\pm$ 0.14	0.85 $\pm$ 0.20	23.9
Dog	10/10	11.4 $\pm$ 1.5	0.52 $\pm$ 0.06	38 $\pm$ 2	11.4 $\pm$ 2.3	12.5 $\pm$ 4.1	71.6 $\pm$ 13.4
Monkey	10/10	35.7 $\pm$ 4.2	1.45 $\pm$ 0.10	30 $\pm$ 1	0.71 $\pm$ 0.24	1.45 $\pm$ 0.29	18.8 $\pm$ 1.4

dogs, but low in rats (table 1). No drug (< 30 nM) was detected in the plasma of monkeys when a 10 mg kg⁻¹ dose was given orally. The estimated bioavailability was 70.3 and 13% in dogs and rats, respectively. Based on blood clearance data, the low bioavailability in rats and monkeys was most likely due to extensive first-pass metabolism during absorption.

To determine whether compound A was subject to significant first-pass elimination, hepatic first-pass metabolism was estimated in rats and monkeys by comparing systemic AUC during portal and femoral (rats) or cephalic vein (monkeys) infusion. When compound A was given at a constant rate of 4 and 20 μ g min⁻¹, respectively, to rats and monkeys, the systemic concentrations of the drug were substantially lower during portal vein infusion than femoral or cephalic vein infusion. The extent of hepatic first-pass effect was estimated to be about 74 and > 95% in rats and monkeys, respectively.

The extent of intestinal first-pass metabolism in rats was determined with the *in situ* isolated intestinal loop method. About 83% of the dose was recovered as unchanged drug after [¹⁴C] compound A (10 mg) was introduced directly into rat jejunum. The extent of intestinal first-pass metabolism was therefore about 17%.

In vitro studies

The blood-to-plasma concentration ratio of compound A was independent of drug concentrations over the range 0.5–20 μ M. The average blood-to-plasma partition ratio was 0.68, 0.55 and 0.70 for the rat, dog and monkey, respectively.

After intravenous administration of compound A, less than 1% of the dose was excreted in urine and bile of rats, dogs and monkeys as unchanged drug over 48 h.

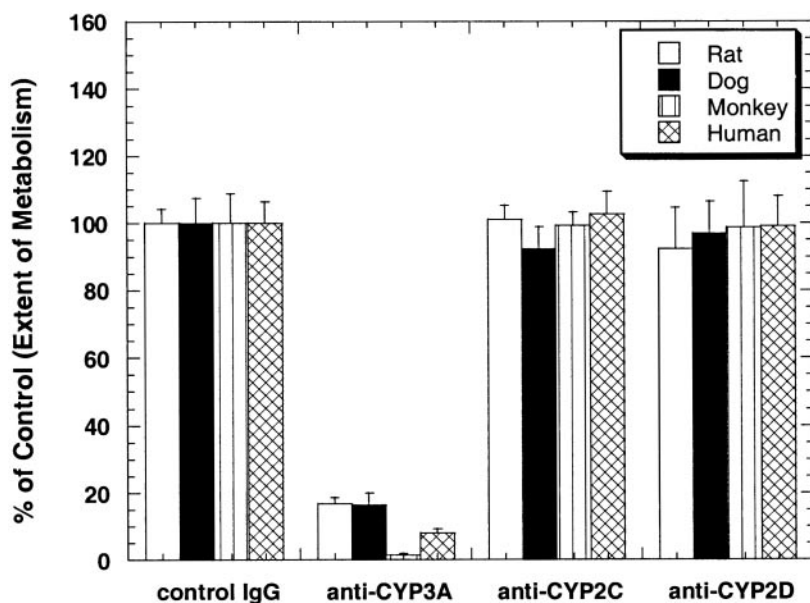


Figure 1. Inhibition of compound A metabolism by antibodies against CYP3A, CYP2C and CYP2D. Data are the mean of three determinations with SD.

Thus, biotransformation was the major route of compound A elimination. Oxidative metabolism, i.e. hydroxylation and *N*-dealkylation, were the primary metabolic pathways of compound A in all three species (unpublished data, Merck Research Laboratories).

Chemical and immuno-inhibition studies showed that the oxidative metabolism of compound A in rats, dogs, monkeys and humans was catalysed mainly by members of the CYP3A subfamily, the same enzymes that mediate indinavir metabolism. Anti-rat CYP3A2 antibody inhibited approximately 83 and 84% of compound A metabolism in rat and dog liver microsomes, and anti-human CYP3A4 inhibited about 98 and 92% of compound A metabolism in monkey and human liver microsomes, respectively (figure 1). On the other hand, antiserum directed against CYP2C and 2D6 had little effect on compound A metabolism. Consistent with these results, ketoconazole, a selective inhibitor of CYP3A enzymes, markedly inhibited (> 85%) the metabolism of compound A in rat, dog, monkey and human liver microsomes. Similarly, indinavir is predominantly cleared in rats, dogs and monkeys via oxidative metabolism, which is mediated mainly by CYP3A enzymes (Lin *et al.* 1996).

Since both indinavir and compound A are metabolized by CYP3A enzymes, the potency of indinavir to inhibit compound A metabolism and vice versa was investigated *in vitro*. Indinavir was a very potent inhibitor of compound A metabolism *in vitro*; the IC₅₀ values of indinavir on compound A metabolism in liver microsomes were low, being 0.10, 0.13, 0.16 and 0.17 µM for rat, dog, monkey and human, respectively. In contrast, compound A was a very weak inhibitor of indinavir metabolism as reflected by IC₅₀ values that were > 20 µM in all species (table 3).

Table 3. UC_{50} values (μM) for indinavir on compound A metabolism and vice versa in rat, dog, monkey and human liver microsomes.

Species	IC_{50} (μM)	
	Indinavir on compound A metabolism	Compound A on indinavir metabolism
Rat	0.10	21.1
Dog	0.13	35.8
Monkey	0.16	20.9
Human	0.17	30.1

In vivo interaction studies

Following oral administration, indinavir enhanced the systemic exposure of compound A in rats, while compound A had little effect on the exposure of indinavir (figures 2 and 3). When indinavir (40 mg kg^{-1}) was given concomitantly to rats with compound A (40 mg kg^{-1}), both the $C_{\max}$ and AUC values for compound A were increased dramatically (by a factor of 17-fold) compared with those when compound A was given alone (table 4). In contrast, the $C_{\max}$ and AUC values for indinavir were not changed by compound A.

Similar to the observations in rats, oral co-administration of indinavir (20 mg kg^{-1}) and compound A (5 mg kg^{-1}) to dogs substantially increased (by a factor of 3–7-fold) both the $C_{\max}$ and AUC values for compound A (table 4).

As in rats and dogs, oral administration to rhesus monkeys of indinavir (20 mg kg^{-1}) and compound A (10 mg kg^{-1}) improved the bioavailability of compound A. In contrast to the undetectable plasma concentrations of compound A when given alone orally, compound A was present in pharmacologically relevant concentrations when it was co-dosed with indinavir (table 4).

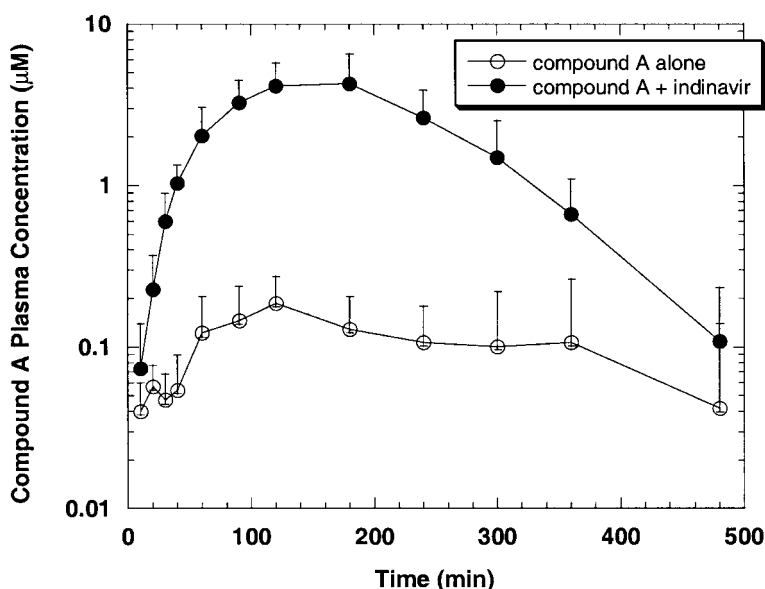


Figure 2. Plasma concentration–time profiles of compound A in rats following oral administration of compound A alone (40 mg kg^{-1}) and both compound A and indinavir (40 mg kg^{-1} of each drug). Data are the mean $\pm$ SD of six determinations.

Table 4. Effects of indinavir on absorption kinetics of compound A in rats, dogs and monkeys (mean $\pm$ SD, $n = 3-6$).

Species	Treatment	$C_{\max}$ (μM)	$T_{\max}$ (min)	$t_{1/2}$ (min)	AUC ($\mu\text{M}\cdot\text{h}$)
Rat	compound A (40 mg kg^{-1} , p.o.)	0.27 ± 0.09	198 ± 123	—	0.93 ± 0.29
	compound A (40 mg kg^{-1} , p.o.) + indinavir (40 mg kg^{-1} , p.o.)	$4.56 \pm 2.01^*$	130 ± 24	80 ± 13	$15.9 \pm 6.9^*$
Dog	compound A (5 mg kg^{-1} , p.o.)	7.6 ± 3.9	27 ± 6	77 ± 26	18.8 ± 17.7
	compound A (5 mg kg^{-1} , p.o.) + indinavir (20 mg kg^{-1} , p.o.)	$21.5 \pm 7.5^*$	130 ± 148	195 ± 111	$133 \pm 70^*$
Monkey	compound A (10 mg kg^{-1} , p.o.)	< 0.03	—	—	—
	compound A (10 mg kg^{-1} , p.o.) + indinavir (20 mg kg^{-1} , p.o.)	1.8 ± 1.0	170 ± 114	69 ± 17	5.9 ± 3.3

* Significantly different ($p < 0.05$) from compound A administration alone.

To assess the route dependence of the enhancement of compound A systemic exposure, indinavir and compound A were co-administrated intravenously. Indinavir enhanced compound A systemic exposure in rats and dogs, but had little effect in monkey AUC (table 5). In rats, the AUC was increased by 50% when compound A was given intravenously (10 mg kg^{-1}) to rats immediately after an oral dose of indinavir (40 mg kg^{-1}) compared with that obtained after dosing compound A intravenously alone. The blood clearance of compound A was decreased from 81.5 to $56.6\text{ ml min}^{-1}\text{ kg}^{-1}$. In dogs, the AUC of compound A was 3-fold higher after an i.v. dose of compound A (2 mg kg^{-1}) immediately following an oral dose of indinavir (20 mg kg^{-1}) than compound A was given alone. The blood clearance was diminished to $3.0\text{ ml min}^{-1}\text{ kg}^{-1}$. In monkeys, the AUC values of compound A were unchanged by the combination treatment.

Discussion

The present study has demonstrated that drug interactions can be used in a beneficial manner to enhance the systemic exposure of a drug of interest. Indinavir, a potent reversible inhibitor of CYP3A enzymes, was shown to increase

Table 5. Effects of indinavir on pharmacokinetics of compound A in rats, dogs and monkeys (mean $\pm$ SD, $n = 36$).

Species	Treatment	CL_B (ml min kg^{-1})	$Vd_{ss,B}$ (1 kg^{-1})	$t_{1/2}$ (min)	AUC ($\mu\text{M}\cdot\text{h}$)
Rat	compound A (10 mg kg^{-1} , i.v.)	81.5 ± 23.5	3.57 ± 1.24	42 ± 17	4.9 ± 1.5
	compound A (10 mg kg^{-1} , i.v.) + indinavir (40 mg kg^{-1} , p.o.)	56.6 ± 27.8	5.21 ± 1.68	67 ± 27	7.6 ± 2.9
Dog	compound A (2 mg kg^{-1} , i.v.)	8.2 ± 1.9	0.49 ± 0.04	54 ± 18	11.9 ± 3.1
	compound A (2 mg kg^{-1} , i.v.) + indinavir (20 mg kg^{-1} , p.o.)	$3.0 \pm 3.2^*$	0.29 ± 0.16	111 ± 31	48.8 ± 33.6
Monkey	compound A (2 mg kg^{-1} , i.v.)	18.3 ± 8.6	0.91 ± 0.21	39 ± 1	4.6 ± 2.1
	compound A (2 mg kg^{-1} , i.v.) + indinavir (20 mg kg^{-1} , p.o.)	18.7 ± 1.3	2.00 ± 0.10	75 ± 3	3.9 ± 0.3

* Significantly different ($p < 0.05$) from compound A administration alone.

substantially plasma levels of compound A, a potent HIV protease inhibitor in rats, dogs and monkeys, when given concomitantly with compound A.

The bioavailability of a drug is determined by the fraction of absorption (f_{abs}) and its hepatic (f_{h}) and intestinal (f_{g}) first-pass metabolism, as shown in equation (1) (Lin 1995):

$$F = f_{\text{abs}} \cdot (1 - f_{\text{g}}) \cdot (1 - f_{\text{h}}). \quad (1)$$

The extent of hepatic and intestinal first-pass effects of compound A in rats was 74 and 17%, respectively. The fraction (f_{abs}) of compound A absorbed in the rat after oral administration was therefore estimated to be about 60% based on equation (1), indicating compound A was reasonably well absorbed. Similarly, compound A underwent extensive hepatic first-pass elimination in monkeys ($f_{\text{h}} > 95\%$). The extent was much greater than expected based on total blood clearance in the species, and the underlying mechanism for this discrepancy remains unclear at the current moment. Nevertheless, the data strongly suggest that the poor oral bioavailability in monkeys was also mainly due to hepatic first-pass metabolism.

Studies with rat, dog and monkey liver microsomes indicated that metabolism of compound A, as was that of indinavir (Lin *et al.* 1996), was mediated almost exclusively by CYP3A enzymes in these species (figure 1). Kinetics studies revealed that indinavir was a very potent inhibitor of compound A metabolism *in vitro*, whereas compound A was a very weak inhibitor of indinavir metabolism. Based on these data, it was concluded that indinavir would inhibit compound A metabolism in rats and dogs but not the other way around.

Although *in vitro* methods such as those employed here are useful tools to identify potential drug-drug interactions, it is still difficult, if not impossible, to predict precisely the magnitude of *in vivo* drug interactions from *in vitro* data (Lin 2000). It was therefore necessary to evaluate the extent of indinavir-compound A interaction *in vivo*. Consistent with the *in vitro* data, indinavir significantly inhibited the metabolism of compound A in rats, resulting in a 17-fold increase in oral AUC of compound A, whereas compound A did not affect indinavir metabolism as indicated by the unchanged indinavir AUC. Since the goal of the present study was to predict the interaction in patients, it is important to confirm that the *in vivo* interaction also occurs in dogs and monkeys. Similar to the case in rats, the systemic exposure of compound A in dogs and monkeys was increased substantially by indinavir following oral co-administration.

Indinavir-compound A interactions were route dependent. The magnitude of enhancement in compound A systemic exposure was significantly greater after an oral dose than an intravenous administration of compound A in all three species. The route dependence of compound A and indinavir interaction was in good agreement with the fact that compound A is an intermediate to high clearance compound. For a high-clearance compound, a decrease in the intrinsic clearance (CL_{int}) by enzyme inhibition would result in more profound increases in AUC after oral than those after intravenous administration as was shown theoretically (Lin 2000) and experimentally (Lin 1996, Tsunoda *et al.* 1999).

In theoretical consideration, the AUC after oral and i.v. administration can be expressed by the following equations based on the well-stirred model (Lin and Lu 1998):

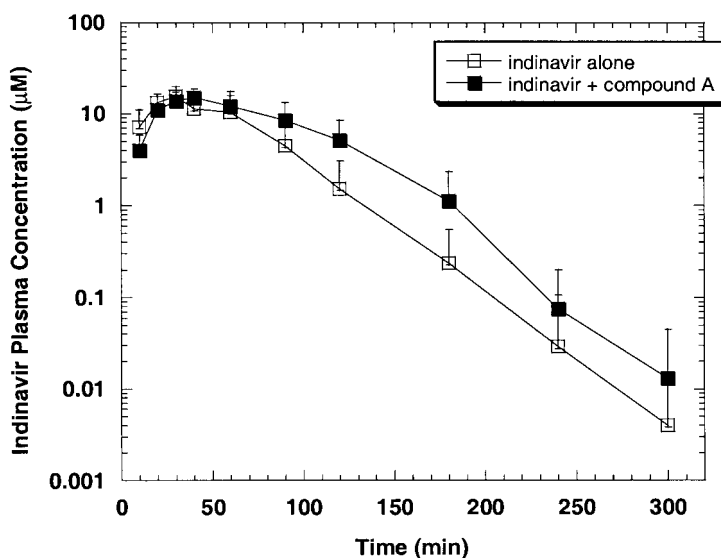


Figure 3. Plasma concentration–time profiles of indinavir in rats following oral administration of indinavir alone (40 mg kg^{-1}) and both compound A and indinavir (40 mg kg^{-1} of each drug). Data are the mean $\pm$ SD of six determinations.

$$\text{AUC}_{\text{i.v.}} = \frac{\text{dose}}{\text{CL}_H} = \frac{\text{dose}}{\frac{Q_H \cdot f_u \cdot \text{CL}_{\text{int}}}{Q_H + f_u \cdot \text{CL}_{\text{int}}}} \quad (2)$$

$$\text{AUC}_{\text{p.o.}} = \frac{F_H \cdot f_{\text{abs}} \cdot \text{dose}}{\text{CL}_H} = \frac{f_{\text{abs}} \cdot \text{dose}}{f_u \cdot \text{CL}_{\text{int}}}, \quad (3)$$

where Q_H is the hepatic blood flow, CL_H is the hepatic clearance, and f_u is the unbound fraction in blood. Therefore, for low clearance drugs, a decrease in the CL_{int} caused by enzyme inhibition will result in an almost proportional increase in the AUC after both oral and i.v. administration. However, for high clearance compounds, a reduction of the CL_{int} will increase the $\text{AUC}_{\text{p.o.}}$ proportionally, but will have little effect on the $\text{AUC}_{\text{i.v.}}$ since the hepatic CL_H is limited by hepatic blood flow. This explains why there is a greater increase in AUC values for compound A after oral administration than after i.v. administration of compound A with indinavir. This type of route-dependent drug–drug interactions have been reported previously for indinavir–ketoconazole (Lin 1996) and midazolam–ketoconazole (Tsunoda *et al.* 1999) combinations.

Similar to the cases in rats and monkeys, compound A exhibits limited bioavailability in humans when dosed alone, and failed to achieve the concentrations in plasma necessary to suppress viral reproduction (Deutsch *et al.* 2000). The clearance of compound A in humans is high, suggesting that the low oral bioavailability is mainly due to extensive metabolism. *In vitro* kinetic studies revealed that the IC_{50} value of indinavir for compound A in human liver microsomes was comparable with those in rat, dog and monkey liver microsomes (table 3). Thus, we predicted that co-administration of indinavir with compound A would substantially enhance the compound A systemic exposure in humans. Indeed, subsequent clinical studies have demonstrated that indinavir dramatically

increase the plasma AUC of compound A (up to 38-fold) (Deutsch *et al.* 2000). At therapeutic doses, the indinavir–compound A combination would allow sufficient systemic exposure of compound A for viral suppression.

In summary, the low oral bioavailability of compound A was mainly due to extensive hepatic first-pass metabolism mediated by CYP3A enzymes; indinavir extensively inhibited compound A metabolism *in vitro*; and as such, it substantially increased compound A plasma levels in all species.

Acknowledgements

The authors thank Ms J. Brunner, Ms K. Lynn, Ms K. Michel, Ms J. Nishime and Ms A. Taylor for technical support in performing animal and metabolism studies; Dr M. Shou for providing the anti-human CYP3A4, 2C and 2D6 antibodies; Mr G. J. Gatto and Dr A. N. Jones for synthesising the deuterated and [^{14}C] compound A; and Dr B. K. Wong for reviewing the manuscript.

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Verapamil: identification of novel metabolites in cultures of primary human hepatocytes and human urine by LC-MSⁿ and LC-NMR

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Received 2 October 2002

1. Verapamil is a well-known and world-wide prescribed calcium antagonist, but it suffers from extensive first-pass metabolism. Although it has been marketed for many years, a complete understanding of its biotransformation in humans is still lacking.

2. The metabolism of verapamil was therefore investigated in cultures of primary human hepatocytes and in extracts of human urine after oral dosing. Identification of metabolites was done with LC-MSⁿ and LC-NMR (600 MHz) to obtain in-depth information on its biotransformation products and definitive proof of the proposed chemical structures of metabolites.

3. Hyphenation of LC-MSⁿ and LC-NMR was shown to be a powerful and effective platform for the identification of metabolites. Indeed, 21 Phase I and 16 Phase II metabolites were identified. Basically, all the Phase II metabolites (glucuronides) and 11 of the Phase I (oxidative) metabolites were not reported previously.

4. New insight into verapamil's biotransformation pathway is provided as well as evidence about its true complexity of metabolic disposal.

Introduction

Verapamil hydrochloride [DL-(5-*N*-(3,4-dimethoxyphenethyl)methylamino-2-(3',4'-dimethoxyphenyl)-2-isopropylvaleronitrile)], a synthetic papaverine derivative, is a well-known calcium channel blocker with anti-anginal, antihypertensive and anti-arrhythmic properties (Eichelbaum *et al.* 1979). More than 250 million dosages are prescribed annually in Germany alone, and the commercial brand consists of a racemic mixture of equal amounts of two optical isomers, i.e. (+)-(*R*)-verapamil and (–)-(*S*)-verapamil. The optical isomers differ considerably in their pharmacological potency with (–)-(*S*)-verapamil being 10–20 times more potent than (+)-(*R*)-verapamil with respect to the negative dromotropic effects on atrioventricular (AV) conduction in man. Verapamil suffers from extensive first-pass metabolism and its biotransformation is stereoselective with the *S*-enantiomer being preferentially subject to oxidation. As a consequence, the bioavailability of the *R*-enantiomer is three times that of the *S*-enantiomer (Vogelsang *et al.* 1984). In general, extensive metabolism results in low drug bioavailability and considerable variability of therapeutic plasma levels, and as a consequence metabolism of verapamil leads to pharmacological inactivation. Thus, patients require frequent dosage of this particular drug. The most common metabolic pathways include

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N-demethylation, O-demethylation and N-dealkylation (McIlhenny 1971, Eichelbaum *et al.* 1979, Nelson *et al.* 1987, Nelson and Olsen 1988). In the past there have been only a few attempts to identify conjugation products of verapamil, although it has been speculated that some of the glucuronides may contribute to the pharmacological and toxicological properties of this particular drug (Miller *et al.* 1968, Olsen *et al.* 1992, Spahn-Langguth and Benet 1992, Ritter 2000). As of today, the only reported glucuronide is that of norverapamil, which was identified in extracts of rat bile (Mutlib and Nelson 1990).

The metabolism of verapamil in microsomal fractions and primary cultures of cardiomyocytes of adult rats and in human heart tissue was studied by Walles *et al.* (2001, 2002). Next to an identification of already known metabolites (norverapamil, the dealkylation product D617 and the two O-demethylation products, PR 22 and D-702), we identified oxidation products of a methoxy group to result in a hemiacetale and formiate derivative (which are intermediates of the final O-demethylation product D702), and oxidation of the N-bound methyl group, which leads to a carbinol amine and further to a formyl derivative (which are intermediates in the formation of norverapamil). In addition, a metabolite formed by direct hydroxylation of verapamil was found. More recently, we investigated the metabolism of verapamil in hepatocytes of rats by LC-MS and collision-induced dissociation and identified 24 Phase I and 14 Phase II metabolites, most of which had not been reported before (Walles *et al.* 2003). The study demonstrated, however, the limitations of the LC-MS approach: Mass spectrometry neither permits the unambiguous localization of the O-demethylation site at a given aromatic ring, nor does it allow the identification of the conjugation sites (the glucuronides of verapamil can be either N- or O-bound). Only high-resolution nuclear magnetic resonance (^1H -NMR) spectroscopy and additional two-dimensional (2D) experiments can provide this information.

Therefore, we used a combination of both methods, LC-NMR and LC-MSⁿ (but also the simultaneous coupling of LC to both MS and NMR) to enable structural elucidation of novel metabolites (Shockcor *et al.* 1996).

We report the unambiguous identification of new Phase I and II metabolites found in culture media of primary human hepatocytes and extracts of human urine after oral (p.o.) dosing. Verapamil metabolites in human urine were previously determined by GC-MS after derivatization (Nelson and Olsen 1988). Our results will be compared with findings from earlier studies, as detailed below.

Materials and methods

Materials and chemicals

Water was purified using a Milli-Q Plus water purification (Millipore, Eschborn, Germany). All chemicals were of analytical reagent grade and purchased from various suppliers: acetonitrile and methanol (Malinkrodt Baker, Deventer, the Netherlands), ammonia acetate (Merck, Darmstadt, Germany), acetic acid (Fluka, Buchs, Switzerland), norverapamil (5-*N*-(3,4-dimethoxyphenethyl) amino-2-(3',4'-dimethoxyphenyl)-2-isopropyl-valeronitrile) (Research Biochemicals International, Natick, MA, USA), racemic verapamil (5-*N*-(3,4-dimethoxyphenethyl)methylamino-2-(3',4'-dimethoxyphenyl)-2-isopropylvaleronitrile) (Lot 56H0925/Sigma-Aldrich, Steinheim, Germany), and β -glucuronidase (367 500 units g⁻¹, Sigma-Aldrich; not sulfatase-free). Deuterium oxide and acetonitrile for LC-NMR experiments were NMR grade (Riedel de Hën, Seelze, Germany). O-demethylverapamil (5-*N*-(3,4-dimethoxyphenethyl)methylamino-2-(3'-methoxy-4'-hydroxyphenyl)-

2-isopropylvaleronitrile) was a kind gift of W. L. Nelson (University of Washington, Seattle, WA, USA).

Studies with human hepatocytes

Isolation and cultivation of primary human hepatocytes. Ethical approval was obtained from the Medical School of Hannover (J. Borlak) and liver specimens were stored in ice-cold physiological saline solution and immediately transported to the laboratory. Vessels visible on the cut surface were cannulated and the liver specimen perfused with 200 ml buffer I containing 8.3 g l^{-1} NaCl, 0.5 g l^{-1} KCl, 2.4 g l^{-1} HEPES and 0.19 g l^{-1} EGTA at pH 7.4 and 37°C . This was followed by perfusion with 200 ml buffer II (8.3 g l^{-1} NaCl, 0.5 g l^{-1} KCl, 2.4 g l^{-1} HEPES) at pH 7.4 and 37°C . Thereafter, collagenase perfusion was performed with 200 ml buffer III containing 100 g collagenase type IV, 3.9 g l^{-1} NaCl, 0.5 g l^{-1} KCl, 2.4 g l^{-1} HEPES and 0.7 g l^{-1} $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$ at 37°C . The collagenase perfusate was recirculated, and flow rates ranged between 20 and 50 ml min^{-1} in adaptation to the size of the specimen. Following perfusion, the liver capsule was carefully removed and the cells harvested by gentle shaking of the liver specimen in ice-cold buffer IV. Buffer IV contained 9.91 g l^{-1} Hanks buffered salt without calcium and magnesium, 2.4 g l^{-1} HEPES and 2.0 g l^{-1} bovine serum albumin. The resulting cell suspension was filtered through a nylon mesh with $100 \mu\text{m}$ pore size and washed three times with buffer IV at 4°C . Viability of the hepatocytes ranged between 85 and 95% as assessed by trypan blue exclusion. On average, $1\text{--}5 \times 10^9$ cells were obtained from one isolation procedure.

Primary human hepatocytes were cultured enclosed in two layers of collagen as described by Dunn *et al.* (1989). A total of 1 ml collagen solution containing 1.1 mg ml^{-1} collagen was used for coating one 60-mm Petri dish (Greiner, Frickenhausen, Germany). The pH of the collagen was adjusted to 7.4 using a $10\times$ DMEM concentrate (Biochrom, Berlin, Germany). Thirty minutes after coating the dishes, 2×10^6 hepatocytes per dish were seeded. Two hours following seeding and attachment of the cells, culture medium was removed together with non-adherent cells. After 24 h in culture, the medium was taken and a second layer of collagen applied on top of the cell layer. After gelation of this second layer, culture medium was added. Culture medium (2 ml) was changed daily and the total volume of cell culture supernatant was 4 ml, including the hydrated collagen matrix volume of 2 ml. Hepatocytes were allowed to recover from the isolation procedure for 5 days and were then treated with $10 \mu\text{M}$ verapamil for 24 h, and supernatant was taken subsequently.

Sample preparation

The cell culture media or urine were deconjugated with $1 \text{ ml } \beta$ -glucuronidase (5 mg ml^{-1} ; 1837 units) solved in 0.01 M ammonium acetate buffer at pH 4.5 for 12 h at 37°C . After incubation, the proteins were precipitated with 6 ml methanol and removed by centrifugation at $1780g$ for 5 min. The resultant supernatant was evaporated to dryness using a gentle stream of nitrogen.

Extraction of metabolites from cell cultures. Verapamil and its metabolites were analysed by solid-phase extraction (SPE) and high-performance liquid chromatography-mass spectrometry (LC-MS).

For SPE, a lipophilic cartridge (RP8 Select B, Merck) was used, which was conditioned with methanol followed by equilibration with water. The sample was loaded onto the cartridge without any organic solvent and washed with 3% methanol to separate any sample matrix. Verapamil and its basic metabolites were eluted with methanol. Eluants were evaporated to dryness and reconstituted in $200 \mu\text{l}$ acetonitrile/ammonium acetate (0.01 M , pH 6.0, 50/50 v/v). Aliquots of $20 \mu\text{l}$ were injected onto the LC-MS system.

Extraction of metabolites from human urine and SPE purification. The metabolites were isolated from human urine using SPE and LC. A total of 160 mg racemic verapamil in the form of two Verahexal[®] 40 tablets each were given as single doses of 80 mg to two healthy male volunteers (27 years, 98 kg; 61 years, 70 kg). The urine was collected for 8 h and pooled (1.3 litres).

Water from urine was removed by evaporation to obtain a final volume of 500 ml, of which 400 ml were purified by SPE. SPE was done with RP8 Select B cartridges (Merck), where 200 mg chemically bonded RP8 material were used as the stationary material. The SPE cartridge was activated with 5 ml methanol and 5 ml water. A total of 30 ml urine was applied and the cartridge was washed with 5 ml water and then eluted with 2 ml methanol. Methanol was evaporated under a gentle stream of nitrogen at room temperature and adjusted to a final volume of $400 \mu\text{l}$.

LC/MS analysis. LC-MS analyses were done on a Waters (Milford, MA, USA) LC instrument (pumps 590) coupled to an Esquire ion-trap mass spectrometer (Bruker-Daltonik, Bremen, Germany) operated under positive-ion electrospray (ESI) conditions in the full scan, MS^2 and in some instances in the MS^3 and MS^4 mode. The nebulizer pressure was set to 40 psi and the dry gas temperature to 350°C and $+3 \text{ kV}$ were applied to the nebulizing capillary. Full mass spectra were acquired by scanning the mass range of m/z 100–700. CID spectra were obtained from the protonated molecules $[\text{M} + \text{H}]^+$. LC

separation was carried out with a gradient elution using an ammonium acetate buffer (0.01 M, pH 6.0) and acetonitrile at a flow of 0.2 ml min^{-1} . The gradient starts with 25% acetonitrile increasing to 50% within 15 min, and further to 75% acetonitrile from 15 to 30 min. From 30 to 55 min, the acetonitrile content remained constant and decreased to 25% from 55 to 60 min. Separation of verapamil and its metabolites was achieved on a $250 \times 2 \text{ mm}$ RP8 select B column with a particle size of $4 \mu\text{m}$ (Merck).

Sample preparation for LC-NMR. Before LC-NMR measurements, the urine extract was prefractionated using analytical LC: a Waters 900 pump at a flow rate of 2 ml min^{-1} was used to separate the glucuronides from the unconjugated metabolites. Separation was achieved on a RP18 column ($250 \times 10 \text{ mm}$ i.d.; Merck). The mobile phase consisted of acetonitrile and 0.01 M ammonium acetate buffer adjusted with acetic acid to pH 6. The flow was split 1:20 before analyte introduction into the MS.

The glucuronides were collected using gradient elution starting with 30% acetonitrile from 0 to 30 min, then rising to 70% from 30 to 60 min and finally to 80% from 60 to 90 min. After 90 min, the percentage of acetonitrile was again decreased to 30%. Fraction 1, isolated from 20 to 60 min, contained the glucuronides. This fraction was concentrated and adjusted to $400 \mu\text{l}$, of which $200 \mu\text{l}$ were used for the LC-NMR experiments.

LC-NMR studies. We used an RP select B column ($125 \times 4 \text{ mm}$, $5 \mu\text{m}$; Merck) with the following LC equipment: an LC-22 pump (Bruker-Saxonia, Leipzig, Germany), a Rheodyne 7725i injection valve (Rheodyne Inc., Cotati, CA) and a Bischoff Lambda-1010-UV-detector (Leonberg, Germany). The outlet of the detector was connected to the flow probe. The mobile phase consisted of acetonitrile/ D_2O (25/75 v/v). Chromatography was done by isocratic elution with a flow rate of 0.3 ml min^{-1} .

^1H -NMR spectroscopy. We used a Bruker Avance DRX 600 spectrometer operated at 600.13 MHz and at 300 K. The spectrometer was equipped with a 4-mm $^1\text{H}/^{13}\text{C}$ inverse flow probe with z -gradient (detection volume $120 \mu\text{l}$).

^1H -NMR stopped-flow spectra were acquired using the Bruker WET sequence lc1wt for multiple solvent suppression. FIDs were collected into 32 k data points (spectral width 9615 Hz) with a flip angle of 90°C ($9.7 \mu\text{s}$) and an acquisition delay of 2.40 s resulting in a total interscan time of 4.50 s. A total of 512 scans were always accumulated. Data were multiplied with an exponential function ($\text{LB}=0.5 \text{ Hz}$) before Fourier transformation. The chemical shifts were referenced to acetonitrile, which was set to 2.00 ppm.

The stopped flow 2D NOESY spectra were acquired with the Bruker pulse sequence lcnosy-wettpdc using the following parameters: the spectral size was $16 \text{ k} \times 256$ data points, the spectral width in F2 was 7183 Hz and 40 increments were used in the F1 dimension, the delay between successive pulses was 2.0 s and the mixing time was set to 0.6 s. The data were zero filled in F1 by a factor of two Fourier transformation.

D/H-back exchange. After recording the spectra, the metabolite-containing solution was collected and distributed on two 2 ml HPLC vials (A, B). The solution of vial B was diluted with ammonia acetate solution (0.01 M, pH 6) in a 1:4 ratio. From both vials, $400 \mu\text{l}$ were injected into the MS system via a syringe pump (flow $100 \mu\text{l min}^{-1}$) and MS^n spectra were recorded.

Recovery experiments. Recovery experiments were done in quadruplicate. For this purpose, the culture media used for human hepatocytes and blank urine were spiked with 50 ng ml^{-1} verapamil and norverapamil. Solid-phase extraction was done as described above, and the resultant eluent was reduced in volume and adjusted to $200 \mu\text{l}$. Measurement of these extracts was done as described above and recoveries were $89 \pm 3\%$, $85 \pm 6\%$, $92 \pm 6\%$ and $88 \pm 3\%$ for verapamil and norverapamil in the culture media of human hepatocytes and urine, respectively.

Quantification. The abundances in table 1 were calculated by comparing the peak ratios of the metabolites relative to that of non-conjugated verapamil (=100%) (table 1). They provided only semiquantitative information on the concentrations of the metabolites relative to that of verapamil, as the ionization efficiency of the various metabolites under ESI conditions may vary.

Results

Structure elucidation of the metabolites by LC- MS^n

The structures of the metabolites were investigated by comparing their MS fragmentation pattern (table 1) with that of verapamil (1). In a first step, the mass

Table 1. Main fragmentation of conjugated and non-conjugated metabolites of verapamil.

No.	Relative abundance ^d (%)		Substituents											Phase I	Phase II	Low abundant (<5%), non-significant ions and neutral losses (in parentheses)						
	Human urine	Human hepatocytes	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	[M + H] ⁺ or <i>m/z</i>	[M + H] ⁺	A ₁ ^e	A ₂ - H	B ₁ ^e	B ₂ + H	B ₃ ^e	C	
1 ^b	100 ^b	100 ^b	OCH ₃	OCH ₃	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₇	H	H	H	H	455	631	150	303	165	-	260	-	216, 238, 262, 337
2 ^b	200	30	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	H	C ₃ H ₇	H	H	H	H	441	453	151	289	165	-	260	398	A ₃ =246
3 ^{b,c}	8	1	-	OCH ₃	OCH ₃	OCH ₃	CH ₃	-	C ₃ H ₇	H	H	H	H	277	453	-	-	-	-	260	234	-
4 ^e	9.5	0.1	-	-	OCH ₃	OH	CH ₃	-	C ₃ H ₇	H	H	H	H	277	453	-	-	-	-	246	234	218
5 ^{b,c}	322	29	-	-	OCH ₃	OCH ₃	CH ₃	-	C ₃ H ₇	H	H	H	H	291	-	-	-	-	-	260	248	-
6 ^b	7b	0.9	OH	OCH ₃	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₇	H	H	H	H	441	617	-	303	151	291	260	248	-
7 ^b	234	0.2	OCH ₃	OCH ₃	OCH ₃	OH	CH ₃	H	C ₃ H ₇	H	H	H	H	441	617	151	289	165	-	246	-	133, 372
8 ^b	234 ^g	n.d.	OCH ₃	OH	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₇	H	H	H	H	441	617	-	303	151	291	260	248	187, 373
9 ^b	234 ^g	n.d.	OCH ₃	OH	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₇	H	H	H	H	441	617	-	289	151	277	246	384	-
10 ^b	12.4	n.d.	OH	OCH ₃	OCH ₃	OH	CH ₃	H	C ₃ H ₇	H	H	H	H	427	603	-	289	151	277	246	384	-
11 ^b	196 ^f	0.2	OH	OCH ₃	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₇	H	H	H	H	427	603	-	275	165	-	260	384	-
12 ^b	196 ^f	n.d.	OCH ₃	OCH ₃	OCH ₃	OH	H	H	C ₃ H ₇	H	H	H	H	427	603	150	275	165	(246)	370	206, 234, 395	
13 ^b	14	0.9	OH	OCH ₃	OCH ₃	OH	H	H	C ₃ H ₇	H	H	H	H	413	-	-	275	151	263	246	230	235
14	0.4	n.d.	OH	OH	OCH ₃	OCH ₃	H	H	C ₃ H ₇	H	H	H	H	413	-	-	-	-	277	260	234	235
15 ^{c,d}	1.2	n.d.	OCH ₃	OCH ₃	OCH ₃	OCH ₃	CH ₃	OH	C ₃ H ₇	H	H	H	H	471	-	-	303	181	291	260	-	234, 248, 453 (-H ₂ O)
16 ^{a,c}	1.4	n.d.	OCH ₃	OCH ₃	OCH ₃	OCH ₃	CH ₃	OH	C ₃ H ₇	H	H	H	H	471	647	-	289	181	291	260	448	453 (-H ₂ O)
17 ^{a,c}	2.5	n.d.	OCH ₃	OCH ₃	OCH ₃	CH ₃	H	OH	C ₃ H ₇	H	H	H	H	457	633	-	289	181	277	260	414	235, 399, 439 (-H ₂ O)
18 ^b	1.2	n.d.	OCH ₃	OCH ₃	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₆ OH	H	H	H	H	471	647	150	319	165	-	-	-	413 453 (-H ₂ O)
19 ^b	24	1.1	0.3	OCH ₃	OCH ₃	OCH ₃	CH ₃ OH	H	C ₃ H ₇	H	H	H	H	471	-	151	-	165	-	260	-	453 (-H ₂ O)
20 ^a	0.3	n.d.	OH	OCH ₃	OCH ₃	OCH ₃	CH ₃	OH	C ₃ H ₇	H	H	H	H	457	633	-	-	167	291	260	-	203, 234, 261, 277, 439 (-H ₂ O)
21 ^a	30 ^a	0.5	n.d.	OCH ₃	OCH ₃	OH	CH ₃	H	C ₃ H ₇	H	H	OH	H	457	633	-	-	-	-	262	-	192, 216, 234, 439 (-H ₂ O), 615
22 ^a	31 ^a	0.6	n.d.	OCH ₃	OCH ₃	OCH ₃	CH ₃	H	C ₃ H ₇	H	H	OH	H	457	633	-	306	181	277	260	414	149, 166, 235, 289, 341, 439 (-H ₂ O)
23 ^a	0.3	n.d.	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	OH	C ₃ H ₇	H	H	H	H	457	633	-	-	181	277	260	-	319, 439 (-H ₂ O)

Values in parentheses are low abundance, about 2%.

n.d., Non-detectable.

^a Position of the hydroxyl group could not be located unambiguously.

^b Metabolite appears also in non-conjugated form.

^c Phenylalkyl moiety lost.

^d Relative peak area of the phase I metabolites (after cleavage with β -glucuronidase) compared with verapamil.

^e Cleavage is accompanied by charge migration.

^f Sum of the abundances of the non-conjugated metabolite (11) and (12).

^g Sum of the abundances of the non-conjugated metabolite (8) and (9).

^h Corresponds to verapamil.

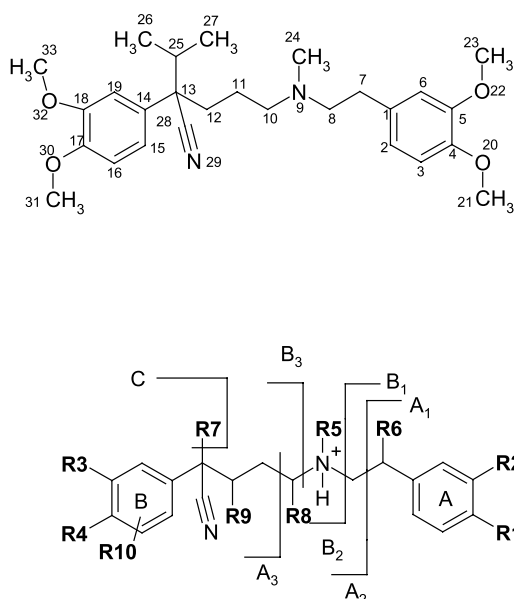


Figure 1. Structure of verapamil and the main MS-fragmentation pathway of verapamil and its metabolites.

of the quasi-molecular ion of each metabolite was compared with that of verapamil to provide a first indication of the structure of the metabolite; in a second step, this proposed structure was confirmed by studying the collision-induced fragments (only ions with a relative intensity of >5% are discussed, if not mentioned otherwise). To facilitate comparisons, we used a nomenclature of metabolite identification identical with that of Walles *et al.* (2003) and independent of species. For instance, we reported the identification of 24 metabolites (termed 2–25) in studies with rat hepatocytes. Many, but not all, have been found in studies with human hepatocytes or human urine. Consequently, metabolite numbers are missing as they were not found in this study. Noticeably, the metabolites (28–32) discussed below were only found in studies with human hepatocyte cultures and human urine, but not with cultures of rat hepatocytes. In addition, metabolites (26) and (27) were only found in cultures of cardiomyocytes or in extracts of microsomes from human heart (Walles *et al.* 2001), but once again are not listed below.

The collision-induced dissociation of protonated verapamil and its metabolites proceeds mainly via some well-defined fragmentation pathways as illustrated in figures 1 and 2 (where it is assumed that the initial protonation occurs at the nitrogen):

- Cleavage of the C-C-bond in the α -position to the nitrogen of the substituted phenylethyl moiety and charge migration (in some instances accompanied by hydrogen transfer) (route A₁ in figure 1) leading to the fragment ion [A₁]⁺ or cleavage of this bond with charge retention and hydrogen transfer to the eliminated neutral, i.e. a substituted toluene (route A₂ in figure 1), leading to the ion [A₂ – H]⁺ (figure 2).

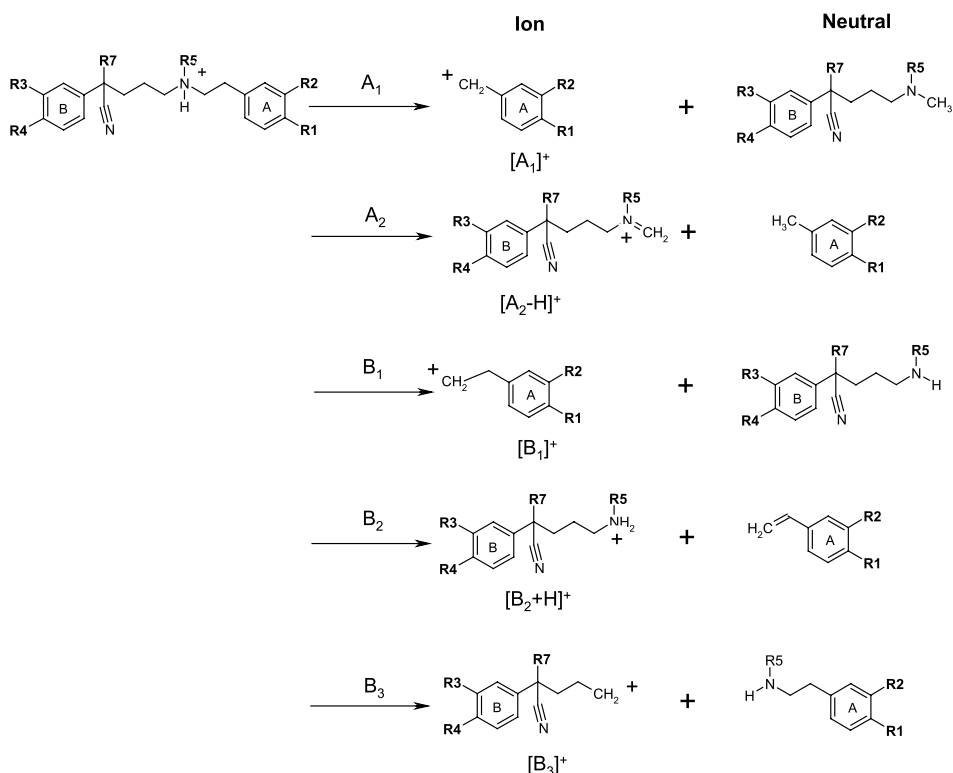


Figure 2. Major collision-induced fragment ions of verapamil and its metabolites.

- Cleavage of the N-C bond of the substituted phenylethyl moiety, charge migration and elimination of a highly substituted phenylbutylamine as neutral (route B₁) leading to the fragment ion $[B_1]^+$ or cleavage of this bond with charge retention and hydrogen transfer onto the nitrogen with elimination of a substituted styrene as neutral (route B₂ in figure 1), leading to the ion $[B_2+H]^+$ (figure 2).
- Alternatively, also cleavage of the N-C bond on the highly substituted phenylbutyl moiety and elimination of substituted phenylethylamine as neutral may occur (B₃), leading to the fragment ion $[B_3]^+$ (figure 2).

It is of considerable interest that the presence of a hydroxy group on the substituted phenylethyl moiety (independent of its location) influenced the fragmentation of the protonated verapamil metabolites: the fragmentation route B₂ is observed only if such a hydroxy group is present in the substituted phenylethyl part of the molecule, while lack of this group leads in general to the fragmentation channel A₁. In contrast, a hydroxyl group on the highly substituted phenylbutylamine moiety apparently has no effect on the fragmentation.

Further, all fragmentation pathways discussed so far led to thermochemically stable, even electron fragment ions by loss of an even electron neutral molecule (even electron rule).

- In contrast, loss of a propyl radical from the protonated molecule or a primary fragment ion (as observed with most metabolites) led to an odd electron

fragment ion (a process that is probably favoured by the adjacent cyano group) (C in figure 1). Note that a competing (thermodynamically more favourable) loss of a propene molecule is also observed in some instances.

- Other cleavages of the alkyl chains (A₃, D and E in figure 1) are observed less frequently. Among the other fragmentation processes, the observation of loss of water provided important structural information. All these additional fragments are listed in the last column of table 1.

Metabolic transformation of verapamil led to characteristic changes in the masses (m/z) of these fragment ions, which (together with the m/z of the quasi-molecular ion) allow the identification of the type and, to a large extent, position of the metabolic transformation, as shown below.

Using this approach, novel Phase I metabolites were identified as summarized in table 1 and 2, which list the m/z of the main fragment ions of these metabolites. If the metabolite was also observed in the conjugated (usually glucuronidated) form, the fragment ions of the glucuronide are listed in table 1 (except for verapamil). Note that the collision-induced loss of the glucuronic acid moiety (176 Da) from these conjugates led to the protonated Phase I metabolite. The MS³ spectra of the $[M - 176]^+$ ions of the glucuronides and the MS² spectra of the corresponding protonated Phase I metabolites are thus almost identical.

In the following, the structure assignment of the newly identified Phase I metabolites, as based on their mass spectrometric fragmentation behaviour, is summarized. (More details have been reported by Walles *et al.* (2003) or are available upon request.)

Verapamil and norverapamil

Most metabolites summarized in table 1 can be rationalized to originate either from verapamil (1) or norverapamil (2), which are, therefore, discussed first (although the N-demethylation leading to (2) is not necessarily the first step). The protonated verapamil (1) fragments via route A₁ (with hydrogen transfer, m/z 150), A₂ (303), B₁ (165) and B₃ (260) (figure 1 and table 1), while with norverapamil the fragmentation route C (m/z 398) is also observed. Oxidative N-demethylation of verapamil giving rise to norverapamil (2) is reflected by a mass shift of the ion $[A_2 - H]^+$ by 14 Da. The fragments of both (1) and (2) were confirmed by comparison with synthetic reference compounds. Verapamil was also observed as glucuronide.

Metabolites formed by oxidative dealkylation and additional demethylation

Metabolites (3) (D-620), (4) (D717) and (5) (D 617) were formed by oxidative dealkylation (loss of the substituted phenylethyl moiety) as shown by the m/z of their quasi-molecular ion and corroborated by their collision-induced fragments as discussed by Walles *et al.* (2003). The masses of the quasi-molecular ions and their collision-induced dissociations allow an unambiguous structure elucidation of metabolites (3–5) with one exception: mass spectrometry does not permit one to determine which of the two methoxy groups of (4) was demethylated. HPLC/NMR experiments of (4) reported below allow this question to be answered.

Metabolites (3–5) were also detected in the conjugated form as glucuronides.

Metabolites formed by oxidative O- and N-demethylation

Metabolites (7–12), (14) and (28) are formed by oxidative demethylation, which can occur one or two times. In part, an additional N-demethylation was observed. Thus, some of these metabolites can be rationalized to originate from verapamil, i.e. (7–10), and others from norverapamil (11, 12, 14, 28), even if N-demethylation is not necessarily the first step. If O-demethylation of verapamil occurs once, a quasi-molecular ion of m/z 441 (14 Da lower than verapamil) is observed, i.e. with (7–9), and if this demethylation occurs twice, a quasi-molecular ion at m/z 427 (2×14 Da lower than verapamil) is found, i.e. with (10). O-demethylation can occur at ring A or B (figure 1). The m/z of the fragment formed via route A_2 and B_1 reveals the position of the O-demethylation as shown in table 1 and discussed in detail by Walles *et al.* (2003). Single and double O-demethylation is also observed for those metabolites derived from norverapamil, i.e. (11, 12, 14, 28). Based on the mass shifts of the quasi-molecular ions and the collision-induced fragments formed via route A_2 or B_1 (as compared with norverapamil) the number of O-demethylations and their site can be derived (table 1).

As mentioned above, the presence of a hydroxyl group at the substituted phenylethyl moiety (here the aromatic ring A) opens up a new decomposition channel, i.e. B_2 , as observed with (7, 9–11, 14, 28). The occurrence of the ion $[B_2 + H]^+$ and its m/z confirms the structure assignment of the O-demethylation products (7, 9–11, 14, 28). This assignment is furthermore corroborated by the m/z of the $[B_3]^+$ ion (shift by 14 Da if ring B is demethylated once, as e.g. with (8) and (14)).

Metabolite (28), which was not found with rat hepatocytes (Walles *et al.* 2003), was only observed with very low abundance, which explains why the fragment ions $[A_2 - H]^+$ and $[B_1]^+$ could not be detected unambiguously. However, the observed fragment ions $[B_2 + H]^+$, $[B_1]^+$ and $[C]^+$ make the structure proposed in table 1 very likely.

While the collision-induced fragments, together with the mass of the quasi-molecular ion, enable us to determine not only the number of O-demethylations, but also the position within the molecule (ring A or B), it is not possible to prove which of the two methyl groups at a given aromatic ring is demethylated using this technique. However, further information is available for (12) from NMR experiments reported below.

For metabolite (8) (D703), a reference compound was available, the fragmentation of which further supported the above structure assignment. Metabolites (7–12) were also observed in the conjugated form.

Metabolites formed by hydroxylation

Within this study, nine metabolites formed by hydroxylation were observed although with low abundance (i.e. 15–18, 24, 29–32). This metabolic route has not been previously described for verapamil, but has recently been found by us with rat hepatocytes. Metabolites (15–18) are discussed first. (15), (16) and (18) are formed by direct hydroxylation of verapamil, which leads to m/z 471 for the quasi-molecular ion, 16 Da higher than that of verapamil. Those derived from norverapamil (17) (but also (31) and (32)), form a quasi-molecular ion of m/z 457, 16 Da higher than that of norverapamil.

If hydroxylation occurs at the substituted phenylethyl moiety, the m/z of the fragment $[B_1]^+$ must be shifted by 16 Da to higher values when compared with both verapamil and norverapamil (as found with (15–17)). The m/z of this fragment is not shifted if hydroxylation occurs at the highly substituted phenylbutyl moiety of the molecule, i.e. with (18), while $[B_1]^+$ is not shifted, as is found with (18). Finally, as discussed above, hydroxylation at the substituted phenylethyl moiety triggers the additional fragmentation according to route B_2 in figures 1 and 2 as observed with (15) and (16). The m/z of the $[B_2 + H]^+$ ions formed via this route confirm this conclusion.

What additional information on the position of the hydroxyl group is available from mass spectrometry? In general, a phenolic hydroxyl group in a protonated molecule is not lost as neutral water upon collision-induced dissociation (but, if at all, as hydroxyl radical), whereas an abundant loss of water is found with alcohols. Consistent with this observation, the protonated metabolites (4, 7–12, 14, 28), which after oxidative demethylation have one or two hydroxyl groups bound to the aromatic ring, do not undergo collision-induced loss of water. In contrast, the protonated metabolites formed by hydroxylation, i.e. (15–18, 24, 29–32) in all instances undergo collision-induced loss of water, strongly suggesting that the hydroxyl group is bound to an aliphatic carbon.

Furthermore, and according to the above discussion, the hydroxyl group in (15–17) showing a loss of water must be bound to the aliphatic part of the substituted phenylethyl moiety, i.e. to C_7 or C_8 . The m/z of the $[A_2 - H]^+$ ions, which comprise C_8 and are not shifted when compared with verapamil (1) or norverapamil (2), prove that the hydroxyl group must be bound to C_7 at least for (15) and (17). (The identification of (16), which shows similar m/z as (15), is less safe. Note that hydroxylation at C_7 leads to a new chirality centre so that (15) and (16) could be diastereomers.)

Finally, the protonated metabolite (18) undergoes loss of 58 Da, i.e. loss of C_3H_6O , from the protonated molecule (fragmentation route C in figure 1), which unambiguously proves a hydroxylation of the isopropyl group. The observed loss of water and the $[A_2 - H]^+$ ions confirm this assignment.

Metabolites (16–18) were also found as glucuronides.

Metabolite (24) is formed by oxidation of the methyl group bound to nitrogen. The quasi-molecular ion of (24) at m/z 471 is 16 Da higher than that of verapamil, which is consistent with a hydroxylation. As the m/z of $[B_1]^+$ and $[B_3]^+$ are not shifted when compared with verapamil itself (i.e. neither the substituted phenylethyl nor the substituted phenylbutyl moiety underwent metabolic transformation), hydroxylation must have occurred at the methyl group bound to nitrogen. The observed loss of water leading to m/z 453 is consistent with a hydroxylation. Thus, (24) represents a carbinolamine, a compound class that is usually very unstable. This metabolite has previously been identified by us in cell cultures of cardiomyocytes and hepatocytes (Walles *et al.* 2001, 2002, 2003), where the structure elucidation has been discussed in more detail.

Metabolites (6, 13, 19–23, 25–28) not listed in table 1 and figure 6 were only found with rat hepatocytes (Walles *et al.* 2003).

With the exception of (28), all metabolites discussed so far have also been found with rat hepatocytes and have been discussed in more detail in Walles *et al.* (2003).

Metabolites (29–32) have only been found in human urine samples and are discussed here for the first time and in more detail. Also found as glucuronides,

they show in their non-conjugated form a quasi-molecular ion of m/z 457, 2 Da higher than verapamil, which points to an oxidative demethylation and additional hydroxylation. In the case of (21), the collision-induced fragment $[B_1]^+$ reveals that demethylation must have taken place at ring A, while the hydroxylation occurred at the substituted phenylethyl moiety, which also explains the occurrence of a fragment $[B_2 + H]^+$ (see above). The m/z 291 of this $[B_2 + H]^+$ ion demonstrates that no metabolic transformation has occurred at the highly substituted phenylbutyl moiety, which is also corroborated by observation of a weak $[B_3]^+$ ion at m/z 260, which is not shifted as compared with verapamil. Both fragments support the conclusion based on $[B_1]^+$. Furthermore, the abundant loss of water from the quasi-molecular ion suggests that the hydroxylation took place in the aliphatic part of the substituted phenylethyl moiety, i.e. at C₇ or C₈. In table 1 and figure 6, a hydroxylation at C₇ is assumed.

Metabolites (30–32) are isomers of the above discussed metabolite (29), but demethylation here occurred at the nitrogen (preceded or followed by a hydroxylation), i.e. the metabolites can be derived from norverapamil. The structure elucidation of (31) and (32) is straightforward: The m/z of the $[B_3]^+$ ion is not shifted as compared with verapamil, which proves unambiguously that no metabolic transformation has occurred at the highly substituted phenylbutyl moiety, which again is supported by the observed m/z 277 of the ion $[B_2 + H]^+$. The occurrence of this $[B_2 + H]^+$ ion demonstrates that hydroxylation took place at the substituted phenylethyl moiety (see above) as also proved by the shift of the $[B_1]^+$ ion to m/z 181 (16 Da higher than that of verapamil). The observed abundant loss of water excludes a hydroxylation of the aromatic ring A, leaving C₇ or C₈ as possible sites for the hydroxyl group. As with metabolite (29), an unambiguous assignment of the hydroxylation site is difficult. The fragment at m/z 306 observed with (31) may be due to the fragmentation route A₂ with a second hydrogen transfer, suggesting that with (31) the hydroxyl group is bound to C₈, while with (32) it is bound to C₇.

A very weak metabolite (30) shows hardly any structure-specific fragments. Based on the observed fragment at m/z 262, assigned as $[B_3]^+$ ion, a demethylation and hydroxylation at ring B is assumed. The two aromatic hydroxyl groups at ring B must be in ortho position as evident from the observed loss of water (Walles *et al.* 2003). The structure of (30) assignment remains tentative.

Identification of metabolites by LC-NMR

LC-MSⁿ allows the identification of several metabolites from human urine and cell culture media. However, in some cases the position of the O-demethylation (C-17 or C-18, and C-4 or C-5) and hydroxylation, as well as the binding sites for glucuronides being N- or O-bound is far from clear. Therefore, we used LC-NMR for the conclusive identification of metabolites of verapamil, mainly (4), (8) and (12), in human urine (for sensitivity reasons, minor components could not be investigated by this approach).

As described above, the SPE extract of the urine was further fractionated off-line by analytical LC to obtain a fraction that mainly contained the glucuronides. This fraction was analysed by LC-NMR and LC-MS. D₂O was used throughout to obtain a better solvent-signal suppression, which at the same time leads to an exchange of all acidic hydrogens in the analytes by deuterium, which incidentally

Table 2. MS data for the metabolites investigated by LC-NMR.

	4-I				4-II				12				8			
	m/z in H ₂ O	m/z in D ₂ O	$N_{D/H}$	m/z in H ₂ O	m/z in D ₂ O	$N_{D/H}$	m/z in H ₂ O	m/z in D ₂ O	m/z in H ₂ O	m/z in D ₂ O	$N_{D/H}$	m/z in H ₂ O	m/z in D ₂ O	$N_{D/H}$	m/z in H ₂ O	m/z in D ₂ O
[M + H] ⁺ / [M + D] ⁺	453	459	5	453	459	5	603	609	617	622	5	617	622	4		
MS ²	277	280	–	277	280	–	427	430	441	443	–	441	443	–		
MS ³	234, 246	237, 247	–	234, 246	237, 247	–	165, 275, 384	166, 277, 387	165, 289	166, 290	–	165, 289	166, 290	–		
MS ⁴	–	–	–	–	–	–	–	–	246	247	–	246	247	–		

$N_{D/H}$, number of exchangeable protons.

Table 3. ¹H-NMR data of verapamil and metabolites.

Proton	Metabolites ^e						
	¹ H-NMR chemical shifts (δ) and coupling constant (Hz)						³ J _{ortho} ^g
	Verapamil ^d	4-I ^c	4-II ^c	8	12-I	³ J _{vicinal}	
	δ	δ	δ	δ	δ		
H-16 (d) ^a	6.920	7.136	7.155	7.151	7.129	—	8.4
H-19 (d)	6.834	6.944	6.995	6.968	6.968	—	—
H-15 (dd)	6.942	7.006	6.925	6.937 ^f	6.985 ^f	—	8.4
H-3 (d)	6.833	—	—	6.925	6.925	—	8.4
H-6 (d)	6.755	—	—	6.837	6.847	—	—
H-2 (dd)	6.719	—	—	6.758	6.778	—	8.4
H-7, H-8, H10 (m)	2.755–3.055	—	—	[—] _b	[—] _b	—	—
H-11 (m)	1.562, 1.232	—	—	[—] _b	[—] _b	—	—
H-12 (m)	2.073–2.174	—	—	[—] _b	[—] _b	—	—
H ₃ -21 (s), H ₃ -23 (s)	3.729	—	—	3.769	3.769	—	—
H ₃ -33 (s)	3.729	3.837	3.848	3.830	3.834	—	—
H ₃ -31 (s)	3.759	—	—	—	—	—	—
H ₃ -24 (s _{breit})	3.745	2.524	2.524	2.657	—	—	—
H-25 (m)	2.152	[—] _b	[—] _b	[—] _b	[—] _b	—	—
H ₃ -27 (d)	1.502	1.118	1.118	1.102	1.102	6.6	—
H ₃ -26 (d)	1.090	0.724	0.724	0.712	0.712	6.6	—
H-1' (d)	0.686	5.040	5.030	4.992	5.015	7.2	—

^a Signal multiplicity: d, doublet; dd, double doublet; m, multiplet; s, singlet.^b Signals overlap with matrix interferences.^c Numeration of the verapamil is kept.^d Solvent composition: acetonitrile-D₃/D₂O 60/40 (v/v); chemical shifts were referenced on acetonitrile-D₃ (1.975 ppm).^e Solvent composition: acetonitrile/D₂O 25/75 (v/v); chemical shifts were referenced on acetonitrile (2.000 ppm).^f Assignment may be interchanged.^g ³J_{meta} couplings are not resolved in the stopped flow LC-NMR spectra.

may be exchanged with hydrogen again (D/H back exchange). This hydrogen/deuterium exchange provides valuable additional information on the structure of the metabolites in MS experiments as discussed below. As a result of the high resolution of the NMR spectra, a complete LC separation of each analyte is not a necessary prerequisite for the subsequent NMR analysis. Consequently, the NMR peak assignment and hence structure elucidation is usually still possible if two or more analytes coelute.

In this study, the glucuronide (4) was well separated from other glucuronides, while those of (8) and (12) coelute. For structure elucidation, the ¹H-NMR data of the metabolites were compared with those of verapamil (table 3) and with already published data (Tetreault and Ananthanarayanan 1993). Methoxy groups were located using a 2D NOESY experiment. The lack of a cross peak in the NOE spectra between a methoxy group and one of the aromatic protons H-3, H-6, H-16 or H-19 indicates that O-demethylation has occurred. This is also confirmed by the presence of a cross peak between the anomeric proton of the glucuronic acid moiety and one of the aromatic protons H-3, H-6, H-16 or H-19 (table 4 and figure 3).

Discussion of the MS and ¹H-NMR spectra of metabolites (4), (8) and (12)

The MS data of the D/H exchange experiments of (4) provided strong evidence for the presence of five exchangeable protons. As the glucuronic acid moiety has

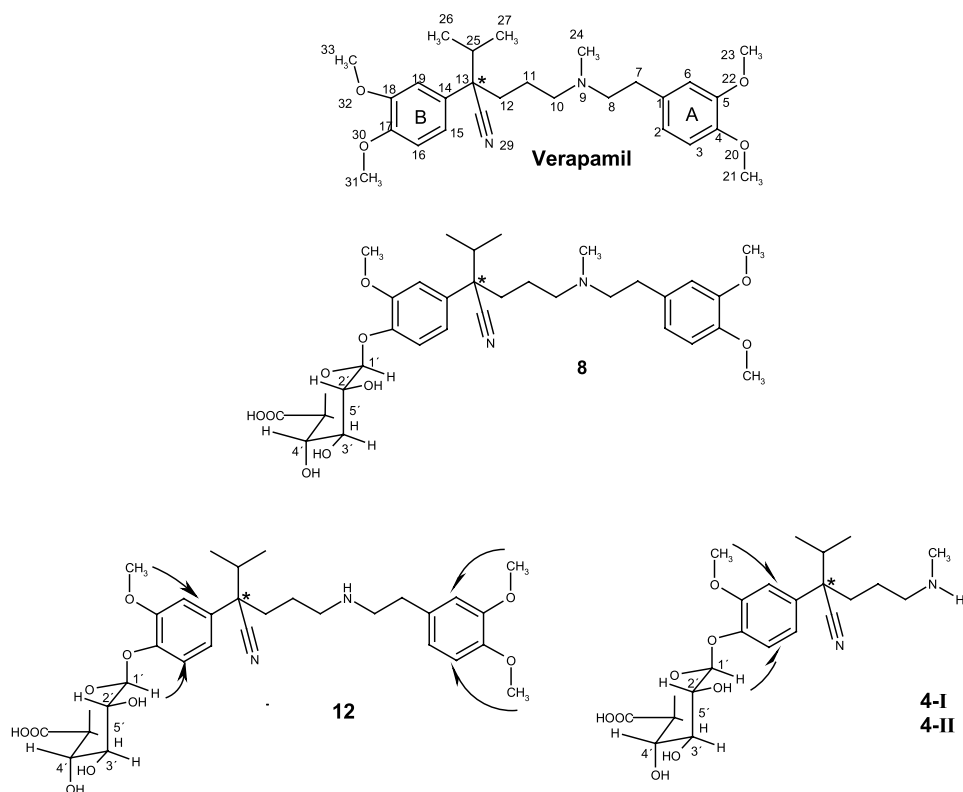


Figure 3. Structures of verapamil and its Phase II metabolites in human urine: the observed cross peaks in the two-dimensional NOESY spectra used for structure elucidation of the metabolites are marked by arrows.

Table 4. Observed cross peaks in 2D WET NOESY-spectra.

Metabolite	Cross peaks						
	H ₃ -33/H-19	H ₃ -31/H-16	H ₃ -23/H-6	H ₃ -21/H-3	H-1'/H-16	H-1'/H-3'	H-1'/H-5'
8	×	—	×	×	×	×	×
12	×	—	×	×	×	×	×
4	×	—	—	—	×	×	×

^a Cross peaks were also observed in long range COSY spectra.

four exchangeable protons after conjugation, there must be an additional exchangeable proton within the molecule, i.e. that bound to nitrogen (figure 3). The introduction of a deuterium label by H/D exchange experiments can also be used for the structure elucidation of unknown metabolites. The protonated molecule of (4) thus shows a neutral loss of 31 amu, which can be due to loss of a methoxy group or loss of the isobaric methylamine molecule.

However, after H/D-exchange of (4) loss of 33 amu from $[M + D]^+ = 280$ to m/z 247 is observed, which proves that loss of deuterated methylamine (and not loss of a methoxy group) had occurred (table 2).

In addition, NMR data of metabolite (4) is given in figure 4, where figure 4a shows the region of the aromatic protons, whereas figure 4b shows the signals of

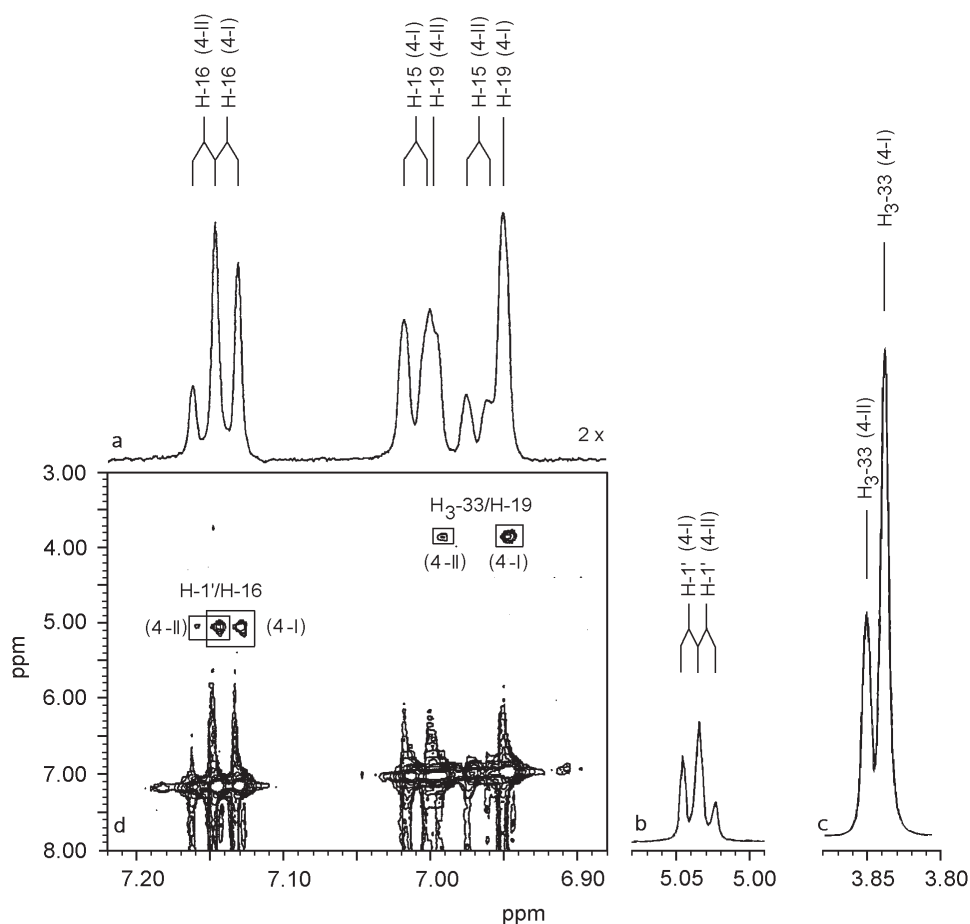


Figure 4. ^1H -NMR data of metabolite (4): (a) aromatic protons, (b) anomeric protons, (c) methoxy groups and (d) 2D NOESY spectra (the numbering of metastables is in parentheses).

the anomeric protons and figure 4c evidences the methoxy protons, while figure 4d displays the partial 2D NOESY spectrum. The ^1H -NMR spectrum shows a double set of signals pointing to the presence of two compounds denominated in figure 4 as (4-I) and (4-II) (intensity ratio 2:1) with similar spectroscopic behaviour, which have an identical fragmentation pattern in the MS (table 2). The signals of the aromatic protons H-15, H-16 and H-19 partly overlap. The lack of the signals of H-2, H-3 and H-6 confirms the oxidative loss of the phenylalkyl chain proposed by the MS experiments described above. At 2.524 ppm, the signals of the N-methyl group are observed (not shown in figure 4), confirming the proposed mechanism from the MS results that no N-demethylation has occurred. Only the signal of one methoxy group of each of the compounds is observed (singlets at 3.837 and 3.848 ppm, respectively, confirming the proposed demethylation of the second methoxy group).

The 2D NOESY spectrum shown in figure 4d shows cross peaks between a methoxy group and H-19 and the anomeric proton H-1' and H-16, but no cross peak between a methoxy group and H-16 (see the scheme in figure 3). This

demonstrates that of the originally two methoxy groups at ring B of verapamil, the methoxy group at C-17 is demethylated and the resulting hydroxyl group is subsequently conjugated with glucuronic acid. As verapamil is a racemic compound, glucuronidation of the enantiomers leads to diastereomers as shown by the double set of signals in figure 4. These diastereomers have the same fragmentation pattern and thus cannot be distinguished by mass spectrometry.

As described above, (8) and (12) coelute in the LC-NMR run and the NMR data thus deliver information on the mixture of both metabolites and perhaps of its diastereomers. Furthermore, the MS data of the D/H back exchange experiments reveal the presence of four exchangeable protons for (8) and five exchangeable protons for (12) (table 2). Consequently, as the glucuronic acid moiety has only four exchangeable protons after conjugation, (12) but not (8) must possess an additional exchangeable proton within the molecule (table 2).

From the MS data we show for (8) an O-demethylation at ring B, and for (12) an additional N-demethylation which is in agreement with the number of exchangeable protons observed in our study and, therefore, both metabolites must be conjugated with glucuronic acid.

The MS data are confirmed by the ^1H -NMR data. The ^1H -NMR spectrum in figure 5 shows the signals of two major components with similar structures (8:12, intensity 60:40). Figure 5a shows that the signals of the aromatic protons H-19, H-16, H-6 and H-2 for the two metabolites partly overlap, whereas the signals of H-3 overlap completely (the assignment of H-15 in table 3 is tentative). This points to a similar structure of the phenylalkyl moiety of both molecules.

In addition, the singlets of the methoxy groups of the two metabolites (8) and (12) partly overlap. The signal at 3.769 ppm shows an intensity of 2×6 protons relative to the aromatic protons, whereas the signals at 3.826 and 3.832 ppm show an intensity of 2×3 protons (figure 5c).

The anomeric protons of the two coeluting glucuronides are observed at 4.992 ppm for (8) and 5.015 ppm for (12) (figure 5b).

At 2.657 ppm, a singlet with the intensity of three protons relative to the signals of the main component (8) is detected (not shown in figure 5). This signal corresponds to the N-methyl-group of (8). As a consequence, (12) must be demethylated at the nitrogen confirming conclusions derived from MS data.

The exact assignment of the methoxy groups can only be determined from 2D NOESY experiment, the results of which are shown in figure 5d. Cross peaks are detected between the protons of the methoxy group at 3.769 ppm and H-6 as well as between the protons of the methoxy group at 3.769 ppm and H-3 for both compounds. Here the methoxy groups H₃-21 and H₃-23 overlap for both compounds. The 2D NOESY spectrum shows no cross peak between a methoxy group and the aromatic proton H-16, but between the anomeric proton H-1' and H-16 for both compounds. These results prove that of the originally four methoxy groups in the verapamil precursor only that bound to C-17 is demethylated and the resulting hydroxyl group is subsequently conjugated with glucuronic acid. An additional N-demethylation occurred for (12).

Discussion

Our study demonstrates that LC/MS in the MSⁿ mode is well suited for the identification of metabolites from cell culture experiments such as human

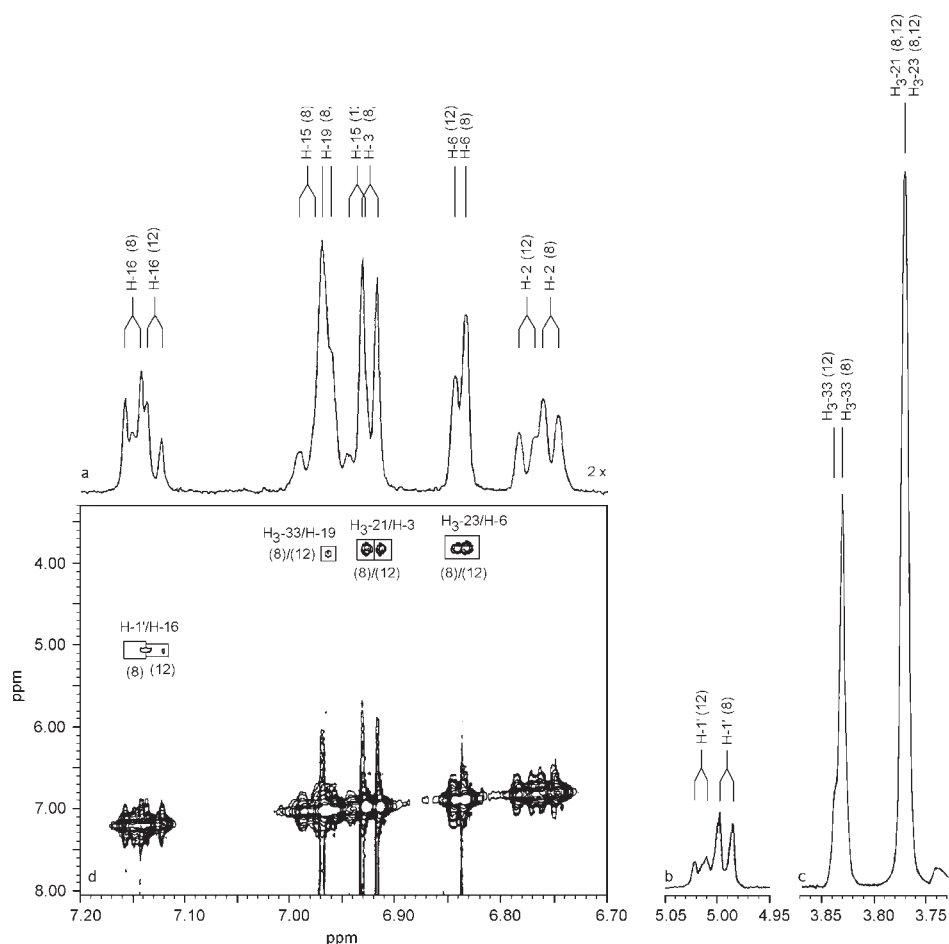


Figure 5. ^1H -NMR data of metabolite (8) and (12): (a) aromatic protons, (b) anomeric protons, (c) methoxy groups and (d) 2D NOESY spectra (the numbering of metastables is in parentheses).

hepatocytes. The collision-induced dissociation of the protonated molecules of the 21 Phase I metabolites identified here can be described by a general fragmentation scheme given in figures 1 and 2. Fragmentation leads to structure-specific ions formed by bond cleavages in the aliphatic moiety of the molecule (with and without hydrogen transfer). This fragmentation predominantly led to thermodynamically stable products, i.e. even-electron ions and neutral molecules (table 1). The observed neutral losses are valuable in particular for the identification of structural features. Loss of water is thus characteristic of a hydroxyl group in the aliphatic moiety of the molecule (except if there are vicinal hydroxyl groups at the aromatic ring). Loss of ammonia during fragmentation proves the presence of a primary amine, loss of methylamine the presence of a secondary amine, loss of acetone is characteristic of a hydroxylation of the isopropyl group.

Proton/deuterium (H/D) exchange experiments can provide additional insight about the number of acidic protons within the molecule, which is valuable information for structural elucidation. Furthermore, these data can be used to confirm the fragmentation pathways of protonated molecules, as described by us.

In the case of verapamil metabolites, the D/H exchange experiments prove that loss of 31 amu corresponds to the subsequent loss of methylamine instead of a methoxy radical (table 2).

Inevitably, the main disadvantage of mass spectrometry is that it cannot provide any information about the position of O-demethylation at one aromatic ring (e.g. C-17 or C-18) or the binding site of the glucuronic acid (N- or O-bound). This information is only available from ^1H -NMR spectroscopy with 2D experiments (like NOESY or long-range COSY). The disadvantage of these 2D experiments is the lack of sensitivity. Using NMR spectroscopy, it was thus not possible to characterize the metabolites in human cell cultures, but only those in human urine, where they are formed in much higher amounts (20–90 μg of the major metabolites per 0.65 litres urine). If for a given metabolite identified in human urine the mass spectra and the retention time are identical to those of other matrices such as cell culture experiments, it is not unreasonable to extrapolate data based on NMR experiments of urine samples to those of other matrices.

In our study, ^1H -NMR spectroscopy has allowed identification of the main Phase II metabolites in human urine with unequivocal identification of the position where O-demethylation and conjugation with glucuronic acid had occurred.

We report more than 20 Phase I metabolites, of which (2) (norverapamil), (3) (D-620), (4) (D-717), (5) (D-617), (7) (D-702), (8–11) and (12) (D-715) have been reported previously (Knoll nomenclature in parentheses). In a previous GC/MS study of human urine, metabolites (2–5, 7, 8, 12) were identified and quantified and their stereoselectivity determined (Nelson and Olsen 1988). These authors furthermore report that 59% of the dose was excreted in urine in 48 h, where about one-half of the detected urinary metabolites was the secondary amine (5). Within our study, a direct quantification of the metabolites was not possible as in most cases reference compounds were not available. Rather, we report in table 1 the peak areas of the metabolites relative to that of non-conjugated verapamil (=100). These peak areas depend on the electrospray ionization efficiency of the individual compounds and thus reflect the relative abundances only semiquantitatively. In agreement with Nelson and Olsen (1988), compounds (2–5, 7, 8, 12) are major metabolites in human urine. A previously reported formyl derivative of (5) identified by Nelson and Olsen was not found by us.

For the first time, we report the identification of metabolites (14–18, 28–32), whereas (24) was already reported by Walles *et al.* (2001). All metabolites are summarized in figure 6, which describes the most likely metabolic pathways of verapamil.

In addition, we show the production of 16 Phase II conjugates observed by us, which have not as yet been reported. Fourteen of these conjugates (4, 11, 12, 7–10, 29–32) are probably O-bound glucuronides. The binding site of the glucuronic acid could not be identified unambiguously by MS, but ^1H -NMR studies with (12) have shown that in case of two possible binding sites (N or O), the oxygen is preferred for conjugation, at least in humans (figure 3).

If the Phase I metabolite has no hydroxyl group, the glucuronides must be N-bound. Two glucuronides are thus most likely to be N-bound: one quaternary N-bound glucuronide (1) (i.e. the glucuronide of verapamil itself) and a secondary N-bound glucuronide (4) (Hawes 1998).

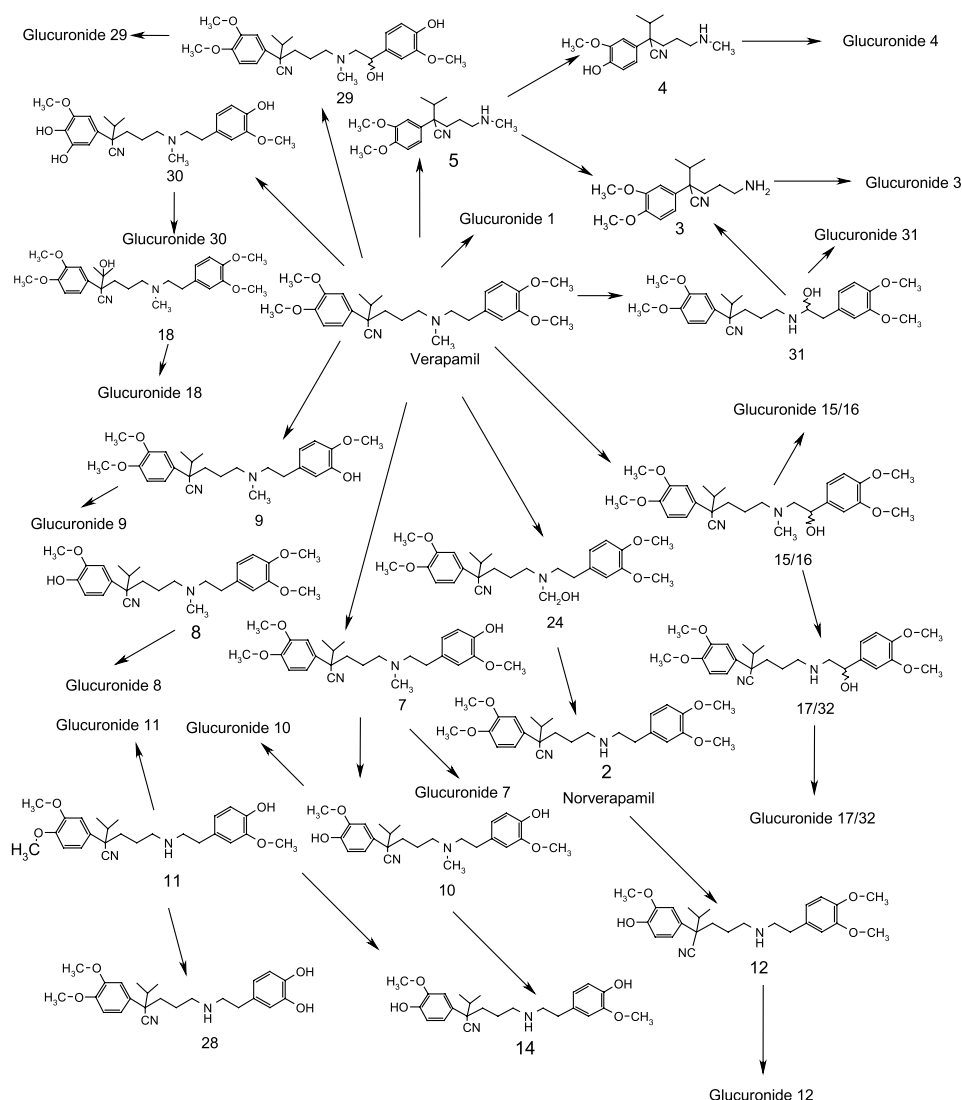


Figure 6. Proposed pathway for the human metabolism of verapamil.

Among the N-glucuronides, one distinguishes between secondary, tertiary and quaternary ammonia glucuronides. Whereas the formation of secondary and tertiary glucuronides is only dependent on the substance, there are species differences in the formation of the quaternary glucuronides (Chiu and Huskey 1998). For tertiary amines like tricyclic antidepressives and antihistaminines, the quaternary glucuronidation is mostly preferred by humans and primates.

The only known glucuronide is the norverapamil glucuronide identified as an ester by GC-MS in bile of rats (Mutlib and Nelson 1990), but has not been observed in our study. One reason could be that only urine and not bile was investigated. Another reason could be the chemical stability of the conjugates.

N-glucuronides of aryl-amines are cleaved under weak acidic conditions even in the absence of enzymes (Sherratt and Damani 1989). In general, the N-glucuronides of primary and secondary amines are very unstable in solutions with a $\text{pH} < 7$. The structure of quaternary ammonia glucuronides resembles that of ester glucuronides. They are stable in neutral and acidic solutions and hydrolyse only under basic conditions. The optimal pH range varies significantly between O-ether and quaternary ammonia glucuronides. The secondary N-glucuronides are hydrolysed under acidic conditions and are stable under basic conditions (Huskey *et al.* 1994). As the hydrolysis conditions vary between the different kinds of glucuronides, some might be cleaved under the chromatographic conditions (pH 6) in our study.

Stereoselectivity in Phase I metabolism has been described before (Schomerus *et al.* 1976, Vogelsang *et al.* 1984, Nelson and Olsen 1988). It is obvious that the glucuronidation rate is also linked to one enantiomeric form of the drug.

It is known that glucuronidation of racemic metabolites leads to diastereoisomers (Dieterle *et al.* 1986, Laethem *et al.* 1995, Meunier and Verbeeck 1999).

For (2), two diastereomers (with identical mass fragmentation patterns) were observed in our study with a 60:40 ratio in the NMR spectrum. It is obvious that the major isomer corresponds to the *S*-glucuronide, whereas the minor isomer corresponds to the *R*-glucuronide of (2). The LC-NMR technique has previously been used to identify diastereoisomeric glucuronides of flurbiprofen (Spraul *et al.* 1993).

For (8) and (12), only one diastereomer was observed in the ^1H -NMR spectrum of (8) and (12), but in addition to the signals of the major compounds, additional small signals were observed. These signals could be caused by the diastereomers of these metabolites. Studies with human liver microsomes have shown that verapamil is extensively metabolized via N-dealkylation, N- and O-demethylation (Nelson *et al.* 1987, Nelson and Olsen 1988). Mechanistic studies have shown that O-demethylation occurs preferably in the following order of position: C-4 > C-17 > C-18 > C-5 (Nelson *et al.* 1987). The ^1H -NMR experiments in this study show that in human urine the methoxy group in position C-17 (ring B) is preferably demethylated and conjugated with glucuronic acid. The hydroxylated metabolites (15–18, 29–32) provide evidence for a completely new concept in the metabolic pathway of verapamil.

In conclusion, LC-MSⁿ is the method of choice for drug metabolism studies with extracts of human hepatocyte cultures because of its high sensitivity and the limited amount of available metabolites. As this method does not allow differentiation between isomers, ^1H -NMR spectroscopy is necessary for an adequate sufficient structural elucidation. As with the available instrumental equipment, the amounts of the minor metabolites in human hepatocytes are too small for ^1H -NMR experiments, a characterization of the major metabolites from human urine is preferable. To verify the identity of the metabolites in other matrices, the retention times and the fragmentation pattern can be used.

LC-NMR can provide rapid structural information as the sample preparation is not as time consuming as that for offline ^1H -NMR. H/D exchange experiments can provide additional valuable information on the structures of newly identified metabolites.

Acknowledgements

The loan of an esquire ion-trap mass spectrometer by Bruker Daltonics, Bremen, Germany, is kindly acknowledged. *O*-demethylverapamil (5-*N*-(3,4-dimethoxyphenethyl)methylamino-2-(3'-methoxy-4'-hydroxyphenyl)-2-isopropylvaleronitrile) was a kind gift of W. L. Nelson (University of Washington, Seattle, WA, USA).

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Metabolism of methyl-(E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (azoxystrobin) in rat

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Received 16 September 2002

1. The metabolic fate of [¹⁴C]-methyl-(E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (azoxystrobin) was determined in the male and female rat following a single oral dose of 1 and 100 mg kg⁻¹ and in surgically prepared, bile duct-cannulated rats following a single oral dose of 100 mg kg⁻¹.

2. Azoxystrobin was extensively metabolized with at least 15 metabolites. There was a sex difference, with females producing more metabolites than males.

3. The two principal metabolic pathways were hydrolysis of the methoxyacid followed by glucuronic acid conjugation and glutathione conjugation of the cyanophenyl ring followed by further metabolism leading to the mercapturic acid. There were also several other minor pathways.

Introduction

Methyl-(E)-2-{2-[6-(2-cyanophenoxy)pyrimidinyl-4-yloxy]phen}-3-methoxyacrylate (azoxystrobin) is a novel broad-spectrum fungicide (Godwin *et al.* 1992, 1994, 1997, Frank and Sanders 1994, Heaney and Knight 1994). Very little has been published previously for the metabolism of this or related compounds (Joseph 1999). Studies are described herein following oral administration of three different radiolabelled forms of [¹⁴C]-azoxystrobin in male and female rats. Two major metabolic pathways and several minor pathways have been elucidated.

Materials and methods

[¹⁴C]-phenylacrylate-labelled azoxystrobin with a specific activity 1.895 GBq mmol⁻¹ (labelled in the right hand ring of figure 1), [¹⁴C]-pyrimidinyl-labelled azoxystrobin with a specific activity 2.020 GBq mmol⁻¹ (labelled in the middle ring of figure 1), [¹⁴C]-cyanophenyl labelled azoxystrobin with a specific activity 2.307 GBq mmol⁻¹ (labelled in the left hand ring of figure 1) and unlabelled azoxystrobin were obtained from Zeneca Agrochemicals (Jealott's Hill Research Station, Bracknell, UK).

Polyethylene glycol (PEG) 600 was obtained from NBS Biologicals (Haverhill, UK). Optiphase-MP, Optisorb '1' and Optisorb '3' were obtained from Canberra Packard (Pangbourne, UK).

Animals

Wistar-derived male and female rats (Alpk:ApfSD strain, bodyweight range 168–364 g) were obtained from the Barriered Animal Breeding Unit (Zeneca Pharmaceuticals). The animals were maintained under controlled environmental conditions (19–23 °C, 40–72% relative humidity, 12 h of light cycled with 12 h of dark). Urinary and faecal metabolite profiles were obtained from two groups of five male and five females given either 1 or 100 mg [U-¹⁴C]-azoxystrobin kg⁻¹ as a single oral dose by

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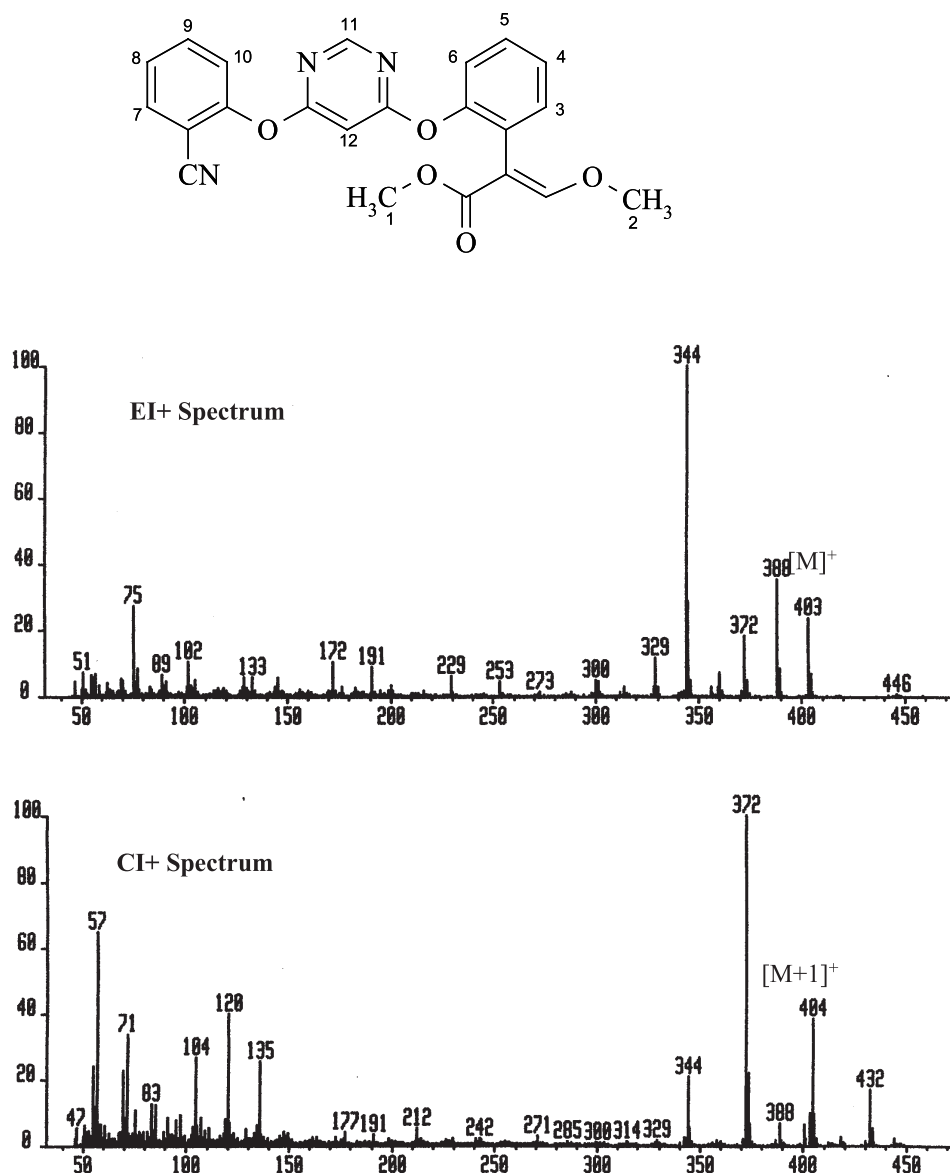


Figure 1. Structure and mass spectra of azoxystrobin.

gavage, using PEG 600 as the dosing vehicle. The dose was administered at 4 ml kg⁻¹ bodyweight and the amount of radioactivity was 4 MBq kg⁻¹.

In addition, to obtain enough mass of material for metabolite identification, three groups of two male and two female rats were bile duct cannulated under Fluothane (Zeneca Pharmaceuticals) anaesthesia. The cannulae were exteriorized and the animals allowed to recover. On the morning following surgery, each group was given a single oral dose of 100 mg of one of the three forms of radiolabelled azoxystrobin kg⁻¹ as described above.

Urine and faeces were collected into containers cooled by solid CO₂. Urine was collected during 0–6 and 6–12 h, faeces were collected during 0–12 h, and urine and faeces during 12–24, 24–36 and 36–48 h and then at 24-h intervals until 144 h after dosing. For the bile duct-cannulated rats, bile was collected at room temperature during 0–6, 6–12, 12–24, 24–28, 28–36 and 36–48 h after dosing, and urine and faeces were collected as described above until 48 h after dosing. All samples were stored at approximately –20 °C before analysis.

Determination of radioactivity

Duplicate weighed aliquots of urine and bile were mixed with Optiphase-MP scintillant (10 ml) and analysed for radioactivity (for 10 min or to a 1% standard deviation of the count) using a Packard Tricarb 2000 CA liquid scintillation counter. Values were corrected for counting efficiency with a [^{133}Ba] external standard. Faeces were ground with an approximately equal quantity of magnesium sulphate until homogeneous. Duplicate weighed aliquots (about 200 mg) were oxidized (Packard Tricarb B306) and $^{14}\text{CO}_2$ absorbed in Optisorb '1' and mixed with Optisorb 'S' scintillant (total volume 10 ml). Radioactivity was analysed by liquid scintillation counting as described above.

High-performance liquid chromatography (HPLC)

Analytical HPLC was used to quantify individual metabolites in bile, urine and faecal extracts and also to compare the isolated metabolites and reference standards. Typical conditions were thus:

- Column: Hichrom HIRPB reverse phase (15 cm $\times$ 4.6 mm i.d.).
- Mobile phase: A = water modified with 0.1% acetic acid; B = acetonitrile modified with 0.1% acetic acid.
- Gradient: 80% A 20% B for 5 min then linearly to 20% A 80% B in 25 min.
- Flow rate: 1 ml min $^{-1}$.
- Detection: UV absorption at 254 nm.
- On-line radiodetection (Model 506 radiochemical detector Berthold Instruments, St Albans, UK).
- Quantitation: 0.3-min fractions were collected using a fraction collector.

Preparative HPLC was carried out to obtain a sufficient mass of isolated metabolites for NMR analysis. Samples of untreated or enzyme-digested bile were subject to preparative HPLC as described above with the following modifications:

- Column: 20 cm $\times$ 10 mm i.d.
- Flow rate: 2.5 ml min $^{-1}$.
- Peak collection: individual peaks were collected into round-bottomed flasks.

Following evaporation, the residues were reconstituted in a small volume of initial mobile phase and re-injected onto the analytical HPLC to assess purity. When required, samples were further purified by preparative HPLC using the same conditions as above.

Mass spectrometry (MS)

- Direct insertion probe MS: a VG 70-70E mass spectrometer (VG Analytical, Wythenshawe, UK) with a model 11-250 data system was used to obtain the mass spectra of azoxystrobin. The samples were introduced directly via the probe. Both electron impact (EI+) and chemical ionization (CI+ with methane as the reagent gas) spectra were recorded (figure 1).
- HPLC-electrospray-MS (HPLC-ESI-MS): analysis was carried out using a Sciex API III Triple Quadrupole Mass Spectrometer fitted with an electrospray (ESI) source (Perkin Elmer, Beaconsfield, UK). The HPLC conditions were the same as described above except that:
 - Column: 15 cm $\times$ 2.1 mm i.d.
 - Flow rate: 250 μl min $^{-1}$.
 - Bile, urine, faecal extracts, purified metabolites and/or reference standards were introduced via HPLC or by direct infusion. Spectra were recorded in positive-ion mode only.
- Tandem mass spectrometry (MS-MS): some samples were analysed by HPLC-ESI-MS-MS or direct MS-MS using the same instrument and conditions as above. The collision cell gas was argon at a pressure of approximately 1 Torr.
- Proton nuclear magnetic resonance spectroscopy (^1H -NMR): purified metabolites or reference standards were dissolved in an appropriate deuterated solvent and proton NMR spectra were obtained using a Bruker WM 400 Spectrometer.

Results

Excretion of radioactivity.

Animals were given a single oral dose of either 1 or 100 mg pyrimidinyl [^{14}C]-labelled azoxystrobin and urine and faeces were collected over 7 days. The excretion of radioactivity is shown (figure 2) for both male- and female-dosed animals.

- Low dose study (1 mg kg $^{-1}$): faecal elimination of radioactivity accounted for 83.2 and 72.6% of the administered dose, and urinary elimination accounted for

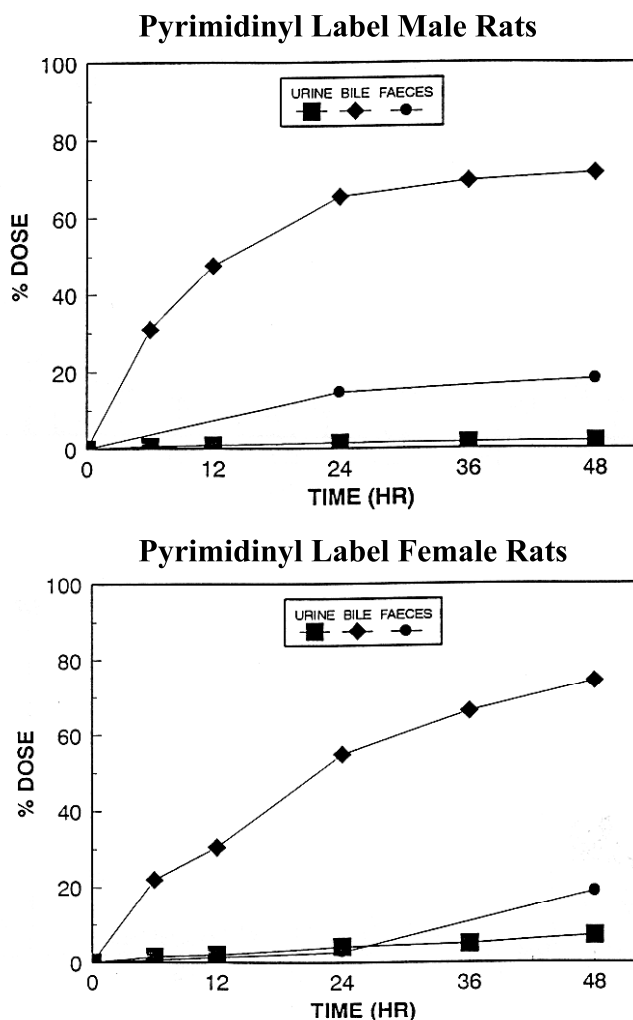


Figure 2. Excretion of azoxystrobin in male and female rats. Data are from bile duct-cannulated rats dosed at 100 mg kg^{-1} .

10.2 and 17.9% of the dose in males and females, respectively. The total recovery of administered radioactivity, including excreta and tissue residues, was 94.1% for males and 91.8% for females.

- High-dose study (100 mg kg^{-1}): faecal elimination of radioactivity accounted for 89.4 and 84.5% of the administered dose, and urinary elimination accounted for 8.5 and 11.5% of the dose in males and females, respectively. The total recovery of administered radioactivity, including excreta and tissue residues, was 98.6% for males and 97.5% for females.

Excretion of radioactivity by bile duct-cannulated animals

Animals fitted with a bile duct cannula were given a single, oral 100 mg kg^{-1} dose of one of the three $[^{14}\text{C}]$ -labelled forms of azoxystrobin and bile, urine and

faeces were collected over 48 h. There were no major differences between the labelled forms. (data not shown):

- Pyrimidinyl label: biliary excretion of radioactivity accounted for 71.6 and 74.2% of the dose in male and females, respectively. The radioactivity in faeces accounted for 18.1 and 18.9% of the dose, and the radioactivity in urine accounted for 2.0 and 7.1% of the dose in males and females, respectively. The total recovery of radioactivity in excreta was 91.6% of the dose for male rats and 100.0% for females.
- Cyanophenyl label: biliary excretion of radioactivity accounted for 56.6 and 62.5% of the dose in male and females, respectively. The radioactivity in faeces accounted for 29.1 and 28.1% of the dose, and the radioactivity in urine accounted for 2.0 and 4.2% of the dose in males and females, respectively. The total recovery of radioactivity in excreta was 87.7% of the dose for male rats and 94.8% for females.
- Phenylacrylate label: biliary excretion of radioactivity accounted for 64.4 and 63.6% of the dose in male and females, respectively. The radioactivity in faeces accounted for 18.1 and 29.6% of the dose, and the radioactivity in urine accounted for 4.4 and 4.0% of the dose in males and females, respectively. The total recovery of radioactivity in excreta was 86.8% of the dose for male rats and 97.2% for females.

Identification of metabolites:

- Compound I (retention time 23.4 min), parent compound, was not present in bile but was the major component present in solvent extracted faeces collected from bile cannulated rats, representing approximately 98% of faecal extracted radioactivity. It co-chromatographed on HPLC with a Zeneca Agrochemicals reference standard of azoxystrobin (5504/01) and was identified by HPLC-ESI-MS in the solvent extract of faeces collected from the high-dose study male rats. The HPLC-ESI-MS spectrum showed a molecular ion $(M+1)^+$ at m/z 404.
- Compound II (retention time 21 min) hydroxy-azoxystrobin, was isolated by preparative HPLC from bile and co-chromatographed on HPLC with a reference standard. The ESI-MS spectrum showed an ion at m/z 420 $(M+1)^+$ and a fragment at m/z 388 $(M-OCH_3)^+$. The NMR spectrum of this metabolite was similar to that obtained from azoxystrobin with the exception of the signals from protons in the cyanophenol ring, whose chemical shifts had changed to δ 7.23, 7.20 and 7.20 ppm with one signal absent. These observed signals were at the chemical shift values expected for a hydroxy substitution in the 8-position of the cyanophenyl ring. This was also detected by HPLC-ESI-MS in urine collected from both bile and non-bile duct-cannulated rats.
- Compound III (retention time 20 min) hydroxy-azoxystrobin, was isolated by preparative HPLC from bile and analysed by HPLC-ESI-MS and NMR. The HPLC-ESI-MS spectrum was the same as obtained for metabolite II. The NMR of this metabolite was weak, however it was possible to see that the spectrum was similar to the spectrum obtained for a reference sample of azoxystrobin, with the exception of the signals from the cyanophenyl ring, whose chemical shift values had changed to δ 7.32, 7.76 and 7.65 ppm with one signal absent. These observed signals were consistent with the chemical shift values expected for a hydroxy substitution in the 10-position of the cyanophenyl

ring. It was also detected by HPLC-ESI-MS in urine collected from both bile and non-bile duct-cannulated female rats.

- Compounds IVa and b (retention times 16 and 18 min), hydroxy-azoxystrobin-glucuronide, were isolated by preparative HPLC. Two isomers of this metabolite were detected. The HPLC-ESI-MS spectrum shows an ion at m/z 596 ($M+1$)⁺ and an NH₄ adduct at m/z 613. The HPLC-ESI-MS spectrum was identical to a reference glucuronide conjugate. The MS-MS data (figure 3) shows the fragmentation pathway to be via the loss of OCH₃ to give m/z 564 ($M-31$)⁺ followed by loss of the glucuronide moiety (176 mass units) to give m/z 388. This then loses C=O to give the base peak at m/z 360 and a loss of CH₃ gives the final fragment at m/z 345. From MS and MS-MS data, the exact position of conjugation of either isomer could not be determined. However, from these data it is assumed that these two isomers are the glucuronide conjugates corresponding to the two hydroxylated metabolites II and III. Both metabolites were also identified by HPLC-ESI-MS in urine collected from both bile duct-cannulated and non-bile duct-cannulated female rats.
- Compound V (retention time 17 min), des-methyl-azoxystrobin-glucuronide, was isolated by preparative HPLC from bile. The HPLC-ESI-MS spectrum showed an ion at m/z 566 ($M+1$)⁺ and a sodium adduct at m/z 588. The HPLC-ESI-MS spectrum was identical to a Zeneca Agrochemicals reference glucuronide conjugate of standard 5504/02. MS-MS data showed daughter ions of mass 566 at m/z 390 (loss of glucuronide moiety), m/z 358 base peak (loss of glucuronide and C=O), m/z 330 (loss of C=O from the base peak) and m/z 301. Endogenous taurocholic acid, also isolated in the sample, limited the amount of information available from the NMR spectrum, but the signal expected at δ 3.5 ppm for the carboxylic OCH₃ protons was clearly absent from the spectrum confirming the position of substitution as being the acid function on the

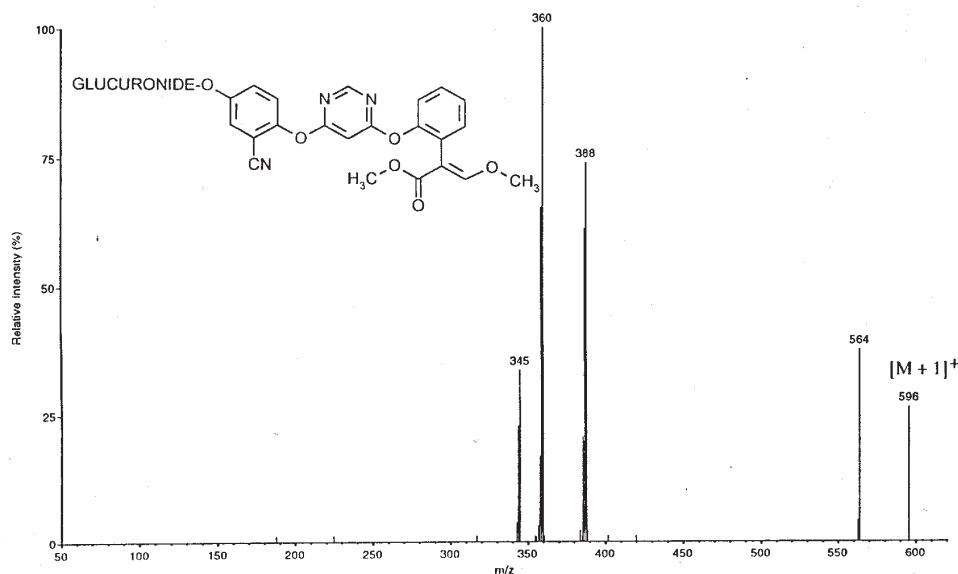


Figure 3. Electrospray tandem mass spectrum of metabolite IV of azoxystrobin (products of m/z 596).

molecule. It was also detected by HPLC-ESI-MS in urine collected from both bile duct-cannulated and non-bile duct-cannulated female rats.

- Compounds VIa and b (retention time 16 and 18 min), two isomers of azoxystrobin-glutathione, were isolated by preparative HPLC from bile. The HPLC-ESI-MS spectrum showed an ion at m/z 708 ($M+1$)⁺. The ESI-MS-MS spectrum showed the loss of a glutathione moiety (product ion at m/z 403). Other daughter ions of mass 709 were observed but could not be assigned to specific fragments. Assignment of the position of glutathione conjugation (*ortho* to CN) in one isomer was based upon the NMR data obtained for the mercapturate (metabolite IX, see below).
- Compound VII (retention time 15 min), a glycine-cysteine conjugate of azoxystrobin, was isolated by preparative HPLC from bile using preparative HPLC after pretreatment with cholylglycine hydrolase in an attempt to remove contaminating taurocholic acid. The HPLC-ESI-MS spectrum showed an ion at m/z 580 ($M+1$)⁺. Assignment of the position of conjugation (*ortho* to CN) was based upon the NMR data obtained for the mercapturate (metabolite IX, see below).
- Compound VIII (retention time 15.5 min), the cysteine conjugate of azoxystrobin, was isolated by preparative HPLC from bile. The HPLC-ESI-MS spectrum showed an ion at m/z 523 ($M+1$)⁺. Assignment of the position of conjugation (*ortho* to CN) was based on the NMR data obtained for the mercapturate (see below).
- Compound IX (retention time 19 min), the mercapturate of azoxystrobin, was isolated by preparative HPLC from bile. An ion was seen at m/z 565 ($M+1$)⁺. This NMR spectrum was free of interference from endogenous taurocholic acid and the resolution was good enough to observe the splitting patterns of the aromatic protons. The signals at δ 7.15, 7.70 and 7.45 ppm correspond to protons in the 8-, 9- and 10-positions in the molecule and their splitting pattern was that expected for *ortho*, meta tri-substituted aromatic ring. There was no signal at approximately δ 7.7 ppm, which is where the signal from the proton at the 7-position would be expected. Therefore, the position of substitution was at the 7-position of the cyanophenyl ring.
- Compound X (retention time 11 min), des-cyanophenyl-azoxystrobin, was isolated by preparative HPLC from bile. The HPLC-ESI-MS spectrum showed an ion at m/z 303 ($M+1$)⁺ and its sodium adduct at m/z 325. A fragment at m/z 271 corresponds to the loss of OCH₃ ($M-31$)⁺. It co-chromatographed on HPLC with a reference standard. The NMR spectrum showed the absence of signals at δ 7.45–7.80 ppm due to the cyanophenyl ring protons, indicating the absence of the cyanophenyl ring from this metabolite. The signals due to the protons on the pyrimidinyl ring at δ 8.08 and 4.49 ppm were still sharp which confirms there was a hydroxy group meta to the remaining ether link in the pyrimidinyl ring. It has the same HPLC retention time as a hydroxypyrimidinol-phenylacrylate reference standard and was also seen by HPLC-ESI-MS in urine collected from both bile and non-bile duct-cannulated female rats.
- Unidentified compound XI (retention time 13.6 min) was isolated by preparative HPLC from bile. No HPLC-ESI-MS spectrum was obtained because the metabolite did not ionize and the only information available from the NMR spectrum showed that the cyanophenyl ring was substituted and that the signals

at $\delta 8.5$ and 6.5 ppm due to the pyrimidinyl ring protons were broadened considerably.

- Compound XII (retention time 14 min), hydroxy-phenylacrylate, was isolated by preparative HPLC from bile. The HPLC-ESI-MS spectrum showed an ion at m/z 209 ($M+1$)⁺. The retention time and HPLC-ESI-MS-MS spectra of this metabolite were the same as those obtained for a reference standard. The NMR spectrum showed clearly that the cyanophenyl and pyrimidinyl rings were not present in this metabolite. The signals due to the acrylate function were still present and the chemical shift values and splitting pattern of the aromatic protons indicated that there was a hydroxy group *ortho* to the acrylate function.
- Compound XIII (retention time 23 min), des-methoxy-methenyl-azoxystrobin, was identified by HPLC-ESI-MS and HPLC-ESI-MS-MS of bile. The HPLC-ESI-MS showed an ion at m/z 362 ($M+1$)⁺ and a sodium adduct at m/z 384. The fragment at m/z 330 corresponds to a loss of OCH_3 ($M-31$). The HPLC-ESI-MS-MS spectrum showed the major daughter ion of mass 362 to be at m/z 302, which corresponds to the loss of the methylated acid function. Other minor fragments occur at m/z 339, 174 and 116. The retention time and HPLC-ESI-MS-MS spectra of this metabolite were the same as those obtained for a reference standard and this metabolite was also seen by HPLC-ESI-MS in urine collected from both bile duct-cannulated and non-bile duct-cannulated female rats.
- Compound XIV (retention time 14 min), hydroxy-des-methoxy-methenyl-azoxystrobin, was identified by HPLC-ESI-MS and HPLC-ESI-MS-MS of bile. The HPLC-ESI-MS spectrum showed an ion at m/z 378 ($M+1$)⁺ and a sodium adduct at m/z 400. The HPLC-ESI-MS-MS daughter ion spectrum showed the base peak at m/z 314. Other daughter ions were observed but could not be assigned to specific fragments.
- Compound XV (retention time 7 min), cyanophenoxy-hydroxypyrimidinol, isolated by preparative HPLC from bile. It was analysed by HPLC-ESI-MS and the spectrum contained an ion at m/z 214 ($M+1$)⁺. It had the same retention time as a reference standard.

Quantitation of metabolites

Metabolites were quantified by injecting samples onto an HPLC column and collecting fractions for liquid scintillation counting. The results for the 100 mg kg^{-1} dose in female rats are shown in figure 4.

Results and discussion

Studies with azoxystrobin (1 and 100 mg kg^{-1}) have shown that up to 90% of the dose was excreted in the faeces. Even after exhaustive Soxhlet extraction with methanol and chloroform/methanol, only about 20% of the radioactivity was extracted from the 1 mg kg^{-1} study and 50% from the 100 mg kg^{-1} study. Whereas 1 mg kg^{-1} dose level faecal extracts show several approximately equally abundant metabolites but no parent compound, the 100 mg kg^{-1} dose level contained primarily parent compound, representing about 30% of the dose. Furthermore, faeces from bile duct-cannulated rats administered 100 mg kg^{-1} azoxystrobin contained only parent compound, whereas the parent was absent from bile. It is clear,

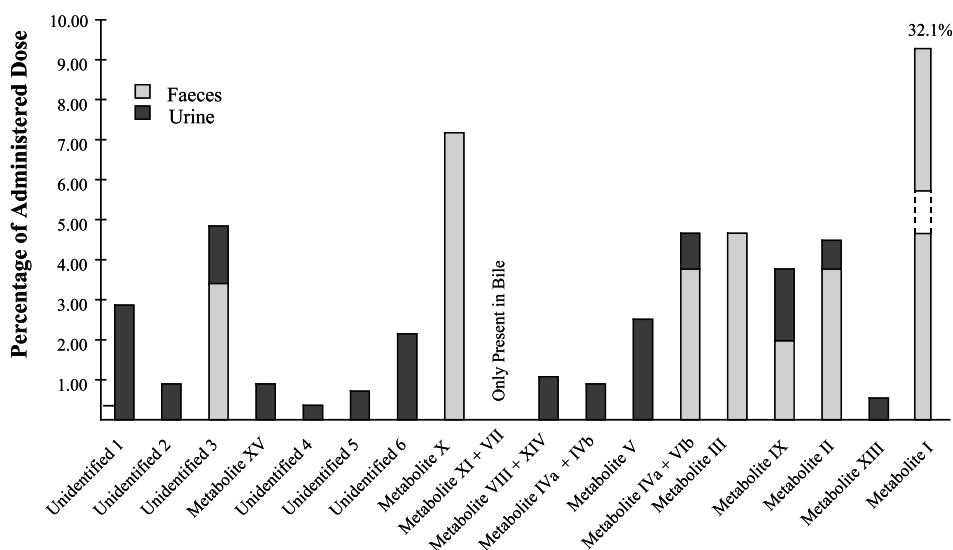


Figure 4. Quantitation data for metabolites in urine and faeces from female rats administered 100 mg kg^{-1} azoxystrobin. Data were from female rats dosed at 100 mg kg^{-1} .

therefore, that the absorption of azoxystrobin was dose-dependent. The absence of parent compound from the faecal extracts from the 1 mg kg^{-1} study suggests that absorption was quantitative and that radioactivity in faeces originated from biliary elimination. In contrast, the amount of parent in the faecal extracts from the 100 mg kg^{-1} study indicates that about 70% of the dose was absorbed.

The difference in the efficiency of extraction of radioactivity from faeces from non-cannulated rats administered 1 or 100 mg kg^{-1} azoxystrobin was explained by parent compound being readily partitioned into the acetonitrile, whereas the more polar biliary metabolites of azoxystrobin were less easily extractable. This was consistent with the almost quantitative efficiency of extraction of radioactivity from faeces of bile duct-cannulated rats, which contained only parent compound.

Since the identification of azoxystrobin metabolites was hindered by the low efficiencies of extraction from faeces, biliary metabolites were investigated instead. Since this route of elimination is important, the metabolic fate of [^{14}C]-phenylacrylate, pyrimidinyl and cyanophenyl azoxystrobin were separately examined in bile duct-cannulated rats given a single oral dose of 100 mg kg^{-1} . This dose level enabled sufficient amounts of metabolites to be separated for identification.

Biliary, urinary and faecal metabolites originating from rats dosed with [^{14}C]-phenylacrylate, pyrimidinyl and cyanophenyl azoxystrobin were compared using TLC. (data not shown) Azoxystrobin was well metabolized, forming at least 15 metabolites. The UV (254 nm) HPLC trace (figure 5) shows the number and retention times of the metabolites. There were no significant differences in the metabolite profiles among the three labelled forms, demonstrating that there was minimal cleavage of the ether linkages between the three chemical rings. Two metabolites, separated by TLC, did show the absence of either the cyanophenyl or phenylacrylate ring, but these were minor biliary components. Furthermore, these two metabolites had identical R_f values for the corresponding reference standards

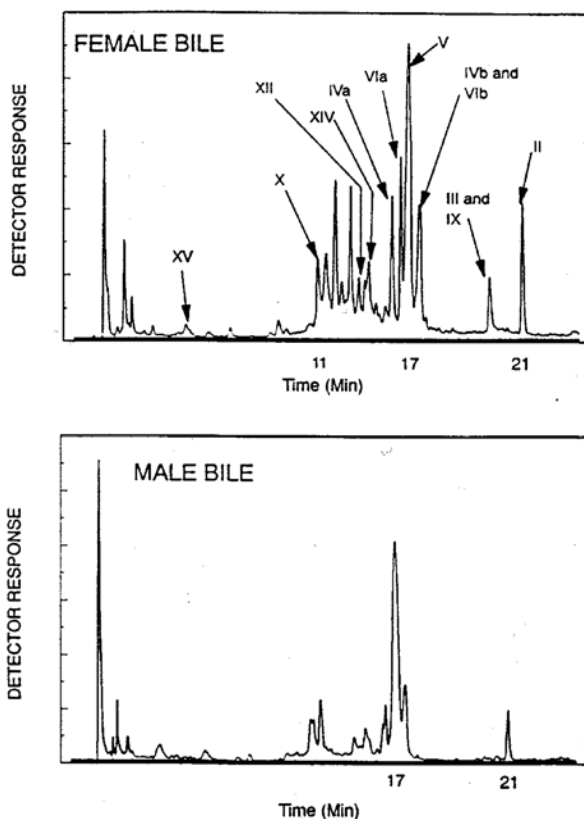


Figure 5. HPLC chromatograph of bile from rats dosed with 100 mg kg^{-1} [^{14}C]-pyrimidinyl-labelled azoxystrobin showing the separation of metabolites.

and were later structurally identified as hydroxy-pyrimidinol-phenylacrylate (metabolite X) and cyanophenoxy-hydroxypyrimidinol (metabolite XV).

The TLC and HPLC analysis of bile revealed a sex difference in metabolism. There were several metabolites excreted in the bile and urine present in smaller amounts or not detected in the male. Comparisons of the HPLC chromatograms of excreta from the low single-dose study (1 mg kg^{-1}) to the high-dose study (100 mg kg^{-1}) and a repeat-dose study (1 mg kg^{-1}) all showed very similar profiles (data not shown). There was no evidence, therefore, that the biotransformation was dose-dependant, although, as stated above, there was evidence of dose-dependent absorption.

Since only minimal cleavage of the ether linkages had occurred, experiments on the metabolism of azoxystrobin were conducted using samples from [^{14}C]-pyrimidinyl azoxystrobin-dosed rats. Bile from female rats was primarily used for the identification of metabolites because more metabolites were present in this case. The presence or absence of these metabolites in male bile and other excreta were examined by comparative chromatography and on some samples by using HPLC-ESI-MS.

All excreted metabolites above 4.5% of the administered dose from bile duct-cannulated rats administered 100 mg kg^{-1} [^{14}C]-pyrimidinyl azoxystrobin were identified. Some metabolites at 0.1% of the dose were also identified. A mean total

of 88% of the excreted dose (80.4% of the administered dose) for males and 82% of the excreted dose (82% of the administered dose) for females was accounted for.

The majority of the metabolites were identified using HPLC-ESI-MS and HPLC-ESI-MS-MS. Other mass-spectroscopic methods were attempted (Thermospray and fast atom bombardment (FAB)), but ESI-MS was the only technique found to produce spectra of intact conjugates. FAB repeatedly gave spectra for endogenous taurocholic acid that had also been isolated with the samples. Although tandem mass spectroscopy (MS-MS) provided further structural information, it could not determine the position of conjugation and this was only possible using NMR. Greater amounts of relatively pure sample are required for NMR analysis compared with MS methods. The isolation of relatively pure compound in sufficient amounts was difficult because many of the metabolites were present as only a small percentage of the dose and because it was difficult to resolve completely many of the metabolites chromatographically. NMR analysis was further hampered by the presence of taurocholic acid in some of the samples. Taurocholic acid is a major constitutive component of bile and has similar chromatographic properties and pK_a to the metabolites of interest. The presence of taurocholic acid in the NMR samples masked the protons in the aliphatic region of the spectrum. Nevertheless, for most metabolites, the aromatic protons were sufficiently distinguishable for the position of conjugation to be determined. Taurocholic acid to some extent was removed from the samples by digesting with choloylglycine hydrolase before NMR analysis, but it was not possible to remove it entirely.

The proposed biotransformation pathway for azoxystrobin is shown in figure 6. Azoxystrobin was extensively metabolized forming at least 15 metabolites, many of which only constituted a few per cent of the dose. The most pronounced metabolic pathway involved the hydrolysis of the methoxyacid and subsequent conjugation with glucuronic acid. In bile duct-cannulated rats administered 100 mg kg⁻¹ [¹⁴C]-pyrimidinyl azoxystrobin, about 30% of the dose was excreted in bile as the acid glucuronide.

Addition of glutathione to the cyanophenyl ring (Halladay *et al.* 1999, Yergey *et al.* 2001) followed by further metabolism leading to the mercapturic acid is also an important metabolic pathway. Two isomers of the glutathione conjugate were detected by HPLC-ESI-MS (metabolites VIa and b) and both isomers had the same molecular weight and identical MS-MS spectra. Similar isomers were not seen, however, for the cysteinyl-glycine, cysteine or mercapturic acid metabolites. Furthermore, there was no evidence for the existence of isomers for any of the glutathione-related metabolites from the NMR data. The glutathione, cysteinyl-glycine, cysteine and mercapturic acid metabolites individually accounted for only a small percentage of the dose. Collectively, however, they accounted for about 10% of the administered dose and therefore represented the next most major metabolic pathway. The precise position of glutathione conjugation could not be determined unequivocally from the NMR spectrum because of interference from taurocholic acid. The final structure of the glutathione, the cysteinyl-glycine and cysteine metabolites was assigned based on the NMR of the mercapturic acid where the levels of the taurocholate impurity were not significant. The mercapturic acid was detected in bile but not in urine, which would suggest that the acetylation of the cysteine conjugate occurred in the liver.

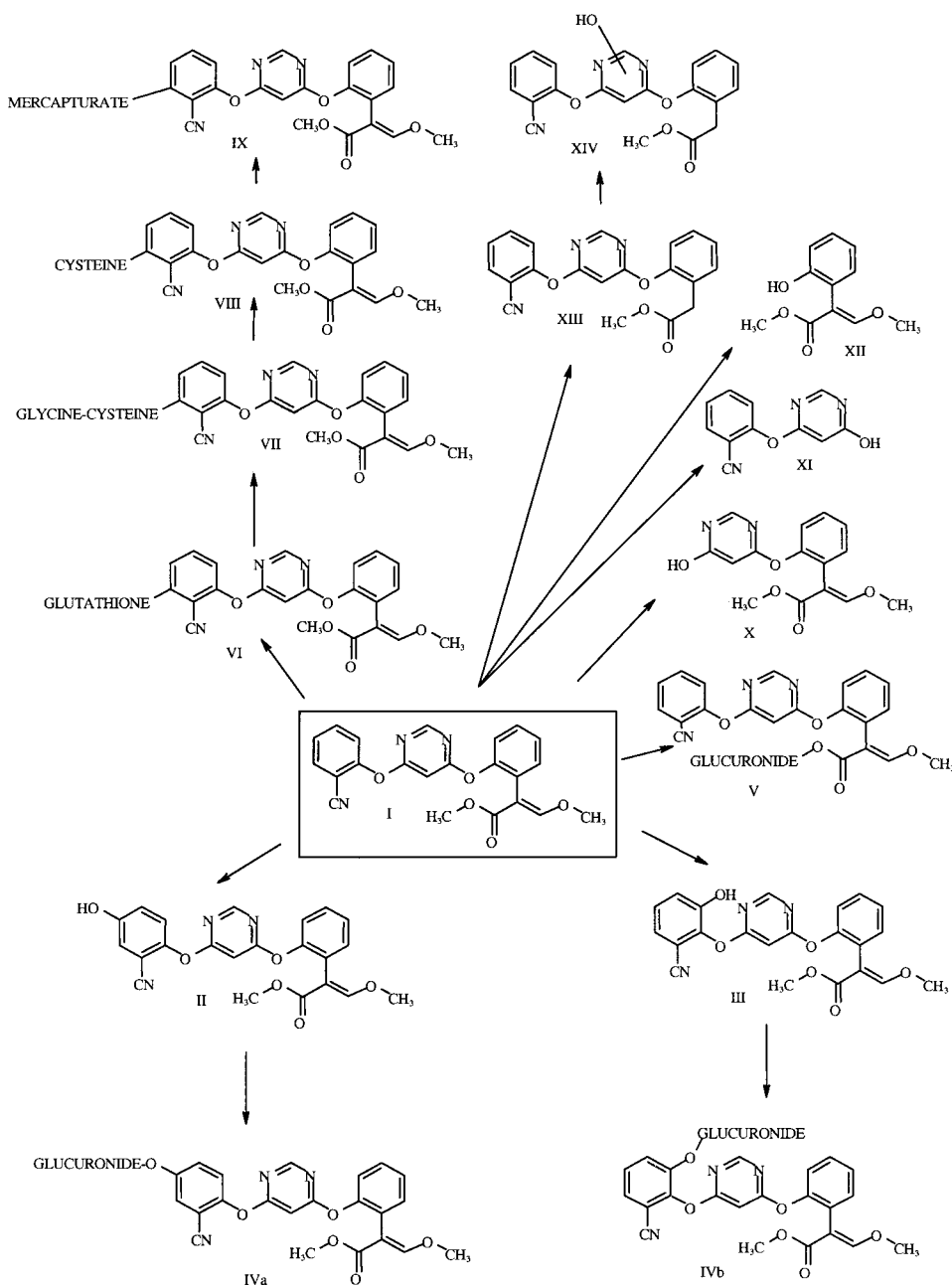


Figure 6. Metabolic pathway of azoxystrobin.

Another route of metabolism was via hydroxylation of the cyanophenyl ring at the 8- or 10-position followed by glucuronic acid conjugation. Two hydroxylated metabolites and two aryl-*O*-glucuronic acid conjugates were present in the bile and urine from rats administered 100 mg kg^{-1} [^{14}C]-pyrimidinyl azoxystrobin. Each of these aryl-*O*-glucuronides accounted for up to 7% of the dose. NMR could not

determine the position of glucuronide conjugation because of taurocholic acid contamination, but the positions of hydroxylation were unequivocally assigned.

There were further minor metabolic pathways involving the acrylate moiety resulting in the formation of the des-methoxy-methenyl-azoxystrobin (metabolite XIII) and its hydroxylated derivative (metabolite XIV). The exact position of hydroxylation was not determined. Metabolite XIV did not co-chromatograph with the hydroxy-des-methoxy-methenyl-azoxystrobin standard (Zeneca Agrochemicals reference standard 5504/24) and it had different MS-MS spectra. It was therefore thought that the hydroxylation had occurred on one of the aromatic rings. At least two possible mechanisms exist to explain the formation of metabolites VIII and XIV. The first involves oxidative cleavage of the C=C double bond followed by cleavage of the methylether moiety. An alternative mechanism involves the formation of the aldehyde via demethylation, followed by oxidation to the acid and decarboxylation.

Three metabolites arising via the cleavage of the ether linkages between the rings were identified. Hydroxy-pyrimidinyl-phenylacrylate (metabolite X) arose from the cleavage of the ether linkage between the cyanophenyl and pyrimidinyl rings. The cyanophenoxy-hydroxypyrimidinol and hydroxy-phenylacrylate metabolites (XV and XII, respectively) arose from cleavage of the ether linkage between the phenylacrylate and pyrimidinyl rings. The hydroxyphenylacrylate metabolite (XII) was not quantified because of the absence of the radiolabelled pyrimidinyl ring.

Conclusion

The absorption of azoxystrobin was dose dependent, but there was no evidence of dose-dependant metabolism. There were no significant differences between the metabolism of the three [^{14}C]-labelled forms of azoxystrobin. There was, however, a significant quantitative difference between the sexes in metabolism. Azoxystrobin was well metabolized, yielding at least 15 metabolites (figure 6). The major pathways were hydrolysis of the methoxyacid and glucuronic acid conjugation forming the acyl-*O*-glucuronide and glutathione conjugation on the cyanophenyl ring followed by further metabolism resulting in the mercapturic acid.

There were further minor metabolic pathways involving the acrylate moiety resulting in the formation of the des-methoxy-methenyl-azoxystrobin (metabolite XIII) and its hydroxylated derivative (metabolite XIV).

Acknowledgements

The authors thank Betty L Flitcroft for acquiring the HPLC-ESI-MS mass spectra from the Perkin-Elmer Sciex API III and Brian Powell for obtaining the HPLC-ESI-MS-MS spectra, both from Zeneca Specialities, Blackley, Manchester, UK. They also thank Brian Wright from Zeneca Pharmaceuticals, Alderley Park, UK, for the acquisition and interpretation of NMR spectra.

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Use of cultured precision-cut rat lung slices to study the *in vitro* induction of pulmonary cytochrome P450 forms

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Received 10 January 2003

1. The aim was to investigate the effects of some cytochrome P450 (CYP) enzyme inducers on CYP1A and CYP2B subfamily forms in cultured precision-cut rat lung slices.

2. Precision-cut lung slices were prepared from male Sprague–Dawley rats and cultured for 24 and/or 48 h in medium containing 0–20 µg ml⁻¹ Aroclor 1254 (ARO), 0–50 µM β-naphthoflavone (BNF) and 0–50 µM benzo(a)pyrene (BP).

3. Treatment with ARO, BNF and BP produced significant increases in lung slice whole homogenate 7-ethoxyresorufin *O*-deethylase activity.

4. Levels of CYP1A1 apoprotein were markedly increased in lung slice microsomes after treatment for 48 h with either 10 µg ml⁻¹ ARO or 5 µM BNF. In contrast, neither ARO nor BNF had any marked effect on levels of CYP2B1/2 apoprotein in 48-h cultured rat lung slice microsomes.

5. Real-time quantitative reverse transcription-polymerase chain reaction methodology (TaqMan®) was used to quantify lung slice CYP1A1 and CYP2B1/2 mRNA levels. Rat lung slice CYP1A1 mRNA levels were increased up to 8.3-fold after treatment for 24 h with 2 and 10 µg ml⁻¹ ARO, 0.5 and 5 µM BNF, and 20 µM BP. In contrast, treatment with 10 µg ml⁻¹ ARO produced only a small 1.6-fold increase in CYP2B1/2 mRNA levels.

6. Precision-cut lung slices are a useful model *in vitro* system for the assessment of the effects of chemicals on pulmonary CYP forms.

Introduction

In recent years, much effort has been devoted to the development of *in vitro* systems to study the metabolism and toxicity of xenobiotics. One potentially very valuable *in vitro* model system is the precision-cut tissue slice technique, which has been applied to various tissues including the liver, kidney, lung, heart and spleen (Parrish *et al.* 1995, Bach *et al.* 1996). For the lung, an agarose embedding procedure has been developed that provides a solid support for this tissue, thus permitting the preparation and culture of precision-cut lung slices (Stefaniak *et al.* 1992, Parrish *et al.* 1995). Major advantages of the tissue slice technique include the maintenance of tissue architecture, so that all cell types are present and the ready application of the technique to different tissues and species (Parrish *et al.* 1995, Bach *et al.* 1996, Lerche-Langrand and Toutain 2000).

Whereas precision-cut liver slices have been extensively used for studies of xenobiotic metabolism and toxicity, comparatively few studies have been

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performed with precision-cut lung slices (Parrish *et al.* 1995, Bach *et al.* 1996, de Kanter *et al.* 1999, 2002, Lerche-Langrand and Toutain 2000). Both mammalian liver and lung contain a variety of cytochrome P450 (CYP) forms, some of which can be induced *in vivo* by the administration of various compounds (Conney 1986, Okey 1990, Baron and Voigt 1993, Nemery and Hoet 1993, Nelson *et al.* 1996). The induction of hepatic CYP forms can be studied *in vitro* using either cultured hepatocytes or precision-cut liver slices (LeCluyse *et al.* 1996, Lake *et al.* 1998, Coecke *et al.* 1999, Lerche-Langrand and Toutain 2000). Unlike the liver, the *in vitro* induction of pulmonary CYP forms does not appear to have been extensively studied. Using Syrian hamster and human lung slices prepared with a tissue chopper (i.e. non-precision cut), Hoet *et al.* (1997) observed an induction of 7-ethoxyresorufin *O*-deethylase activity after treatment with two known CYP1A inducers. In studies with precision-cut lung slices, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced the activity of the CYP1A marker 7-ethoxyresorufin *O*-deethylase in human lung slices (Slezak *et al.* 1998) and benzo(a)pyrene (BP) to induce CYP1A1 mRNA levels in rat lung slices (Hand *et al.* 2000).

The aim of the present study was to evaluate further the usefulness of cultured precision-cut rat lung slices to study the *in vitro* induction of pulmonary CYP forms. The CYP forms studied comprised members of the CYP1A and CYP2B subfamilies. CYP1A1 induction was evaluated by determining levels of mRNA, apoprotein and enzyme activity. For CYP2B1/2 (i.e. CYP2B1 and CYP2B2), levels of mRNA and apoprotein were determined. In this study, lung slice mRNA levels were determined employing real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) technology (TaqMan[®]). The CYP form inducers studied comprised the polychlorinated biphenyl mixture Aroclor 1254 (ARO), β -naphthoflavone (BNF) and BP. Previous studies have demonstrated that while BNF and BP induce CYP1A forms in the rat, ARO induces a range of CYP forms including members of the CYP1A, CYP2B, CYP3A and other subfamilies (Conney 1986, Okey 1990).

Materials and methods

Materials

Tissue culture materials were obtained from Life Technologies Ltd (Paisley, UK), SeaPrep[™] agarose from Flowgen Instruments (Sittingbourne, UK) and benzo(a)pyrene, β -naphthoflavone, 7-ethoxyresorufin, resorufin, dicumarol, NADPH and Tris from Sigma-Aldrich Co. (Poole, UK). Aroclor 1254 was the generous gift of the Monsanto Chemical Co. (St Louis, MO, USA). Enhanced chemiluminescence reagents for Western immunoblotting and an antibody to rat CYP2B1/2 were obtained from Amersham Pharmacia Biotech UK Ltd (Little Chalfont, UK), whereas an anti-peptide antibody to rat CYP1A1 was the generous gift of Dr R. J. Edwards and Professor A. R. Boobis (Imperial College School of Medicine, London, UK). The CYP2B1/2 antibody was stated by the manufacturer not to cross react with CYP1A1, CYP3A and CYP4A1; whereas the specificity of the CYP1A1 antibody has been described by Edwards (1998). QIAGEN RNA^{later}[™], QIAGEN RNeasy[®] mini kits and QIAGEN Omniscript[™] Reverse transcriptase kits were obtained from QIAGEN Ltd (Crawley, UK) and TaqMan[®] Universal Master Mix Reagents, RNase inhibitor and random hexamer primers from Applied Biosystems (Warrington, UK). The oligonucleotide primers and probes for rat CYP1A1, CYP2B1/2 and β -actin were synthesized by Applied Biosystems.

Animals

Male Sprague-Dawley rats were obtained from Harlan Olac (Bicester, UK) and were allowed free access to R&M No. 1 laboratory animal diet (Special Diets Services, Witham, UK) and water. The animals were housed in mesh-floored cages in accommodation maintained at 22 $\pm$ 3°C with a relative humidity of 40–70% and allowed to acclimatize to these conditions for at least 7 days before use.

Rats (10–14 weeks old) were killed by exsanguination under sodium pentobarbitone anaesthesia (60 mg kg⁻¹, i.p.). The lungs were immediately excised and placed in ice-cold oxygenated (95% O₂/5% CO₂) Earle's balanced salt solution (EBSS) containing 25 mM D-glucose, 50 µg ml⁻¹ gentamicin and 2.5 µg ml⁻¹ fungizone.

Preparation of lung slices

The trachea was cannulated and the lungs instilled at 37°C with a 0.75% (w/v) agarose solution under a constant hydrostatic pressure of 20 cm (Placke and Fisher 1987, Stefaniak *et al.* 1992, Price *et al.* 1995). After allowing the agarose to gel at 4°C, tissue cylinders were prepared with an 8-mm motor-driven tissue-coring tool. From the tissue cylinders lung slices (around 500 µm thickness) were prepared in cold oxygenated EBSS containing the above additions using a Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL, USA). Tissue slice thickness was determined as described by Price *et al.* (1998).

Culture of lung slices

Lung slices were floated onto Vitron (Vitron, Inc., Tucson, AZ, USA) type C titanium roller inserts (two slices/insert) and cultured in glass vials at 37°C containing 1.7 ml culture medium employing a Vitron dynamic organ culture incubator. The culture medium consisted of Minimal Essential Medium Alpha containing 0.1 µM insulin, 0.1 µM dexamethasone, 50 µg ml⁻¹ gentamicin and 2.5 µg ml⁻¹ fungizone. Lung slice cultures were maintained at 37°C under an atmosphere of 95% air/5% CO₂. After 1 h, the medium was replaced with fresh medium and subsequently the medium was changed every 24 h. The test compounds were dissolved in dimethyl sulphoxide (DMSO) and added to the culture medium so that the final DMSO concentration was 0.4% (v/v) in all vials including the control (no test compound) cultures.

Biochemical investigations

After culture, lung slices were washed in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, homogenized (two slices in 2 ml) in this medium by sonication (Beaman *et al.* 1993) and the homogenates stored at -80°C before analysis. 7-Ethoxyresorufin *O*-deethylase activity was determined at 37°C in spectrofluorimeter cuvettes containing 2 µM substrate, 8 µM dicumarol, 250 µM NADPH, lung slice homogenate and 50 mM Tris-HCl buffer, pH 8.4, in a final volume of 2 ml. Resorufin formation was monitored with time at wavelengths of 535 nm excitation and 582 nm emission. Protein content was determined by the method of Lowry *et al.* (1951) employing bovine serum albumin as standard. Lung slice homogenates were centrifuged at 10 000 *g* av. for 15 min to obtain the post-mitochondrial supernatant fraction and subsequently at 158 000 *g* av. to separate the microsomal fraction from the cytosol. Microsomal fractions were resuspended in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, and 2 mM EDTA, and aliquots stored at -80°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lung slice microsomal fractions (7.5 µg protein/lane) and Western immunoblotting were performed as described by Lake *et al.* (1996) with a 4% (w/v) acrylamide stocking gel and a 7.5% (w/v) acrylamide resolving gel employing a mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Studies were performed with antibodies to both rat CYP1A1 and CYP2B1/2. Immunoreactive bands were detected with enhanced chemiluminescence reagents employing the manufacturer's instructions.

RNA extraction from lung slices

After culture, lung slices were washed in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, and individual slices stored in QIAGEN RNeasyTM RNA stabilization reagent. Total RNA was extracted from cultured rat lung slices using QIAGEN RNeasy[®] mini kits and eluted in 30 µl RNase-free water.

Reverse transcription

The reaction mixture (final volume of 20 µl) was prepared with final concentrations as follows: 4 units Omniscript Reverse Transcriptase, 1 × RT buffer, 10 units RNase inhibitor, 1 µM random hexamer primers, 0.5 mM each of dATP, dGTP, dCTP and dTTP, and 8 µl of the total RNA extract. The RT reaction was performed for 60 min at 42°C and then 5 min at 93°C to stop the reaction.

Design of TaqMan[®] primers and probes

Rat CYP and β-actin primers and probes (table 1) were designed using Primer ExpressTM v.1.5 software (Applied Biosystems). The RNA sequences of CYP1A1, CYP2B1/2 and β-actin were obtained from GenBank with accession numbers X00469, M37134 and NM019212. Nucleotide primers and probe sequences were checked against the NCBI BLAST database to ensure specificity for the

Table 1. TaqMan® primers and probe sequences for rat CYP1A1, CYP2B1/2 and β-actin.

Gene	Primers/probes	Location	5' → 3' sequence
CYP1A1	forward	750–772	TGAGTTTGGGGAGGTTACTGGTT
	reverse	838–818	TGAAGGCATCCAGGGAAGAGT
	probe	777–808	ATACCCAGCTGACTTCATTCCCTATCCTCCGTT
CYP2B1/2	forward	434–453	CCCAATGTTTGGTGAGGAA
	reverse	511–490	CTGTGATGCACTGGAAGAGGAA
	probe	461–482	AATCCCAGGGAGCCCCACTGGA
β-actin	forward	623–604	CGTTCAGCTGTGGTCAAGAA
	reverse	517–534	CTGCCACACGCCATCATG
	probe	596–572	CCACGCTCGGTGAGGATTTTCATCA

selected gene. This analysis confirmed that the selected primers and probe set for CYP2B1/2 would detect both CYP2B1 and CYP2B2.

Real-time quantitative PCR

Real-time quantitative PCR for rat CYP and β-actin mRNA levels was performed employing a TaqMan® 7700 sequence detector system. The reaction mixture (final volume of 25 µl) for PCR was prepared with final concentrations as follows: 1 × Universal Master Mix Reagents (Applied Biosystems; containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dATP, dGTP, dCTP, dUTP, passive reference and optimized buffer components), 5 µl of the products of the RT reaction in RNase-free water and 75–300 nM of each primer and probe for each rat CYP and β-actin mRNA assay. The PCR reaction was performed for 40 cycles: denaturation at 95°C for 15 s and anneal/extension at 60°C for 1 min. The levels of the CYP mRNAs determined were expressed as a ratio to β-actin (co-amplified in a duplex reaction), which was employed to normalize RNA loading.

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance. Comparisons between means were made using the least significant difference test.

Results

Precision-cut rat lung slices of around 500 µm thickness were prepared and cultured for 3, 24 and 48 h. Previous studies have reported that this slice thickness is optimal for lung slices (Parrish *et al.* 1995, Bach *et al.* 1996). Whole rat lung slices were used to determine mRNA levels, whereas lung slice whole homogenates and microsomal fractions were used for CYP enzyme activity and apoprotein studies, respectively.

Effect of CYP inducers on 7-ethoxyresorufin O-deethylase activity in cultured rat lung slices

Rat lung slices were cultured with 0.2–20 µg ml⁻¹ of the polychlorinated biphenyl mixture ARO for 24 and 48 h. Compared with rat lung slices cultured in control medium (DMSO only treated), ARO treatment resulted in a significant induction of 7-ethoxyresorufin O-deethylase activity (figure 1). The greatest increase in enzyme activity was observed at an ARO concentration of 10 µg ml⁻¹. Overall, there was little difference in levels of 7-ethoxyresorufin O-deethylase activity after treatment with 0.2–20 µg ml⁻¹ ARO for either 24 or 48 h. Hence, subsequent enzyme induction studies were performed using either 24- or 48-h cultured rat lung slices.

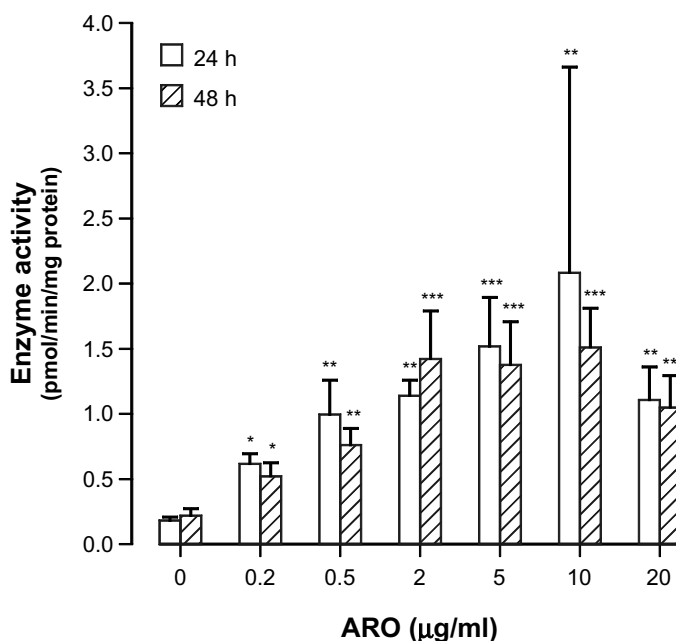


Figure 1. Effect of treatment with Aroclor 1254 (ARO) on 7-ethoxyresorufin *O*-deethylase activity in cultured rat lung slices. Precision-cut rat lung slices were cultured in medium containing 0 (control, DMSO only) and 0.2–20 $\mu\text{g ml}^{-1}$ ARO for either 24 or 48 h. Enzyme activity was determined in lung slice whole homogenates. Data are the mean $\pm$ SEM of three experiments. Values significantly different from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Rat lung slices were treated with 0–50 μM BNF for 24 h. 7-Ethoxyresorufin *O*-deethylase activity was significantly increased by treatment with 0.2–5 μM BNF, whereas no effect was observed with 20 and 50 μM BNF (figure 2). No studies were undertaken to ascertain if high concentrations of BNF were cytotoxic to cultured rat lung slices. 7-Ethoxyresorufin *O*-deethylase activity was also significantly increased by treatment of rat lung slices with 2–50 μM BP for 48 h (figure 3). The greatest increase in enzyme activity was observed with 20 μM BP, although similar levels of enzyme induction were observed in rat lung slices treated with 5 and 50 μM BP (figure 3).

Effect of CYP inducers on CYP1A1 and CYP2B1/2 apoprotein levels in cultured rat lung slices

Rat lung slices were cultured in control medium (DMSO only treated) or medium containing either 5 μM BNF or 10 $\mu\text{g ml}^{-1}$ ARO for 48 h. Microsomal fractions were prepared from 48 h control and CYP inducer treated slices and also from freshly cut rat lung slices. Western immunoblotting of rat lung slice microsomes was performed with antibodies to rat CYP1A1 and CYP2B1/2 (i.e. to CYP2B1 and CYP2B2). The specificity of the antipeptide antibody to CYP1A1 has already been described (Edwards 1998), whereas the CYP2B1/2 antibody readily detected immunoreactive CYP2B1/2 in liver microsomes from sodium phenobarbitone-treated, but not control, rats (data not shown). CYP2B1/2, but not CYP1A1, apoprotein was readily detectable in freshly cut and 48-h cultured

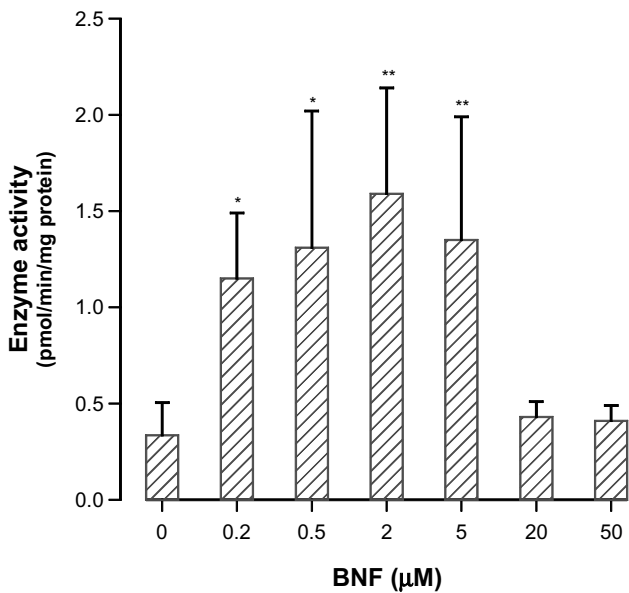


Figure 2. Effect of treatment with β -naphthoflavone (BNF) on 7-ethoxyresorufin *O*-deethylase activity in cultured rat lung slices. Precision-cut rat lung slices were cultured in medium containing 0 (control, DMSO only) and 0.2–50 μ M BNF for 24 h. Enzyme activity was determined in lung slice whole homogenates. Data are mean $\pm$ SEM of three to four experiments. Values significantly different from control: * p < 0.05; ** p < 0.01.

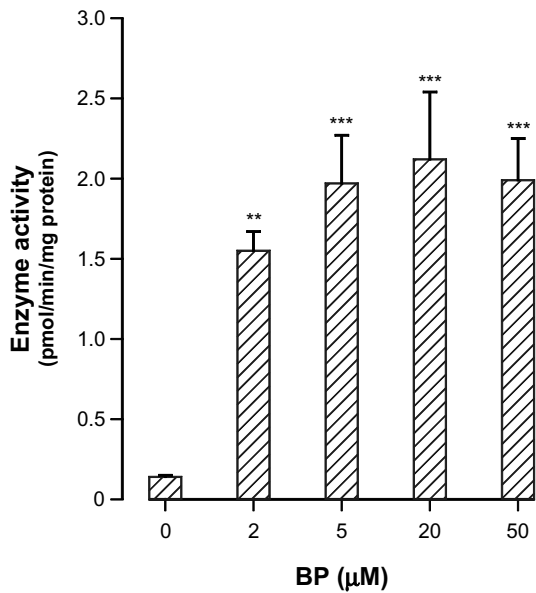


Figure 3. Effect of treatment with benzo(a)pyrene (BP) on 7-ethoxyresorufin *O*-deethylase activity in cultured rat lung slices. Precision-cut rat lung slices were cultured in medium containing 0 (control, DMSO only) and 2–50 μ M BP for 48 h. Enzyme activity was determined in lung slice whole homogenates. Data are the mean $\pm$ SEM of three to four experiments. Values significantly different from control: ** p < 0.01; *** p < 0.001.

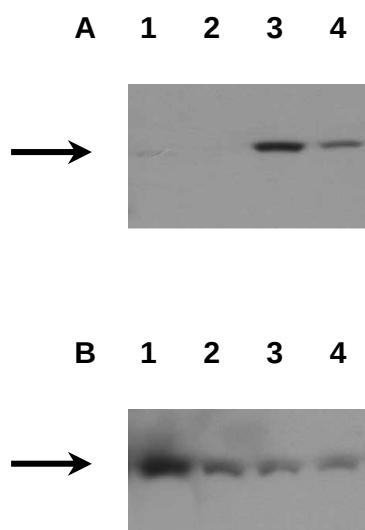


Figure 4. Effect of treatment with β -naphthoflavone (BNF) and Aroclor 1254 (ARO) on levels of CYP1A1 and CYP2B1/2 apoproteins in freshly cut and cultured rat lung slices. Immunoblots of microsomal fractions from freshly cut and 48-h cultured precision-cut rat lung slices incubated with antibodies to rat CYP1A1 (A) and CYP2B1/2 (B). Microsomal fractions ($7.5\mu\text{g protein/lane}$) were prepared from pooled freshly cut lung slices (lane 1), and from lung slices cultured for 48 h in control medium (DMSO only, lane 2) and medium containing either $5\mu\text{M}$ BNF (lane 3) or $10\mu\text{g ml}^{-1}$ ARO (lane 4).

control rat lung slice microsomes (figure 4). Compared with 48-h cultured control rat lung slice microsomes, CYP1A1 apoprotein levels were markedly induced by treatment with both BNF and ARO (figure 4A). In contrast, neither BNF nor ARO appeared to have any marked effect on levels of CYP2B1/2 apoprotein (figure 4B).

Effect of CYP inducers on CYP1A1 and CYP2B1/2 mRNA levels in cultured rat lung slices

Levels of CYP1A1 and CYP2B1/2 (i.e. both CYP2B1 and CYP2B2) mRNAs were determined by real-time quantitative RT-PCR methodology (TaqMan[®]) (table 1). To normalize for RNA loading, levels of CYP1A1 and CYP2B1/2 mRNAs were expressed as a ratio to β -actin mRNA, which was co-amplified in a duplex reaction. None of the CYP inducers examined had any significant effect on levels of β -actin mRNA in cultured precision-cut rat lung slices (data not shown).

Rat lung slices were cultured in control medium (DMSO only treated) or medium containing 2 and $10\mu\text{g ml}^{-1}$ ARO, 0.5 and $5\mu\text{M}$ BNF, and 2 and $20\mu\text{M}$ BP for 3 and 24 h. After 3 h, CYP1A1 mRNA levels were significantly increased by 2.6–2.8-fold by treatment with 0.5 and $5\mu\text{M}$ BNF and with 2 and $20\mu\text{M}$ BP (figure 5A). Levels of CYP1A1 mRNA were significantly increased in rat lung slices treated with 2 and $10\mu\text{g ml}^{-1}$ ARO, 0.5 and $5\mu\text{M}$ BNF, and $20\mu\text{M}$ BP for 24 h (figure 5B). At the highest concentrations of the three CYP inducers

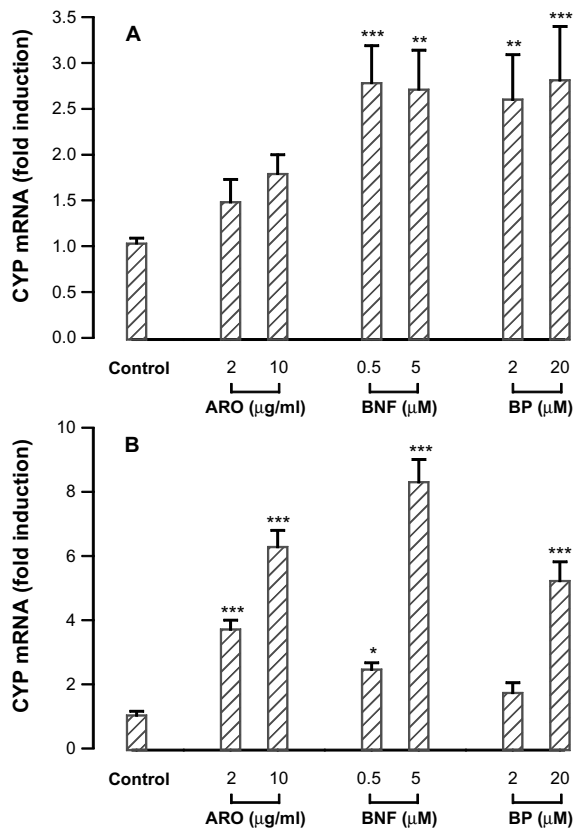


Figure 5. Effect of treatment with Aroclor 1254 (ARO), β -naphthoflavone (BNF) and benzo(a)pyrene (BP) on levels of CYP1A1 mRNA in cultured rat lung slices. Precision-cut rat lung slices were cultured in medium containing 0 (control, DMSO only), 2 and 10 $\mu\text{g ml}^{-1}$ ARO, 0.5 and 5 μM BNF, and 2 and 20 μM BP for either 3 (A) or 24 (B) h. Total RNA was extracted from whole lung slices and levels of CYP1A1 mRNA were determined by real-time quantitative RT-PCR methodology (TaqMan[®]). To normalize for RNA loading, levels of CYP1A1 mRNA were expressed as a ratio to β -actin mRNA, which was co-amplified in a duplex reaction. Data are the mean $\pm$ SEM of three experiments. In each experiment, six control and four slices for each concentration of ARO, BNF and BP were analysed. Values significantly different from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

examined, the increases in CYP1A1 mRNA levels ranged from 5.2- to 8.3-fold (figure 5B).

The effect of treatment with 2 and 10 $\mu\text{g ml}^{-1}$ ARO on CYP2B1/2 mRNA levels in 3- and 24-h cultured rat lung slices was also evaluated. After 3 h, levels of CYP2B1/2 mRNA were significantly reduced by treatment with 2 and 10 $\mu\text{g ml}^{-1}$ ARO (figure 6A). While treatment with 2 $\mu\text{g ml}^{-1}$ ARO for 24 h had no significant effect on levels of CYP2B1/2 mRNA cultured rat lung slices, a small 1.6-fold increase was observed after treatment with 10 $\mu\text{g ml}^{-1}$ ARO (figure 6B).

Discussion

The aim of the present study was to evaluate the usefulness of cultured precision-cut rat lung slices to study the induction of pulmonary CYP forms. Rodent lung contains a range of CYP forms, including members of the CYP1A,

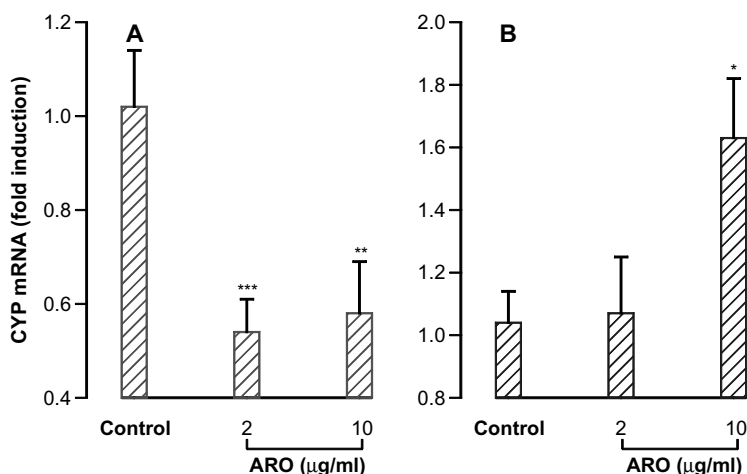


Figure 6. Effect of treatment with Aroclor 1254 (ARO) on levels of CYP2B1/2 mRNA in cultured rat lung slices. Precision-cut rat lung slices were cultured in medium containing 0 (control, DMSO only), 2 and 10 $\mu\text{g ml}^{-1}$ ARO for either 3 (A) or 24 (B) h. Total RNA was extracted from whole lung slices and levels of CYP2B1/2 mRNA were determined by quantitative real-time RT-PCR methodology (TaqMan[®]). To normalize for RNA loading, levels of CYP2B1/2 mRNA were expressed as a ratio to β -actin mRNA, which was co-amplified in a duplex reaction. Data are the mean $\pm$ SEM of three experiments. In each experiment, six control and four slices for each concentration of ARO were analysed. Values significantly different from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

CYP2A, CYP2B, CYP2E, CYP3A, CYP4A, CYP4B and other subfamilies (Arinç 1993, Baron and Voigt 1993, Nemery and Hoet 1993, Verschoyle *et al.* 1993, Debri *et al.* 1995, Lake and Lewis 1996, Lee *et al.* 1998). Pulmonary CYP forms are known not to be uniformly distributed throughout all lung cell types. For example, cell separation and immunocytochemical studies have demonstrated that non-ciliated bronchiolar epithelial cells (Clara cells) and type II alveolar epithelial cells contain relatively high levels of CYP forms (Voigt *et al.* 1990, Baron and Voigt 1993, Devereux *et al.* 1993, Nemery and Hoet 1993).

The present study focussed on two rat pulmonary CYP forms: CYP1A1 and CYP2B1/2. Whereas levels of CYP1A1 are low in control rat lung, this CYP form is markedly inducible following *in vivo* treatment with known CYP1A inducers (Godden *et al.* 1987, Keith *et al.* 1987, Voigt *et al.* 1990, Arinç 1993, Baron and Voigt 1993, Nemery and Hoet 1993, Lee *et al.* 1998). Unlike CYP1A1, CYP2B1 is a major constitutive CYP form in rat lung; whereas many reports suggest that CYP2B2 is either expressed at very low levels or is absent from rat lung (Keith *et al.* 1987, Törnquist *et al.* 1988, Imaoka *et al.* 1989, Voigt *et al.* 1990, Arinç 1993, Nemey and Hoet 1993, Nims and Lubet 1996). Hence, it is likely that the levels of CYP2B1/2 mRNA and apoprotein determined in this study primarily constitute CYP2B1 mRNA and protein.

The results clearly demonstrate that CYP1A1 is readily inducible in cultured precision-cut rat lung slices. The treatment of rat lung slices with ARO, BNF and BP for 24 and/or 48 h resulted in significant increases in 7-ethoxyresorufin *O*-deethylase activity. Whereas levels of CYP1A1 apoprotein were not detectable in either freshly cut or 48-h cultured lung slice microsomes, CYP1A1 apoprotein was readily detectable in microsomes from rat lung slices cultured for 48 h in

medium containing either ARO or BNF. In addition, real-time quantitative RT-PCR (TaqMan[®]) was employed to determine CYP1A1 mRNA levels in cultured rat lung slices. Whereas treatment with BNF and BP produced small increases in CYP1A1 mRNA levels after 3 h, treatment with ARO, BNF and BP for 24 h resulted in significant increases in CYP1A1 mRNA levels. Overall, these results are in agreement with previous *in vivo* studies and one *in vitro* study where CYP1A inducers have been shown to induce levels of CYP1A1 in rat lung (Godden *et al.* 1987, Keith *et al.* 1987, Arinç 1993, Baron and Voigt 1993, Nemery and Hoet 1993, Verschoyle *et al.* 1993, Lee *et al.* 1998, Hand *et al.* 2000).

In contrast to CYP1A1, levels of CYP2B1/2 (presumably primarily CYP2B1) apoprotein and mRNA were not markedly inducible in cultured precision-cut rat lung slices. As would be expected for a constitutively expressed CYP form, levels of CYP2B1/2 apoprotein were readily detectable in freshly cut lung slice microsomes. However, in 48-h cultured lung slice microsomes, levels of CYP2B1/2 apoprotein did not appear to be inducible by ARO or by treatment with BNF. The treatment of lung slices for 3 h with ARO resulted in a decrease in levels of CYP2B1/2 mRNA, whereas after 24 h a small increase was observed after treatment with 10 but not with 2 µg ml⁻¹ ARO. Whereas the effect of ARO treatment on lung slice CYP2B-dependent enzyme activities was not investigated in this study, the results suggest that CYP2B1/2 is not markedly inducible in cultured rat lung slices. Although further studies with additional CYP2B inducers could be performed, the present findings are in agreement with previous *in vivo* studies where CYP2B inducers have been shown to have little or no effect on levels of CYP2B1/CYP2B2 in rat lung (Godden *et al.* 1987, Keith *et al.* 1987, Imaoka *et al.* 1989, Arinç 1993, Baron and Voigt 1993, Nemery and Hoet 1993, Nims and Lubet 1996, Lee *et al.* 1998).

Whereas CYP1A1 was clearly inducible in cultured rat lung slices, in more limited studies little effect was observed on CYP2B1/2. The present studies could be extended to examine the effects of chemicals on other CYP subfamily forms present in rat lung. Another possible application of precision-cut lung slices may be to evaluate the cell-specific effects of xenobiotics by employing immunocytochemical techniques. For example, previous *in vivo* studies have demonstrated the induction of CYP1A1 and CYP3A1 in rat lung type II alveolar epithelial and/or Clara cells (Keith *et al.* 1987, Baron and Voigt 1993, Debri *et al.* 1995). Moreover, the use of this *in vitro* model system could help reduce the usage of experimental animals. At present, in order to assess the effects of xenobiotics on pulmonary CYP forms, animals are treated *in vivo* with the test chemicals. The lungs are then removed for either determination of CYP enzyme activities in subcellular fractions or for immunocytochemical procedures (Baron and Voigt 1993, Verschoyle *et al.* 1993).

In summary, precision-cut lung slices have been previously employed to study the metabolism and toxicity of xenobiotics. The results of the present study demonstrate that precision-cut lung slices may also be employed to evaluate the effects of chemicals on pulmonary CYP forms.

Acknowledgements

The authors are grateful to the UK Food Standards Agency (Contract No. T01011) and the European Union (EU BIOTECH Contract No. BI04-CT97-2145) for financial support for these studies.

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Relative receptor expression is a determinant in xenobiotic-mediated CYP3A induction in rat and human cells

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Received 6 January 2003

1. Species differences in xenobiotic-mediated transcriptional activation of *CYP3A* genes are known to exist. These differences are proposed to be due, in part, to host cell differences.

2. Host cell effects were investigated by trans-species transient transfection of reporter genes containing either the rat CYP3A23 or human CYP3A4 proximal promoters into human HepG2 and rat FaO and H4IIEC3 hepatoma cells. HepG2 and FaO cells supported activation of both CYP3A constructs by xenobiotics in a species-specific manner, whereas H4IIEC3 cells were non-permissive.

3. The mRNA complement of the cell lines was then quantified by semiquantitative RT-PCR for adult CYP3As (CYP3A23, CYP3A4/5), steroid hormone receptors (constitutive androstane receptor, glucocorticoid receptor- α , pregnane X receptor) and transcription factors (Hepatic nuclear factor 4 α , retinoid X receptor).

4. Principal component analysis of absolute receptor levels demonstrated a wide scattering, with no coherent pattern. In contrast, PCA of relative receptor ratios segregated H4IIEC3 cells from all other samples.

5. The observation is confirmed that species differences in response to xenobiotics are a result of host cell environment. In addition, new evidence is provided to support the hypothesis that in addition to individual receptor activation profiles, the relative abundance of steroid hormone receptors that control *CYP3A* gene expression play an important role in this observed species difference.

Introduction

The cytochrome P450 (CYP) enzymes comprise a superfamily of haem-thiolate proteins present in both prokaryotes and eukaryotes, and are responsible for the majority of primary oxidative metabolism of chemicals, both endogenous and xenobiotic (Nelson *et al.* 1996). One subfamily, CYP3A, is extensively involved in the metabolism of environmental chemicals and drugs, and are amongst the most abundant monooxygenases in mammalian liver and intestine. For example, CYP3A4 is the major CYP3A in adult human liver and small intestine, comprising up to 30% of the total CYP content (Watkins 1992), and is involved in the metabolism of approximately 50% of drugs in clinical usage that undergo oxidation (Cholerton *et al.* 1992). On this basis, CYP3A4 can be seen to be an important enzyme with respect to the action and duration of drugs and their metabolites in man; in rats, its orthologue is CYP3A23 (Brown *et al.* 1997).

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The regulation of the CYP3A subfamily is complex. Many compounds that are substrates for these enzymes also increase their activity, mainly by transcriptional activation (Ogg *et al.* 1999), although this may also be achieved through stabilization of the mRNA or protein products (Waxman and Azaroff 1992). Marked species differences in response to CYP3A inducers exist. For example, rifampicin is a potent inducer of *CYP3A4* expression but has no effect on *CYP3A23* gene expression *in vivo* or *in vitro* (Kocarek *et al.* 1995). As new drug entities are currently initially tested for metabolism and toxicity in rodent species, these species-dependent regulatory aspects need to be fully dissected to allow proper, informative testing to be carried out.

Barwick *et al.* (1996) investigated these differences in transcriptional activation through trans-species reporter gene assays using the 5' flanking sequences of rat CYP3A23, human CYP3A4 or rabbit CYP3A6 coupled to a CAT reporter gene. Rifampicin increased CAT activity for all the constructs transfected into rabbit but not rat primary hepatocytes. This indicated that in addition to gene promoter structure, the host cellular environment dictates the activation of *CYP3A* gene expression. Human CYP3A4 induction profiles resemble most closely those of rabbit CYP3A6, however selected inducers, including spirinolactone, cyproterone acetate and clotrimazole have been reported to induce both rat and human CYP3A but not rabbit (Kocarek *et al.* 1995).

One cause of these host cell effects may be as a result of species differences in receptor activation profiles. The pregnane X-receptor (PXR) has been identified as the major receptor responsible for transcriptional activation of CYP3A gene expression (Lehmann *et al.* 1998). In addition, transgenic humanization of mice with the human PXR confers human-like induction profiles, suggesting that the activation profile of this receptor influences species-specificity of the xenobiotic-mediated activation of *CYP3A* gene expression (Xie *et al.* 2000a). However, such a mechanism cannot be responsible for all species specificity for a number of reasons. Other cellular factors such as constitutive androstane receptor (CAR) (Sueyoshi *et al.* 1999) and GR α (Pascussi *et al.* 2000a, b) are known to influence xenobiotic-mediated transcription induction, either through competition for ligand/DNA binding, or through cooperative activation of PXR expression. In addition, binding sites distal to the PXRE have been shown to influence some induction pathways, suggesting protein-protein interactions within the *CYP3A4* proximal promoter also play a role (El-Sankary *et al.* 2002). Therefore, regulation of CYP3A genes in a species-dependent manner is likely to be due to a complex series of interactions, integrating all of these factors to create a coordinated net response to xenobiotic exposure.

To examine whether the host cell effects observed between rabbit and rat hepatocytes extend to human cells, we used our previously validated *in vitro* reporter gene assay approach (Ogg *et al.* 1999), coupled with semiquantitative RT-PCR. Reporter genes containing either *CYP3A4* or *CYP3A23* proximal promoter regions, linked to a secretory alkaline phosphatase reporter gene (SEAP), were transfected into human HepG2 and rat H4IIEC3 and FaO hepatoma cell lines and treated with known CYP3A inducers, to identify species differences in activation of *CYP3A* gene expression due to host cellular environment. To complement this analysis, the receptor complement of the above cell lines was characterized by RT-PCR to examine if differences in receptor expression between the species may contribute to the observed specificity in activation of *CYP3A* gene expression.

Materials and methods

Chemicals

Unless otherwise stated all reagents were of molecular biology grade and purchased from Sigma (Poole, UK).

Plasmids

The secretory placental alkaline phosphatase reporter gene pSEAP2-promoter (hereafter pSV40) was purchased from Clontech (Oxford, UK). The CYP3A4 reporter gene (hereafter p3A4) contained approximately 1 kb (−1087 to 57 bp) of the CYP3A4 5' flanking sequence into pSV40. The CYP3A23 reporter gene (hereafter p3A23) contained approximately 1.6 kb (−1606 to −11 bp) of the CYP3A23 5' flanking sequence into pSV40. All plasmids were grown in *Escherichia coli* strain TOP10F' (Invitrogen, Paisley, UK) and purified using QIAGEN endo-free maxi preps (Dorking, UK) according to the manufacturer's instructions.

Cell culture

All cell culture media and supplements were purchased from Gibco BRL (Invitrogen). HepG2 cells (ECACC85011430), FaO (ECACC89042701) and H4-II-EC3 (ECACC85061112) were obtained from the European Collection of Animal Cell Cultures (ECACC, CAMR, UK). Dexamethasone (Dex), rifampicin (Rif) and dimethylsulphoxide (DMSO) were of cell culture quality and purchased from Sigma. HepG2 and H4-II-EC3 cells were cultured using minimal essential media with Earle's salts supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 100 µg ml^{−1} gentamycin and 10% Australasian foetal bovine serum. FaO cells were cultured using a 1:1 mixture of nutrient mixture F10 (Ham) with L-glutamine and Dulbecco's modified eagle media supplemented with 100 µg ml^{−1} gentamycin and 5% foetal bovine serum. To maintain phenotypic consistency, cell lines were used for experiments used only up to passage 13 after receipt from ECACC.

Transient transfection

Transient transfection of the HepG2 cells was based upon the calcium phosphate precipitation methodology of Jordan *et al.* (1996) and described in detail by Ogg *et al.* (1999). Briefly, HepG2 cells in medium were seeded into 96-well plates (Nunc, Hereford, UK) at a concentration of 0.6×10^6 cells ml^{−1} (final volume 120 µl/well) and incubated for 24 h in a humidified container at 37°C in 5% CO₂. Transfection mixes were prepared on ice with a standard precipitation formation time of 1 min. Plasmid DNA, p3A4, p3A23 or pSV40 (empty vector control) were used at a standard of concentration of 12.5 µg ml^{−1} of transfection mixture (equivalent to 1.5 µg/well). Twenty-four hours after transfection, cells were exposed to the xenobiotic, as described below. H4-II-EC3 and FaO cells were transiently transfected using TransFast[®] (Promega, UK) 2.5 ng µl^{−1} plasmid DNA and a charge ratio of 3:1 were used and the manufacturer's technical manual followed.

Dexamethasone and rifampicin were dissolved in DMSO and used in final concentrations of 5, 10 and 50 µM, concentrations previously shown to encompass $I_{\max}$ and EC₅₀ for induction in HepG2 cells (El-Sankary *et al.* 2001). Xenobiotics were dissolved so that final solvent concentration was 0.1% and solutions were prepared fresh on the day of dosing. Medium was removed from wells and stored at −20°C for later assay of SEAP activity. Fresh medium was then added to the wells and xenobiotic solution and solvent controls added as required. Each experimental condition was carried out in eight separate wells. Following 48 h of exposure to xenobiotic or solvent control, the medium was removed and stored at −20°C for subsequent measurement of SEAP activity.

SEAP activity determination

Aliquots of cell culture medium (25 µl/well) were transferred into 96-well Optiplates (Canberra Packard, Pangbourne, UK). Endogenous alkaline phosphatase activity was deactivated by heat treatment of the medium at 65°C for 30 min. SEAP activity was then assayed using the AURORA chemiluminescence system (ICN, Basingstoke, UK), according to the manufacturer's protocol. Chemiluminescent output was measured using a LumiCount automated plate reader (Canberra Packard).

Data analysis of transfection data

SEAP activity was determined before xenobiotic addition and 48 h after xenobiotic addition for p3A4, p3A23 and pSV40. A specific chemical effect (SCE), related to normalized fold induction, was calculated from these values as the ratio of the relative change over time of each plasmid in the presence or absence of xenobiotic; the statistical significance of this value over solvent control was then

determined as described in Plant *et al.* (2000). $I_{\max}$ (representing the overall maximal induction produced, encompassing ligand–receptor, receptor–receptor, receptor translocation and receptor–DNA interactions caused by the inducer) and EC_{50} (concentration producing 50% of the $I_{\max}$) were calculated using non-linear regression analysis (GraphPad Prism v.2.0), and an indication of the overall effect of the xenobiotic calculated by dividing $I_{\max}$ by EC_{50} (Inductive Ability, IA).

mRNA isolation

mRNA was extracted from male 240 g Wistar rat liver and HepG2 (passage 8 from receipt), H4-II-EC3 (passage 8 from receipt) and FaO (passage 7 from receipt) cell lines using the PolyATract system 1000 (Promega), according to the manufacturer's recommendations. Integrity of the mRNA was confirmed on a 2% agarose gel and the concentration and purity determined using a GeneQuant II spectrophotometer (LKB, Sweden).

RT-PCR analysis of selected candidate genes

cDNA sequences of target genes were obtained from the NIH gene database (<http://www.ncbi.nlm.nih.gov/web/search/index.html>) and specific primer pairs designed, spanning intron–exon boundaries (Vector NTI 4.0, InforMax, Inc., Frederick, MD, USA) (table 1). As the primers for human CYP3A4 did not distinguish between CYP3A4 and CYP3A5, the derived value represents the sum of the expression of these two mRNAs.

RT-PCR was carried out in a single tube (50 µl) reaction using Ready to Go RT-PCR beads (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions using a 100 ng cell line and rat liver mRNA prepared, as described above, and 1 ng adult human liver poly A+ mRNA (Clontech). Specificity of amplification was checked via sequencing (ABI 373, Applied Biosystems, Warrington, UK) and then kinetic analysis (10–42 cycles) was performed to determine the location of the mid-exponential phase for each target and products were sequenced to ensure specific amplification of the target gene.

For the semiquantitative analysis of each target gene, RT-PCR reactions were carried out in triplicate for each sample to reduce the effect of intertube RT-reaction (Kolls *et al.* 1993). All RT-PCR products were analysed on 2% agarose gels containing $0.5 \mu\text{g ml}^{-1}$ ethidium bromide and band densities calculated using a VDS Imagemaster system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Data analysis of RT-PCR data

Principal component analysis (PCA) was also performed on both the target gene mRNA levels and on ratios of each target gene mRNA level to every other target gene mRNA level (calculated using an adapted Latin square method), using the Multivariate Statistical Package (MVSP v.3.13, Kovach Computing Services, Pentraeth, UK).

Results

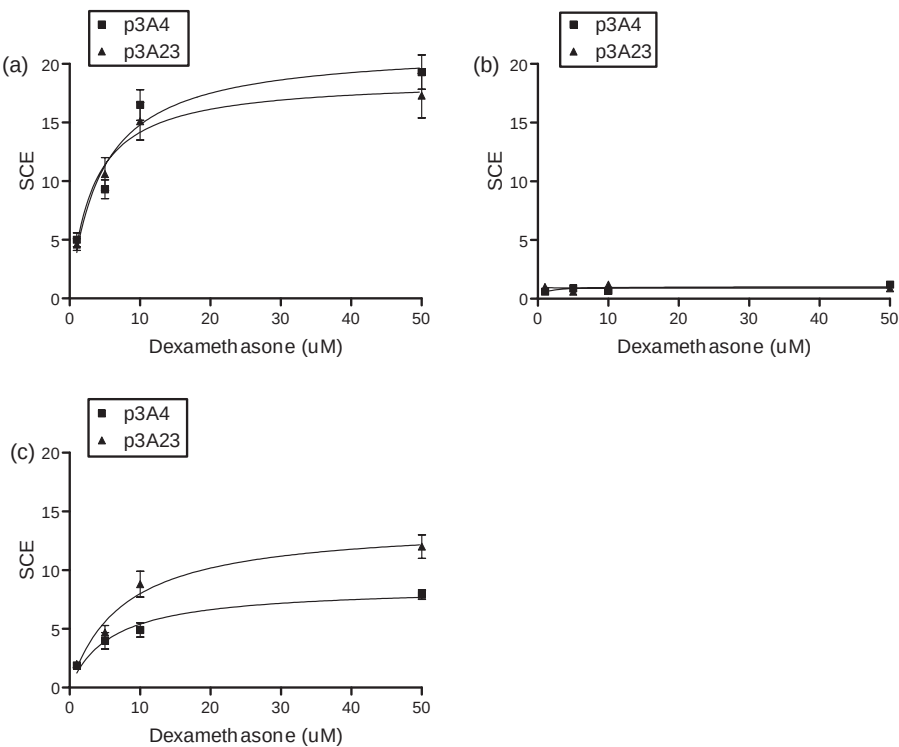
Transient transfections

The validated *in vitro* reporter gene assay was used to examine the activation of SEAP reporter gene expression under the control of either human (p3A4) or rat (p3A23) *CYP3A* proximal promoter in rat and human hepatoma cell lines by dexamethasone and rifampicin. HepG2 and FaO cells were competent for the dose-dependent xenobiotic-mediated activation of both reporter genes by dexamethasone, whereas H4IIIEC3 cells showed no inductive response with either reporter gene (figure 1). Maximal observed induction ($I_{\max}$) was higher in FaO cells for both constructs (21.4 and 18.7 for p3A4 and p3A23, respectively) compared with HepG2 cells (8.6 and 14.0 for p3A4 and p3A23, respectively), whereas the calculated EC_{50} did not alter (figure 1). As expected, rifampicin was seen as a species-specific inducer with only HepG2 cells competent to support significant rifampicin-mediated activation of reporter gene expression. Both p3A4 and p3A23 reporter genes were induced by rifampicin in HepG2 cells, and produced broadly equivalent $I_{\max}$ and EC_{50} (figure 2).

Table 1. Primers and reaction conditions for semi-quantitative RT-PCR.

Gene	Accession	Upstream Primer	Downstream Primer	Anneal (° C)	Cycles
CYP3A4/5	M18907	TGGACAGAGCCTGAGAAGTTCC	ATTCAGGCTCCACTTACGGTGC	58	24
h PXR	AF061056	CAAGCGGAAGAAAAGTGAACG	CTGGTCCTCGATGGGCAAGTC	58	30
h CAR	NM_005122	CACTTTTCATGGTACTGCAAG	GCAGTTTTCGTAGTGTTC	58	28
h GRα	M10901	GTCTGAAGAGCCAAGAGC	GTGATGACGACTCAACT	60	24
h RXRα	NM_002957	GAGAAGTCTATGCCGTCC	GCCTAAGAGATGTGTTGTCACC	58	28
h HNF4α	Z49825	GAGATGAGCCGGGTGTCCAT	TCTCCTGCAACAGGTTGTCA	60	24
h SRC-1	U90661	AGTTCTCCAGTCCTCAGG	GCTCATTTGACAGCACGGCATAAA	58	28
CYP3A23	X96721	TGGCCAGAGCCTGAGGAATTTC	TTCAGCAGAACTCCTTGAGGGA	55	16
r PXR	AF151377	GATCAAGAGGAAGAAGAGGG	ATCTGGTCCTCGATAGGCAG	55	21
r CAR	AF133095	TGCTCACACACTTTGTCAGAT	CTGGATGCGATGGATTTCAT	55	25
r GRα	M14053	AAGGTCTGAAGAGCCAAGAG	TGTTGACTCAACTGCTTCTG	55	21
r RXRα	NM_012805	CAAGCAGCAGACAAGCAGCT	GCAGTACGCTTCTAGTGATG	58	26
r HNF4α	X57133	GAGATGAGCCGGGTGTCCAT	TCTCCTGCAACAGGTTGTCA	55	22

Primer pairs used for semi quantitative RT-PCR of human (h) and rat (r) mRNA are listed, along with accession number for the sequences these primer pairs were designed to. Reaction conditions (PCR annealing temperature and PCR cycles for mid-exponential phase) are also included.

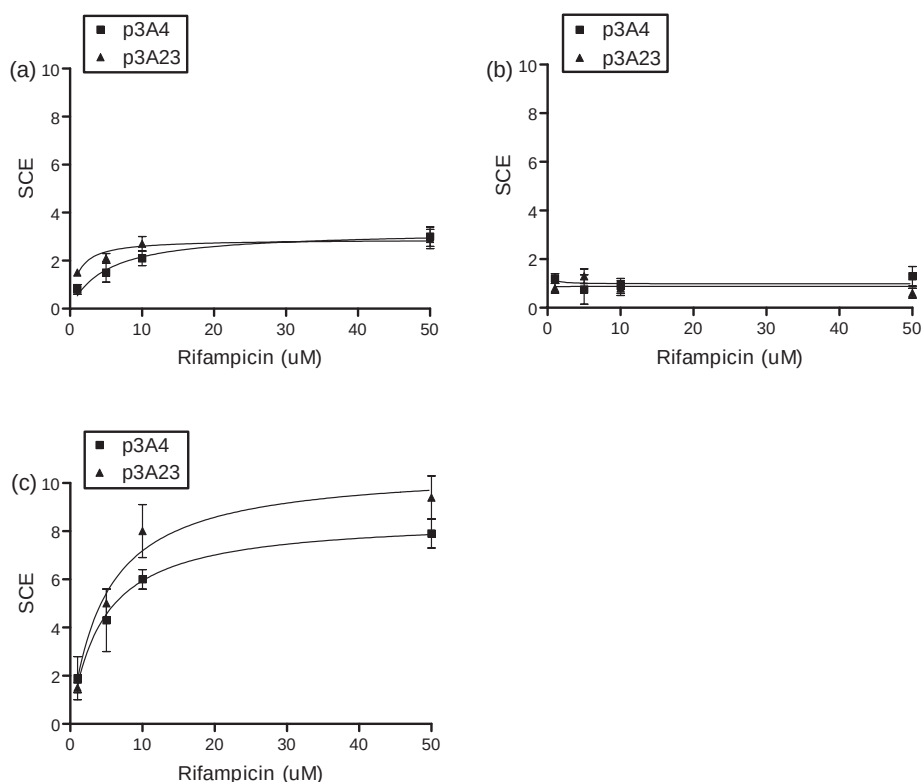


Cell line	Species	$I_{\max}$		EC_{50}		IA	
		p3A4	p3A23	p3A4	p3A23	p3A4	p3A23
FaO	rat	21.4 $\pm$ 2.6	18.7 $\pm$ 1.0	4.5 $\pm$ 1.9	3.2 $\pm$ 0.7	4.7	5.8
H4IIEC3	rat	DNF		DNF		DNF	
HepG2	human	8.6 $\pm$ 0.9	14.0 $\pm$ 1.4	5.9 $\pm$ 1.4	7.5 $\pm$ 2.3	1.4	1.9

Figure 1. Trans-species induction of CYP3A23 and CYP3A4 reporter genes by dexamethasone. HepG2, FaO or H4IIEC3 cells were transfected with p3A4, p3A23 or pSV40 reporter gene constructs as described in the Materials and methods. Cells were exposed to varying concentrations of dexamethasone and SCE, $I_{\max}$, EC_{50} and IA were calculated as also described. (a–c) Induction in HepG2, FaO and H4IIEC3 cells, respectively. Each datum point is the mean of eight separate determinations, and error bars represent compound variation. Results are representative of experiments undertaken on at least two separate occasions. DNF, did not fit; as no statistically significant induction was observed a dose–response curve may not be generated or $I_{\max}$ and EC_{50} calculated.

Cell characterization

To delineate the mechanisms underlying the aforementioned intra- and inter-species variation, the mRNA complement of the major xenosensing steroid hormone receptors (CAR, GR α and PXR) and transactivating factors (HNF4 α and RXR α) believed to regulate CYP3A expression, as well as the major adult human and rat CYP3As (CYP3A4/5 and CYP3A23, respectively) were characterized using semiquantitative RT-PCR. Figure 3 shows the mRNA levels of CYP3A4/5 and CYP3A23 in cell lines and adult human or rat liver. Both human liver and HepG2 cells express CYP3A4/5 mRNA, although levels in



Cell line	Species	$I_{\max}$		EC_{50}		IA	
		p3A4	p3A23	p3A4	p3A23	p3A4	p3A23
FaO	rat	3.2 ± 0.3	2.9 ± 0.2	5.0 ± 1.5	1.1 ± 0.4	0.6	2.6
H4IIEC3	rat	DNF		DNF		DNF	
HepG2	human	8.6 ± 0.4	10.6 ± 1.0	4.4 ± 0.7	4.8 ± 1.5	1.9	2.2

Figure 2. Trans-species induction of CYP3A23 and CYP3A4 reporter genes by rifampicin. HepG2, FaO or H4IIEC3 cells were transfected with p3A4, p3A23 or pSV40 reporter gene constructs as described in the Materials and methods. Cells were exposed to varying concentrations of dexamethasone and SCE, $I_{\max}$, EC_{50} and IA calculated as also described. (a–c) Induction in HepG2, FaO and H4IIEC3 cells, respectively. Each datum point is the mean of eight separate determinations, and error bars represent compound variation. Results are representative of experiments undertaken on at least two separate occasions. DNF, did not fit; as no statistically significant induction was observed a dose–response curve may not be generated or $I_{\max}$ and EC_{50} calculated.

human liver were approximately 143-fold higher. In contrast, both rat hepatoma cell lines contained no detectable CYP3A23 mRNA, demonstrating a fundamental divergence from the adult liver phenotype.

To examine the hypothesis that receptor complement was an important factor in determining cell line competence with respect to xenobiotic-mediated induction of CYP3A reporter genes, the expression levels of the major xenosensing steroid hormones and other factors previously implicated in control of CYP3A gene

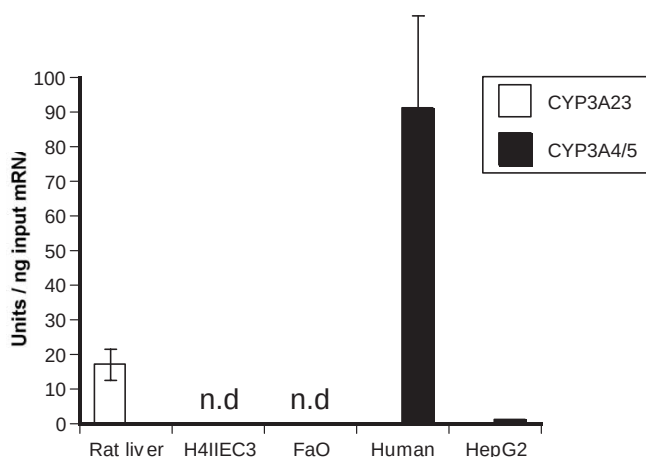


Figure 3. Expression of adult CYP3A species in hepatocyte cell lines. mRNA was extracted from HepG2, FaO, H4IIEC3 cells and rat liver as described in the Materials and methods. Levels of human CYP3A4/5 or rat CYP3A23 were measured by semiquantitative RT-PCR in these extract, plus commercial human mRNA. Signal intensity was normalized to per nanogram of input mRNA and error bars represent the standard error of the mean from three separate extractions. Statistical significance was measured using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$. n.d., Below the detection limit of the system.

expression were examined. All cellular factors examined were expressed in both human liver and HepG2 cells (figure 4). However, as was seen with expression of CYP3A4/5, levels of these factors was consistently and substantially higher in human liver compared with HepG2 (mean difference, 126-fold), with the exception of HNF4 α levels, which were low in both human liver and HepG2 cells. In comparison, rat liver expression of these factors was of the same order of magnitude as that seen in FaO and H4IIEC3 cells (figure 5).

Principal component analysis (PCA)

PCA is a technique for reducing the dimensionality of datasets for compression or recognition purposes and is increasingly used to examine large biological datasets (Hoglund *et al.* 2002). This dimensional reduction permits graphical representation of the data, allowing relationships between the components to be drawn.

PCA was used to compare the mRNA levels of the five main receptors between the cell lines and their liver counterparts and to indicate any similarities in receptor mRNA profiles. A representation of the analysis is shown in figure 5a, with little clustering being evident. To reflect the increasing evidence that there is a large degree of interdependence of xenosensing factors in controlling CYP expression, the amount of these factors *relative* to each other was also examined. Latin square tables were produced to calculate the abundance of each receptor mRNA compared with the other receptors. PCA was carried out on these relative values and the results are shown in figure 5b. Human liver, rat liver, FaO cells and HepG2 cells cluster together, whereas H4IIEC3 cells are well separated from this group.

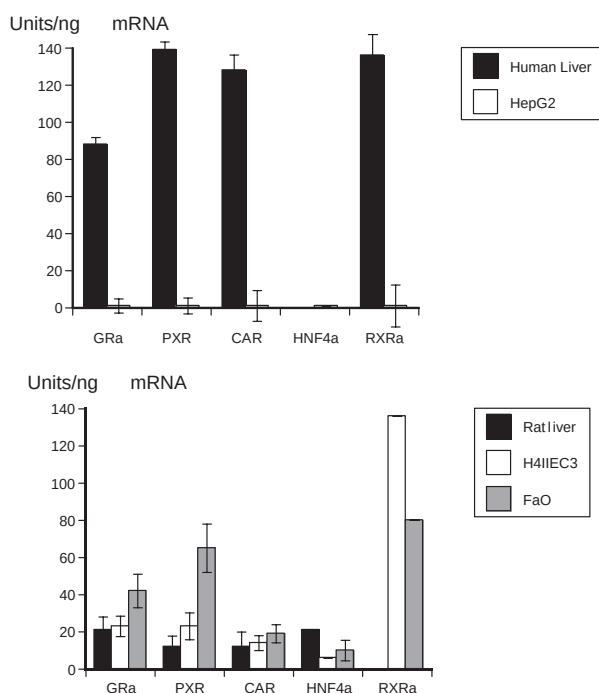


Figure 4. Expression of transcription factors in cell lines and adult livers. mRNA was extracted from HepG2 cells as described in the Materials and methods. Levels of CAR, GR α , PXR, RXR α , HNF4 and SRC-1 were measured by semiquantitative RT-PCR in this extract, plus commercial human mRNA. Signal intensity was normalized to per nanogram of input mRNA and error bars represent the standard error of the mean from three separate extractions. Statistical significance was measured using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

Discussion

With the increasing use of *in vitro* assays in early drug discovery/development, it is important to understand fully the molecular mechanisms behind the observed results. This is important both to validate these assays as accurate models of the *in vivo* response and to allow a greater understanding of observed species differences, thereby enabling confident extrapolation to the clinic. Through the use of cross-species gene transfection assays and the assessment of the receptor profiles of commonly used hepatoma cell lines, the importance of these features was examined when determining the response of these cells to xenobiotics.

In agreement with the literature, activation of CYP3A reporter genes was demonstrated to occur in a species-dependent manner, with specificity of activation being determined by the host cell (Barwick *et al.* 1996). The observations of Barwick *et al.* were also extended to show that this host-cell effect occurred in human cells as well as rat and rabbit. In making such a comparison, however, note that this work uses hepatoma cell lines whereas Barwick *et al.* used primary cell lines. Thus, the observed differences between rat and human cells may reflect individual differences between cell lines and not a species-dependent effect. However, initial experiments with a second human hepatoma cell line, HuH7, have provided results very similar to those observed in the HepG2 cells, strongly suggesting that the observed differences are indeed due to species-dependent variations. In addition, it was demonstrated that FaO cells can support a larger

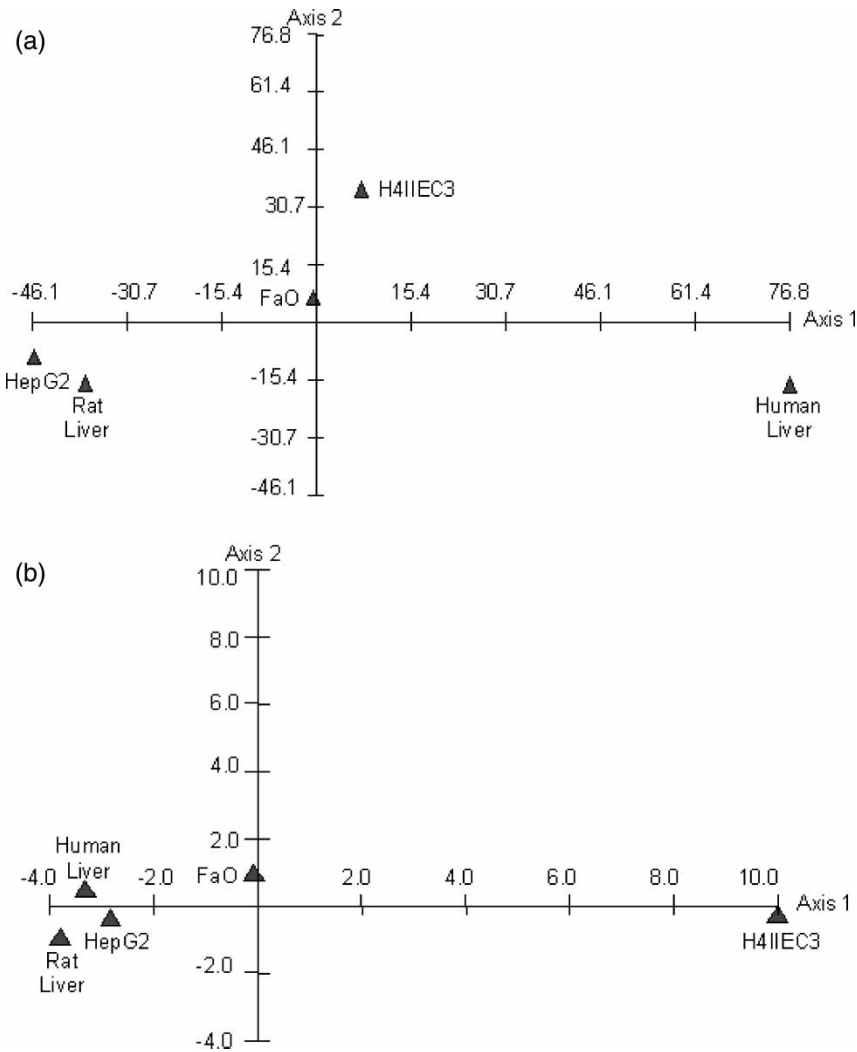


Figure 5. Principal component analysis of receptor expression in rat and human liver and HepG2, FaO and H4IIEC2 cell lines. Semiquantitative RT-PCR was used to determine the levels of CAR, GR α , PXR, RXR α and HNF4 α in HepG2, FaO and H4IIEC3 cell lines and rat and human liver as described in the Materials and methods. PCA was carried out on the absolute levels (per nanogram of input mRNA) of (a) individual factors or (b) relative expression to each factor as also described.

induction of dexamethasone-mediated CYP3A gene expression than HepG2 cells. This is in agreement with the published *in vivo* data, demonstrating that dexamethasone-mediated activation of CYP3A gene expression is more marked in rats than humans (Schuetz *et al.* 1993, Ghosal *et al.* 1996). Rifampicin, a known potent inducer of human CYP3A4 but not rat CYP3A23 *in vivo* (Wrighton *et al.* 1985), caused transcriptional activation of both CYP3A4 and CYP3A23 reporter gene constructs in HepG2 cells, while only poor activation was observed in FaO cells.

These experimental data confirm that species specificity in response to xenobiotics is due to host cell effects and not to promoter structure, as trans-species

induction may be observed, and extends these data to include human cells. The molecular mechanism underlying this difference has not been fully elucidated, but is likely to be a combination of two factors. First, differences in the activation profiles of xenobiotics for their cognate receptors in each species could result in differential activation of promoters containing similar response elements in different species. Second, the relative ratio of these transactivating factors, which act as both activators and inhibitors of *CYP3A* gene expression, may vary between species. Such variability might alter the net effect of a xenobiotic on transcriptional activation of *CYP3A* gene expression.

PXR has been identified as the major xenosensing steroid hormone responsible for induction of *CYP3A* genes (Kliewer *et al.* 1998). As stated above, one possible explanation for species-specific response to xenobiotics is differing activation profiles of PXR between species. Xie *et al.* (2000a) provided strong evidence for such a hypothesis through the humanization of xenobiotic-mediated *CYP3A* induction in transgenic mice containing the human PXR. Hence, it seems probable that at least some of the species specificity in drug response is due to the species-specific activation profiles of individual steroid hormone receptors. However, it is unlikely that a species difference in PXR activation profiles is the sole mechanism underlying the observed species differences for a number of reasons. *CYP3A* proximal promoters appear to be highly complex, with multiple binding sites for transcription factors, and an alternative scenario involved the interactions between these factors playing a role in determining the net effect of xenobiotic response. Experimental evidence exists to support such a hypothesis at two levels. First, CAR and PXR are known to compete for both ligands binding and subsequent binding to the PXRE (Xie *et al.* 2000b). Second, transcription factor-binding sites distinct from the PXRE have been shown to modulate xenobiotic-mediated activation of *CYP3A4* gene expression (El-Sankary *et al.* 2002). Indeed, Tirona *et al.* (2003) highlighted the importance of HNF4, the expression of which is measured in the present study, in regulating *CYP3A* expression. Through transgenic studies, they demonstrated that HNF4 α plays a fundamental role in *CYP3A* basal expression, and the PXR/CAR-mediated transcriptional activation caused by xenobiotics. While the *cis*-acting element identified for HNF4 α lies within the distal enhancer, and hence was not studied in the present reporter gene assays, this demonstrated interdependence of HNF4 α and PXR/CAR underlines the potential importance of relative expression levels of transcription factors in determining both basal *CYP3A* and xenobiotic-mediated transcriptional activation.

Reporter gene assays indicated that FaO, but not H4IIEC3, cells were capable of supporting transcriptional activation of *CYP3A* reporter genes. Examination of the differences between the receptor complements of these two cell lines may shed further light on the mechanisms underlying the cell type and species-specific responses observed. One possibility is that H4IIEC3 cells are so divergent from native rat liver cells that they are incapable of supporting any reporter gene experiments. However, this has been shown not to be the case as H4IIEC3 cells have been used successfully for different reporter gene assays by other groups (Beurton *et al.* 1999, Costeas and Chinsky 2000). This suggests therefore that any disruption is specific to regulation of *CYP3A* gene expression, and an obvious cause of this would be a lack of PXR. However, as can be seen in figure 4, PXR is present in FaO and H4IIEC3 cells at approximately equivalent levels. Indeed,

PCA analysis on the absolute levels of the mRNA species measured does not segregate FaO and H4IIEC3 cells. However, PCA analysis of the *relative* transcript levels quite clearly distinguishes H4IIEC3 cells from FaO and rat liver. These data would therefore suggest that xenobiotic-mediated induction of CYP3A reporter genes in H4IIEC3 cells is disrupted due an alteration in the relative levels of transcription factors, and hence the relative input each has into determining the net effect of a xenobiotic on activation of reporter gene expression. One caveat to such a statement is that such measurements are made at the mRNA level and not protein. Differing translational efficiencies between transcripts may mean that the ratios seen at the mRNA level differ to those seen at the protein level. However, while such a caveat should be borne in mind, as the analysis is comparing the same transcripts in different cell lines, it can be presumed that the translational efficiencies are the same in all cell lines. Hence, while the actual ratios may alter between the mRNA and protein level, the relevance of that ratio will not change.

While PCA demonstrates that relative receptor levels differ between cell lines and *in vivo*, it does not provide any light about the possible mechanisms underlying such differences. One plausible explanation would be differing levels of basic cellular processes (e.g. nutrient transport, metabolic capacity or xenobiotic transporter availability), which could affect the stimuli present within a cell type, and thus the receptor profile expressed.

One point of interest is the low level of expression of CYP3A in HepG2 and FaO cells, despite their competence in supporting reporter gene assays. These assays suggest that all the fundamental components required for transcriptional activation of *CYP3A* are present within the cells, and yet they do not express genomic *CYP3A* to any appreciable degree, and any expression is not inducible by xenobiotics (data not shown). In HepG2 cells, the EC_{50} calculated for rifampicin-mediated induction (approximately 5 μ M for both p3A4 and p3A23) are significantly higher than the values given for rifampicin activation of PXR (1–2 μ M), its presumed cognate receptor (Lehmann *et al.* 1998). These values, however, are similar to those seen for p3A4 induction by rifampicin in HepG2 cells (El-Sankary *et al.* 2001), with EC_{50} only approaching 1 μ M when co-transfection with expression plasmids for GR α and PXR are included in the experiment. Coupled with the low expression of PXR in HepG2 cells, this may suggest that PXR does not act as the main receptor for rifampicin-mediated CYP3A induction in HepG2 cells. A potential alternative receptor would be CAR, present in HepG2 cells at elevated levels. This hypothesis is further supported by the reported reciprocal activation of CAR and PXR in cultured primary hepatocytes, transgenic mice and reporter gene assays (Moore *et al.* 2000, Xie *et al.* 2000b). Such an alternate pathway may not be capable of supporting constitutive genomic expression of *CYP3A*. Alternatively, the chromatin structure surrounding the *CYP3A* gene clusters in these cell lines may be altered to a more repressive conformation, decreasing genomic expression of the adult CYP3As.

In conclusion, the host cellular environmental effects as first observed by Barwick *et al.* (1996) are more complex than the initial conclusions drawn. Whereas partial species specificity is determined by the activation profile of individual steroid hormone receptors such as PXR (Xie *et al.* 2000a), relative receptor expression levels are also important in determining CYP3A responsiveness to xenobiotics.

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***In vitro* activation of 7-benzoyloxyresorufin O-debenzylation and nifedipine oxidation in human liver microsomes**

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Received 8 January 2003

1. The effects of substrate concentration and enzyme source (human liver microsomes and recombinant cytochrome P450s, CYP) on the activation of 7-benzoyloxyresorufin *O*-debenzylation and nifedipine oxidation were investigated.

2. 7-Benzoyloxyresorufin *O*-debenzylase activity in human liver microsomes was inhibited by a monoclonal antibody against CYP2B6 and a polyclonal antibody against CYP3A2 by 53–69 and 19–44%, respectively, suggesting that CYP2B6 and CYP3A4 mainly catalyse 7-benzoyloxyresorufin *O*-debenzylation in human liver microsomes.

3. 7-Benzoyloxyresorufin *O*-debenzylase activity at 0.2–5 μ M substrate concentrations in human liver microsomes was increased by the addition of α -naphthoflavone, quinidine, testosterone and progesterone, and the $V_{\max}$ of 7-benzoyloxyresorufin *O*-debenzylation increased with increasing α -naphthoflavone concentrations, whereas the K_m remained constant. Additionally, 7-benzoyloxyresorufin *O*-debenzylation by recombinant CYP3A4 was increased by the addition of α -naphthoflavone, testosterone and progesterone but not by quinidine, whereas no chemicals tested could activate the *O*-debenzylation of 7-benzoyloxyresorufin by CYP2B6.

4. The K_m for nifedipine oxidation activity by CYP3A4 decreased by the addition of progesterone, whereas the $V_{\max}$ remained constant. Quinidine and testosterone increased 7-benzoyloxyresorufin *O*-debenzylase and nifedipine oxidase activities, respectively, in human liver microsomes, whereas activation was not observed in CYP3A4.

5. The results suggest that *in vitro* activation patterns are substrate dependent and that selection of the enzyme source can influence the activation phenomenon.

Introduction

Cytochrome P450s (P450 or CYP) comprise a superfamily of enzymes that catalyse the oxidation of a wide variety of xenobiotic chemicals including drugs, carcinogens and steroids (Gonzalez 1990, Guengerich 1992, Niwa *et al.* 1998, Rendic 2002). In humans, CYP3A4 is the most abundant P450 enzyme present in the liver (Shimada *et al.* 1994) and it plays a significant role in the biotransformation of more than half the drugs in use today (Smith *et al.* 1998a, Guengerich 1999).

7-Benzoyloxyresorufin is widely used as a substrate in the *in vitro* study of drug-metabolizing enzyme activity in liver microsomes from man and animals and in the human HepG2 hepatoma cell line (Doostdar *et al.* 1993, Burke *et al.* 1994, Weaver *et al.* 1999, Umehara *et al.* 2002). Ono *et al.* (1996) reported that 7-benzoyloxyresorufin was *O*-debenzylated only by CYP2B6 among nine human

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P450s studied, and Gervot *et al.* (1999) reported that 7-benzyloxyresorufin *O*-debenzylation activity by CYP2B6 was the highest among 11 human P450s studied. Therefore, 7-benzyloxyresorufin *O*-debenzylation is the most commonly used measure of specific activity for CYP2B6 in *in vitro* studies (Hanna *et al.* 2000, Katoh *et al.* 2000, Umehara *et al.* 2002). On the other hand, Waxman *et al.* (1991) reported that among 11 human P450s examined, CYP2B6, 2C8, 2D6, 3A3, 3A4 and 4A11 catalyse the *O*-debenzylation of 7-benzyloxyresorufin. Additionally, for the high-throughput assay for P450 inhibition, Crespi *et al.* (1997) developed microtitre plate-based fluorometric assays for the activities of five principal P450s, where 7-benzyloxyresorufin was used as a substrate for CYP3A4. However, recent studies have reported that 7-benzyloxyresorufin *O*-debenzylase activity is activated by several chemicals including α -naphthoflavone, budesonide and astemizole (Kenworthy *et al.* 1999, Stresser *et al.* 2000).

Nifedipine is a dihydropyridine calcium antagonist that has been widely used for the treatment of hypertension and other cardiovascular diseases. The oxidation of nifedipine to the corresponding pyridine analogue is catalysed by CYP3A4 in humans (Guengerich *et al.* 1991), and nifedipine has been widely used as an *in vitro* substrate probe for CYP3A4 activity.

Although a number of investigators have proposed mechanisms for the activation of CYP3A-catalysed oxidative metabolism, including an allosteric site model and a two-site model (Shou *et al.* 1994, Ueng *et al.* 1997, Korzekwa *et al.* 1998, Ludwig *et al.* 1999), the detailed mechanism is still unknown (Hollenberg 2002). Therefore, the current paper describes an *in vitro* investigation into 7-benzyloxyresorufin *O*-debenzylation in microsomes from cells transfected with human P450 cDNAs and human liver microsomes, and the effects of substrate concentrations and enzyme source (human liver microsomes and recombinant P450s) on the activation of oxidative metabolism, including 7-benzyloxyresorufin *O*-debenzylation and nifedipine oxidation.

Materials and methods

Materials

Resorufin, 7-benzyloxyresorufin, 7-ethoxyresorufin, quinidine hydrochloride, NADP⁺, glucose 6-phosphate (G6P), and glucose 6-phosphate dehydrogenase (G6PDH) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). α -Naphthoflavone and ketoconazole were obtained from Ultrafine Chemicals Ltd (Manchester, UK). Testosterone and progesterone were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Monoclonal antibody against CYP2B6 and antiserum inhibitory to CYP3A4 were obtained from Gentest Corp. (Woburn, MA, USA) and Daiichi Pure Chemicals Co. (Tokyo, Japan), respectively. All other reagents were of the highest purity commercially available.

Human liver microsomes and microsomal fraction specifically expressing human P450

Pooled microsomes from 12 human livers were obtained from Human Biologic, Inc. (Phoenix, AZ, USA). Microsomes from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5 and 4A11, and control microsomes from wild-type insect cells were purchased from Gentest.

Assay for 7-benzyloxyresorufin *O*-debenzylase and 7-ethoxyresorufin *O*-deethylase activities

The incubation mixture consisted of human liver microsomes (0.1 mg ml⁻¹) or microsomes from insect cells containing recombinant P450s (4–20 pmol ml⁻¹, for 7-benzyloxyresorufin *O*-debenzylase activity), 0.01–10 μ M 7-benzyloxyresorufin or 0.25 μ M 7-ethoxyresorufin, an NADPH-generating system (2 mM NADP⁺, 10 mM G6P, 5 mM MgCl₂, 1 U ml⁻¹ G6PDH), and 100 mM phosphate buffer

(pH 7.4) in a final volume of 0.5 ml. Incubation was carried out at 37°C for 10 min, and the reaction was terminated by the addition of 0.25 ml ice-cooled methanol. After the mixtures were shaken and centrifuged at 11 500 *g* for 4 min; resorufin in the supernatant was determined by HPLC. The HPLC system consisted of a Waters model 600E System Controller, Waters model 600F, Waters model 717 Autosampler, 821FPS Intelligent Spectrofluorometer (JASCO Corporation, Tokyo, Japan), Chromatopac C-R7A (Shimadzu Corporation, Kyoto, Japan), and an analytical column Inertsil ODS-3 (5 μ m, 4.6 $\times$ 150 mm; GL Science, Inc., Tokyo, Japan) equipped with a TSKguardgel ODS-80T's cartridge (3.2 $\times$ 15 mm, Tosoh Co., Tokyo, Japan). The column temperature was set at 40°C. The mobile phase consisted of 20 mM potassium phosphate buffer (pH 7.0)/methanol (50:50), and the flow rate was 1 ml min⁻¹. The fluorescence intensity was determined at an excitation wavelength of 575 nm and emission of 595 nm. Calibration curves were linear for resorufin concentrations from 1 to 100 nM. When 1, 2.5, 20 and 80 nM resorufin was added to the reaction mixture, the coefficients of variation were 4.8, 2.5, 0.5 and 0.6%, respectively.

Assay for nifedipine oxidase activity

The incubation mixture consisted of 0.05 mg ml⁻¹ human liver microsomes or microsomes from insect cells containing recombinant P450s (10 pmol ml⁻¹), 3–100 μ M nifedipine, an NADPH-generating system (2 mM NADP⁺, 10 mM G6P, 5 mM MgCl₂, 1 U ml⁻¹ G6PDH), and 100 mM phosphate buffer (pH 7.4) in a final volume of 0.5 ml. Incubation was carried out at 37°C for 10 min, and the reaction was terminated by the addition of 3 ml ethyl acetate. After 50 μ l of 2 μ g ml⁻¹ *p*-hydroxybenzoic acid isopropyl ester was added as an internal standard, the mixture was shaken for 10 min and centrifuged at 1900 *g* for 5 min. The organic phase (2.5 ml) was evaporated under nitrogen and the residue was dissolved immediately in 200 μ l HPLC mobile phase. The same HPLC system and column as described above were used except that the UV-detector was set at 254 nm. The column temperature was set at 40°C. The mobile phase was water/methanol (55:45) as eluent A and water/methanol (20:80) as eluent B. Gradient conditions were 0–14 min, 0% B; 14–14.1 min, 0–100% B (linear gradient); 14.1–22 min, 100% B; 22–22.1 min, 100–0% B (linear gradient); and 22.1–29 min, 0% B. Flow rate was held at 1 ml min⁻¹ for 14 min and increased to 1.4 ml min⁻¹ at 14.1 min. Calibration curves were linear for oxidized nifedipine concentrations from 0.1 to 10 μ M. When 0.1, 0.2, 2 and 8 μ M oxidized nifedipine were added to the reaction mixture, the coefficients of variation were 2.1, 3.0, 5.4 and 2.8%, respectively.

Assay for debrisoquine 4-hydroxylase activity

The incubation mixture consisted of 0.5 mg ml⁻¹ human liver microsomes, 100 μ M debrisoquine, an NADPH-generating system (2 mM NADP⁺, 10 mM G6P, 5 mM MgCl₂, 1 U ml⁻¹ G6PDH), and 100 mM phosphate buffer (pH 7.4) in a final volume of 0.5 ml. Incubation was carried out at 37°C for 60 min and the reaction was terminated by the addition of 70 μ l of 70% perchloric acid. After the mixtures were shaken and centrifuged at 11 500 *g* for 5 min, 4-hydroxydebrisoquine in the supernatant was determined by HPLC. The same HPLC system and column as described above were used except that the fluorescence intensity was determined at an excitation wavelength of 210 nm and emission of 295 nm. The column temperature was set at 40°C. The mobile phase was 20 mM perchlorate buffer (pH 2.5)/methanol (81:19) as eluent and 20 mM perchlorate buffer/methanol (20:80) as eluent B. Gradient conditions were 0–12 min, 0% B; 12–12.1 min, 0–100% (linear gradient); 12.1–20 min, 100% B; 20–20.1 min, 100–0% B (linear gradient); and 20.1–28 min, 0% B. Flow rate was held at 1 ml min⁻¹ for 12 min and increased to 1.4 ml min⁻¹ at 12.1 min. Calibration curves were linear for 4-hydroxydebrisoquine concentrations from 0.1 to 5 μ M. When 0.1, 0.25, 1, and 4 μ M of 4-hydroxydebrisoquine was added to the reaction mixture, the coefficients of variation were 6.1%, 3.7%, 1.0%, and 1.1%, respectively.

Immunochemical inhibition of 7-benzyloxyresorufin O-debenzylation

The antiserum inhibitory to human CYP3A4 used was the polyclonal antibody raised in rabbits against rat CYP3A2 with more than 90% inhibition of testosterone 6 β -hydroxylation catalysed by human CYP3A4, but it did not inhibit the activities of CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6 and 2E1 (Daichi Pure Chemicals). Immunochemical inhibition was examined by pre-incubation of human liver microsomes (0.05 mg protein) at room temperature for 30 min with various amounts of monoclonal antibody against CYP2B6 and antiserum inhibitory to CYP3A4, and corresponding serum (total volume of antibody or serum, 20 μ l for CYP2B6 or 30 μ l for CYP3A4). After pre-incubation, the NADPH-generating system and 7-benzyloxyresorufin (0.2 or 5 μ M) were added (final volume of 0.5 ml) and incubated at 37°C for 10 min. Resorufin formation was determined as described above.

Data analysis

In preliminary experiments, the linearity of reaction with protein concentration and incubation time was confirmed for each assay condition. All data were analysed using the average of duplicate determinations. The criterion for inhibition or stimulation was that the activity in the presence of chemicals decreased or increased more than 20% of the control activity. $V_{\max}$ and K_m for 7-benzyloxyresorufin *O*-debenzylation, 7-ethoxyresorufin *O*-deethylation, nifedipine oxidation and debrisoquine 4-hydroxylation were determined by fitting to Michaelis-Menten kinetics by non-linear regression analysis (MULTI; Yamaoka *et al.* 1981).

Results

7-Benzyloxyresorufin *O*-debenzylation by microsomes from cells transfected with human P450 cDNAs and human liver microsomes

7-Benzyloxyresorufin *O*-debenzylation activities of microsomes from cells expressing human P450s are summarized in figure 1. At 5 μM substrate concentration, 7-benzyloxyresorufin *O*-debenzylation was catalysed most efficiently by CYP1B1. CYP1A1 showed 28% of the activity by CYP1B1, and the activities by other P450s except for CYP2C9 were 0.6–9.8% of the activity by CYP1B1. The activity by CYP2C9 was negligible (less than 5 $\text{pmol min}^{-1} \text{nmol}^{-1}$ P450).

Kinetic parameters for 7-benzyloxyresorufin *O*-debenzylase activities by CYP1A1, 1B1, 2B6, 3A4 and human liver microsomes were further investigated (table 1). CYP1B1 exhibited the lowest K_m and the highest $V_{\max}$, and CYP1A1 had a twofold higher K_m and a twofold lower $V_{\max}$ than CYP1B1. Therefore, the $V_{\max}/K_m$ for CYP1B1 was four times higher than that for CYP1A1. The K_m for CYP2B6 and CYP3A4 were 99–137 times higher than for CYP1B1, and the $V_{\max}$ were nine to 12 times lower than for CYP1B1; the $V_{\max}/K_m$ for CYP2B6 and

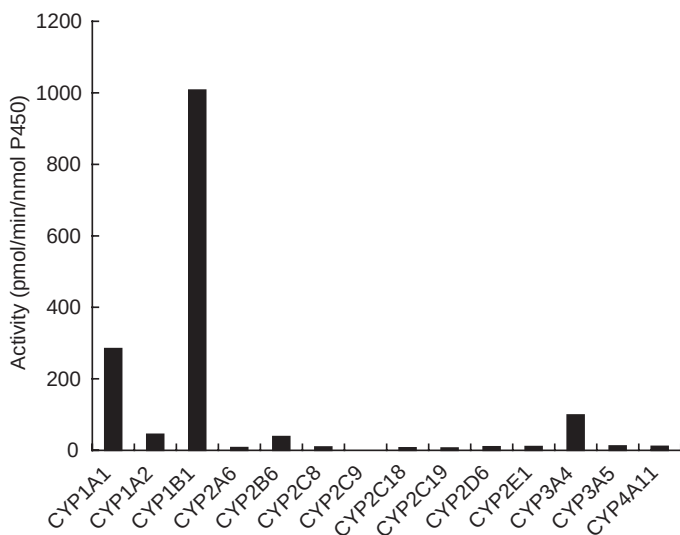


Figure 1. 7-Benzyloxyresorufin *O*-debenzylation in microsomes from cells transfected with human P450 cDNAs. 7-Benzyloxyresorufin at 5 μM substrate concentration was incubated with microsomes from cells transfected with human P450 cDNAs (20 pmol ml^{-1}) at 37°C for 10 min in the presence of an NADPH-generating system. Results are averages of duplicate determinations.

Table 1. Kinetic parameters for the O-debenzylation of 7-benzyloxyresorufin by recombinant P450s and human liver microsomes.

Enzyme source	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{nmol}^{-1}$ P450 or mg^{-1} protein*)	$V_{\max}/K_m$ ($\mu\text{l min}^{-1} \text{nmol}^{-1}$ P450 or mg^{-1} protein*)
CYP1A1	0.039	367	9299
CYP1B1	0.020	790	40 375
CYP2B6	2.74	67	24
CYP3A4	1.98	87	44
Human liver microsomes	0.67	28*	42*

*7-Benzyloxyresorufin at 0.01–10 μM substrate concentration was incubated with microsomes from cells transfected with human P450 cDNAs (4 pmol ml^{-1} for CYP1B1, 10 pmol ml^{-1} for CYP1A1 or 20 pmol ml^{-1} for CYP2B6 and CYP3A4) or human liver microsomes (0.1 $\text{mg protein ml}^{-1}$) at 37°C for 5 min (CYP1A1 and CYP1B1), 10 min (CYP2B6 and CYP3A4) or 20 min (human liver microsomes) in the presence of an NADPH-generating system. Data are derived from duplicate determinations.

CYP3A4 was 0.06–0.11% of that for CYP1B1. The K_m and $V_{\max}$ for human liver microsomes were 0.67 μM and 28 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein, respectively.

Immunochemical inhibition of 7-benzyloxyresorufin O-debenzylation

The effect of monoclonal antibody against CYP2B6 and antiserum inhibitory to CYP3A4 on 7-benzyloxyresorufin O-debenzylation activity by human liver microsomes was investigated at 0.2 μM (below the K_m for CYP2B6 and CYP3A4) and 5 μM (above the K_m for CYP2B6 and CYP3A4) as substrate concentrations (figure 2). Monoclonal antibody against CYP2B6 inhibited 7-benzyloxyresorufin O-debenzylase activity at 0.2 and 5 μM substrate concentrations by 53–56 and 60–69%, respectively. 7-Benzyloxyresorufin O-debenzylase activity at 0.2 and 5 μM substrate concentrations was inhibited by antibody inhibitory to CYP3A4 by 34–44 and 19–23%, respectively.

Effect of several chemicals on the O-debenzylation of 7-benzyloxyresorufin and nifedipine oxidation by human liver microsomes

The effect of α -naphthoflavone, testosterone, progesterone, quinidine and ketoconazole on 7-benzyloxyresorufin O-debenzylase and nifedipine oxidase activities in human liver microsomes was investigated (table 2). Additionally, the effects of the chemicals on these activities were compared with that of related activities; 7-ethoxyresorufin O-deethylase and debrisoquine 4-hydroxylase activities. The K_m for 7-ethoxyresorufin O-deethylation, nifedipine oxidation and debrisoquine 4-hydroxylation by human liver microsomes were 0.22, 12.2 and 83.9 μM , respectively (data not shown). Therefore, the effects of several chemicals on nifedipine oxidase were estimated at 10 μM (chosen to approximate the K_m) and 100 μM (the activity at this concentration is comparable with the $V_{\max}$) as substrate concentrations, and the effects on 7-ethoxyresorufin O-deethylase and debrisoquine 4-hydroxylase activities were estimated at 0.25 and 100 μM substrate concentrations, respectively (chosen to approximate the K_m of each). 7-Benzyloxyresorufin O-debenzylase activity was increased by the addition of α -naphthoflavone, quinidine, testosterone and progesterone by 2.3–2.9, 1.9–2.6, 1.8–1.9 and 3.4–4.9-fold, respectively, and the rates of activation by α -naphthoflavone were

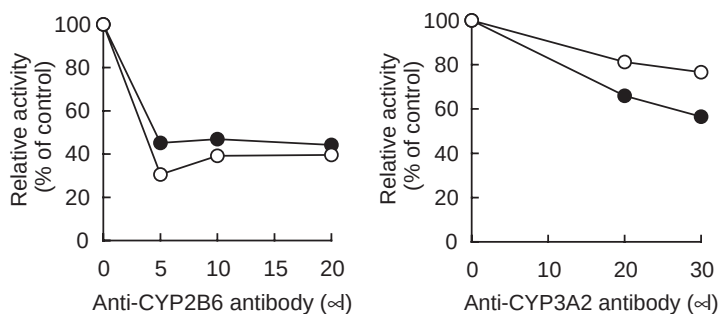


Figure 2. Effect of monoclonal antibody against CYP2B6 and antiserum inhibitory to CYP3A4 on the *O*-debenzylation of 7-benzyloxyresorufin by human liver microsomes. 7-Benzyloxyresorufin at 0.2 μ M (○) or 5 μ M (●) substrate concentration was incubated with human liver microsomes (0.1 mg protein ml⁻¹) at 37°C for 10 min in the presence of an NADPH-generating system. Results are averages of duplicate determinations.

constant at 0.2–5 μ M substrate concentrations, whereas ketoconazole, a potent inhibitor of CYP3A4/5 (Baldwin *et al.* 1995, Newton *et al.* 1995), inhibited the activity by 45–61%. Additionally, testosterone and progesterone increased nifedipine oxidase activity at 10 μ M substrate concentration by 1.4–1.5-fold, whereas the activity at 100 μ M substrate concentration was not affected. On the other hand, these chemicals on 7-ethoxyresorufin *O*-deethylase and debrisoquine 4-hydroxylase activities were without effect, except that α -naphthoflavone, a potent inhibitor of CYP1A1/2 (Bourrie *et al.* 1996), and quinidine, a potent inhibitor of CYP2D6 (Otton *et al.* 1988, Newton *et al.* 1995), inhibited 7-ethoxyresorufin *O*-deethylase and debrisoquine 4-hydroxylase activities, respectively.

The effect of α -naphthoflavone on the kinetic parameters for 7-benzyloxyresorufin *O*-debenzylation by human liver microsomes was further investigated (table 3). In the absence of α -naphthoflavone, K_m and V_{max} for 7-benzyloxyresorufin *O*-debenzylation were 0.43 μ M and 16.7 pmol min⁻¹ mg⁻¹ protein, respectively. The V_{max} of 7-benzyloxyresorufin *O*-debenzylase activity increased with increasing α -naphthoflavone concentrations, reaching a plateau at 5 μ M α -naphthoflavone, whereas the K_m remained constant.

Effect of several chemicals on 7-benzyloxyresorufin O-debenzylation and nifedipine oxidation by CYP3A4 and CYP2B6

The effects of several chemicals on 7-benzyloxyresorufin *O*-debenzylation and nifedipine oxidation activities by CYP3A4 and CYP2B6 are summarized in table 4. 7-Benzyloxyresorufin *O*-debenzylase activities at both 2 μ M (chosen to approximate the K_m) and 10 μ M substrate concentrations were increased by the addition of α -naphthoflavone, testosterone and progesterone, whereas no activation by quinidine was observed. None of the chemicals tested activated the *O*-debenzylation of 7-benzyloxyresorufin by CYP2B6. Ketoconazole (2.5 μ M) inhibited 7-benzyloxyresorufin *O*-debenzylation activities by CYP3A4 and CYP2B6 by 62 and 49%, respectively. Nifedipine oxidation activity at 10 μ M substrate concentration by CYP3A4 was slightly increased by progesterone, whereas the other chemicals did not produce any change on enzyme activity.

The effects of progesterone and testosterone on the kinetic parameters for nifedipine oxidase activity by CYP3A4 were investigated further (table 5). In the

Table 2. Effect of several compounds on metabolic activities in human liver microsomes.

Addition	Activity (% of control)						
	Substrate:	7-Benzoyloxyresorufin		7-Ethoxyresorufin	Nifedipine		Debrisoquine
Substrate concentration (μM):	0.2	2	5	0.25	10	100	100
α-Naphthoflavone (20 μM)	287	NT	227	24	NT	63	115
Testosterone (20 μM)	192	NT	180	108	143	119	105
Progesterone (10 μM)	382	491	342	115	155	113	117
Quinidine (20 μM)	243	263	186	103	64	57	<25
Ketoconazole (0.5 μM)	54	NT	42	NT	NT	NT	NT
Ketoconazole (2.5 μM)	55	NT	39	NT	NT	NT	NT

7-Benzoyloxyresorufin, 7-ethoxyresorufin, nifedipine and debrisoquine were incubated with human liver microsomes and an NADPH-generating system at 37°C in the absence or presence of several chemicals. The corresponding control activities of 7-benzoyloxyresorufin O-debenzylation at 0.2, 2 and 5 μM, 7-ethoxyresorufin O-deethylation, nifedipine oxidation at 10 and 100 μM, and debrisoquine 4-hydroxylation were 6.93, 15.41, 19.20, 14.53, 1381, 2688 and 13.21 pmol min⁻¹ mg⁻¹ protein, respectively. Data are derived from duplicate determinations. NT, not tested.

Table 3. Effect of α -naphthoflavone on 7-benzoyloxyresorufin *O*-debenzylase activity catalysed by human liver microsomes.

α -Naphthoflavone (μM)	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$)	$V_{\max}/K_m$ ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$)
0	0.43	16.7	39.4
1	0.67	33.0	49.5
5	0.33	57.2	176.2
20	0.44	50.5	114.5

7-Benzoyloxyresorufin at 0.2–5 μM substrate concentration was incubated with human liver microsomes (0.1 mg ml^{-1}) and an NADPH-generating system at 37°C for 10 min in the absence or presence of various concentration of α -naphthoflavone. Data are derived from duplicate determinations.

absence of these steroids, the K_m and $V_{\max}$ for nifedipine oxidation were 7.92 μM and 43.6 $\text{nmol min}^{-1} \text{nmol}^{-1}$ P450, respectively. The K_m for nifedipine oxidase activity decreased with the addition of progesterone, whereas the $V_{\max}$ remained unchanged. No effect of testosterone on nifedipine oxidation was observed.

Discussion

Ono *et al.* (1996) reported that 7-benzoyloxyresorufin at 15 μM substrate concentration was *O*-debenzylated only by CYP2B6 among nine human P450s examined in expressed vaccinia virus-infected HepG2 cells. Additionally, Gervot *et al.* (1999) reported that 7-benzoyloxyresorufin *O*-debenzylase activity at 1 μM substrate concentration by CYP2B6 was the highest of 11 human P450s expressed in yeast, suggesting that 7-benzoyloxyresorufin is a very good substrate for CYP2B6, with a K_m of approximately 0.2 μM . On the other hand, Waxman *et al.* (1991) reported that among 11 human P450s expressed in vaccinia virus-infected HepG2 cells, CYP2B6, 2C8, 2D6, 3A3, 3A4 and 4A11 catalysed the *O*-debenzylation of 7-benzoyloxyresorufin (10 μM substrate concentration) at low but measurable rates. The current study investigated 7-benzoyloxyresorufin *O*-debenzylation by 14 P450s in both microsomes from cells expressing human P450s and human liver microsomes (figure 1). At 5 μM substrate concentration, 7-benzoyloxyresorufin *O*-debenzylation was catalysed most efficiently by CYP1B1, followed by CYP1A1. Additionally, CYP1A1 and CYP1B1 exhibited a lower K_m and a higher $V_{\max}$ than CYP2B6 and CYP3A4 (table 1). CYP1A1 and CYP1B1 were expressed predominantly in extrahepatic organs, including lung and kidney, respectively (Smith *et al.* 1998b). Therefore, 7-benzoyloxyresorufin *O*-debenzylation at low substrate concentrations could provide a good measure of the activity of CYP1B1 and/or 1A1 *in vitro* in human extrahepatic organs.

Burke *et al.* (1994) reported that 7-benzoyloxyresorufin *O*-debenzylation in human liver microsomes was inhibited by antibody against CYP3A2 by 22–98%, indicating that this reaction is catalysed mainly by CYP3A but also involves other P450s with considerable variation in the extents of involvement of different P450s. The present study demonstrated that monoclonal antibody against CYP2B6 inhibited 7-benzoyloxyresorufin *O*-debenzylase activity by 53–69%, whereas 7-benzoyloxyresorufin *O*-debenzylase activity was inhibited by antibody inhibitory to CYP3A4 by only 19–44% (figure 2). On the other hand, 7-benzoyloxyresorufin *O*-debenzylation was catalysed most efficiently by recombinant CYP1B1 and CYP1A1 (figure 1 and table 1). Shimada *et al.* (1994) reported that the relative

Table 4. Chemical modulation of 7-benzoyloxyresorufin O-debenzylase and nifedipine oxidase activities catalysed by CYP3A4 and CYP2B6.

Addition	7-Benzoyloxyresorufin O-debenzylase activity (pmol min ⁻¹ nmol ⁻¹ P450)		Nifedipine oxidase activity (nmol min ⁻¹ nmol ⁻¹ P450)	
	CYP3A4		CYP2B6	
	2	10	2	10
Substrate concentration (μM):				
Control	45.9 (100)	96.5 (100)	29.3 (100)	21.5 (100)
α-Naphthoflavone (5 μM)	427.3 (930)	NT	NT	NT
α-Naphthoflavone (20 μM)	287.7 (627)	178.4 (185)	17.5 (60)	12.1 (57)
Testosterone (20 μM)	583.6 (1271)	390.7 (405)	22.5 (77)	25.0 (117)
Progesterone (10 μM)	1288.6 (2806)	726.4 (753)	NT	29.1 (136)
Progesterone (20 μM)	1302.8 (2837)	NT	23.6 (81)	NT
Quinidine (20 μM)	50.5 (110)	77.3 (80)	29.5 (100)	17.1 (80)
Ketoconazole (2.5 μM)	17.4 (38)	NT	14.9 (51)	NT

7-Benzoyloxyresorufin or nifedipine was incubated with microsomes from cells transfected with human P450 cDNAs (20 pmol ml⁻¹ for 7-benzoyloxyresorufin O-debenzylase activity or 10 pmol ml⁻¹ for nifedipine oxidase activity) and an NADPH-generating system at 37°C for 10 min in the absence or presence of several chemicals. Results are the averages of duplicate determinations; and values in parentheses are the percentage of control. NT, not tested.

Table 5. Effect of progesterone and testosterone on nifedipine oxidase activity catalysed by CYP3A4.

Addition	K_m (μM)	$V_{\max}$ ($\text{nmol min}^{-1} \text{nmol}^{-1}$ P450)	$V_{\max}/K_m$ ($\text{ml min}^{-1} \text{nmol}^{-1}$ P450)
Control	7.92	43.6	5.50
Progesterone (10 μM)	5.40	43.3	8.03
Testosterone (20 μM)	6.97	43.5	6.24

Nifedipine at 3–100 μM substrate concentration was incubated with recombinant CYP3A4 (10 pmol ml^{-1}) and an NADPH-generating system at 37°C for 10 min in the absence or presence of progesterone or testosterone. Data are derived from duplicate determinations.

CYP3A4/5, 2C8/9/18/19, 1A2, 2E1, and 2A6 contents in 60 human samples were 29, 18, 13, 7 and 4%, respectively, and that CYP2B6 level is only 0.2%. Additionally, CYP1B1 and CYP1A1 appear to be expressed at very low levels in human liver (Murray *et al.* 1993, Smith *et al.* 1998b). Based on the respective proportion of these P450 isoforms and their specific turnover for 7-benzyloxyresorufin *O*-debenzylation determined on recombinant P450s, as well as the results in immunochemical inhibition, CYP2B6 and CYP3A4 appear mainly to catalyse in 7-benzyloxyresorufin *O*-debenzylation in human liver.

Shou *et al.* (1994) reported that α -naphthoflavone increased the $V_{\max}$ of phenanthrene metabolism without changing the K_m , and that phenanthrene decreased the $V_{\max}$ of α -naphthoflavone metabolism without changing the K_m , suggesting that both substrates (or substrate and activator) are simultaneously present in the active site. On the other hand, Ludwig *et al.* (1999) reported that quinidine and hydroquinidine decreased the K_m and increased the $V_{\max}$ of CYP3A4-mediated meloxicam 5'-methyhydroxylation, which was consistent with a mixed-type non-essential activation, and proposed that CYP3A4 possesses at least two different substrate-binding sites. Additionally, Domanski *et al.* (2000) reported that α -naphthoflavone decreased the K_m of progesterone 6 β -hydroxylation by CYP3A4 and its F304W, L211F/D214E, and F304W/L211F/D214E mutants without changing the $V_{\max}$. In the present study, it was observed that 7-benzyloxyresorufin *O*-debenzylase activity by human liver microsomes was increased by the addition of α -naphthoflavone, quinidine, testosterone and progesterone; the rates of activation by α -naphthoflavone were constant at 0.2–5 μM substrate concentrations (table 2), and that the $V_{\max}$ of 7-benzyloxyresorufin *O*-debenzylase activity increased with increasing α -naphthoflavone concentration, whereas the K_m remained unchanged (table 3). These results strongly suggest that the chemicals, including α -naphthoflavone, quinidine, testosterone and progesterone, increased the metabolic capacity ($V_{\max}$) of 7-benzyloxyresorufin *O*-debenzylation without changing the affinity (K_m) as well as phenanthrene metabolism (Shou *et al.* 1994).

In contrast, testosterone and progesterone increased the nifedipine oxidase activity at 10 μM substrate concentration around the expected K_m in human liver microsomes by 1.4–1.5-fold, whereas the activation was not observed at 100 μM substrate concentration (table 2). Additionally, the K_m of nifedipine oxidation activity by CYP3A4 decreased by the addition of progesterone, whereas the $V_{\max}$ remained unchanged (table 5). These results suggest that progesterone increased the affinity of nifedipine oxidation without changing the capacity, and this activation pattern was different from that in 7-benzyloxyresorufin

O-debenzylation, indicating that *in vitro* activation patterns are substrate-dependent. It has been shown that the addition of progesterone and testosterone slightly increased nifedipine oxidation activity by human liver microsomes and CYP3A4 (Niwa *et al.* 1998, Wang *et al.* 2000), whereas nifedipine oxidase activity by CYP3A4 is not affected by testosterone and progesterone (Kenworthy *et al.* 1999, Stresser *et al.* 2000). Therefore, one possibility for the non-inhibitory effect of testosterone and progesterone on nifedipine oxidation is that activation by these steroids may be involved. It has been shown that the metabolic activities of some of the CYP3A4 substrates are not competitively inhibited by the addition of other CYP3A4 substrates (Kenworthy *et al.* 1999, Stresser *et al.* 2000), and several laboratories have proposed that CYP3A4 possesses at least two different substrate-binding sites (Korzekwa *et al.* 1998, Ludwig *et al.* 1999). Further detailed investigations are required to confirm individual substrate/effector pairs whether no inhibition was observed due to the slight activation or to more than two distinct substrate-binding sites.

In this study, the stimulation of nifedipine oxidation by testosterone was observed only in human liver microsomes, but not in CYP3A4 (tables 2 and 4). Quinidine also increased 7-benzoyloxyresorufin O-debenzylase activity in human liver microsomes by 1.9–2.6-fold (table 2), whereas the activities by recombinant CYP2B6 and CYP3A4 were not activated by quinidine (table 4). Kenworthy *et al.* (1999) and Stresser *et al.* (2000) reported that 7-benzoyloxyresorufin O-debenzylation, as well as nifedipine oxidation and testosterone 6 β -hydroxylation, by recombinant CYP3A4 was inhibited by the addition of quinidine at 10–30 μ M concentrations. On the other hand, meloxicam 5'-methylhydroxylation by human liver microsomes and recombinant CYP3A4 is activated by quinidine and hydroquinidine, although the activation is not observed in nifedipine oxidation and testosterone 6 β -hydroxylation by recombinant CYP3A4 (Ludwig *et al.* 1999). Additionally, diclofenac 5'-hydroxylation by human liver microsomes and recombinant CYP3A4 is activated by quinidine (Ngui *et al.* 2000). Therefore, these results suggest that the selection of enzyme source (liver microsomes or recombinant P450) as well as the substrate can affect the activation. Emoto *et al.* (2001) reported that nifedipine oxidase activity was increased by the addition of α -naphthoflavone to human liver microsomes, whereas few effects of α -naphthoflavone were observed in human intestinal microsomes, suggesting that CYP3A enzymes in liver and intestine might have different characteristics. The mechanism for the effect of the enzyme sources is still unknown.

We demonstrated the stimulation of nifedipine oxidation by testosterone and progesterone *in vitro*. Lasker *et al.* (1984) reported flavone-stimulated zoxazolamine metabolism both *in vitro* and *in vivo* when untreated or phenobarbital-treated rats were used. Additionally, Tang *et al.* (1999) reported that the decrease in plasma concentrations of diclofenac after combined doses of diclofenac and quinidine in monkey are taken to reflect increased hepatic clearance of the drug, presumably resulting from the stimulation of CYP3A-catalysed oxidative metabolism. Importantly, activation has also been shown to occur *in vivo*, at least in rat and monkey. Whether a similar drug–drug interaction occurs in humans remains to be determined.

In conclusion, it has been demonstrated that *in vitro* activation patterns are substrate-dependent, and that the selection of enzyme source (liver microsomes or recombinant P450) can affect the activation.

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Dexamethasone decreases plasma levels of the prochiral fenbendazole and its chiral and achiral metabolites in sheep

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Received 16 September 2002

1. The effect of co-administration of either short- or long-acting formulations of DXM on hepatic function and the plasma pharmacokinetic behaviour of prochiral fenbendazole (FBZ) and its metabolites was evaluated in sheep.

2. Neither DXM treatment markedly affected any of the biochemical markers of hepatic function tested. In contrast, both formulations significantly modified the plasma pharmacokinetic behaviour of FBZ and its metabolites.

3. Plasma FBZ concentrations and the associated area under the time–concentration curves were significantly lower, although the plasma detection period was longer (72 versus 48 h) in the DXM pretreated animals compared with those given FBZ alone.

4. DXM also appeared to alter the pattern of FBZ absorption, possibly through effects on abomasal pH. The shape of the plasma concentration–time curves for oxfendazole (OFZ) and fenbendazole sulphone (FBZSO₂) were similar to FBZ, raising the possibility that DXM treatment may have altered the liver biotransformation of the parent drug.

5. The concentrations of the (+) chiral metabolite of OFZ were significantly lower in DXM pretreated animals compared with those given FBZ alone. The trend was similar for the (–) antipode, although the differences between DXM pretreated and non-pretreated animals were not statistically significant.

Introduction

Benzimidazole (BZD) compounds are extensively used in veterinary and human medicine in the chemotherapy of many parasite diseases. Optimization of the use of BZDs in domestic animals, as well as in man, is pivotal for reducing the selection of resistance and maintaining efficacy against parasites. Over recent years, several investigators have attempted to evaluate a variety of factors that could potentially enhance the bioavailability of BZD molecules at sites of parasite infection. The increasing prevalence of anthelmintic resistance globally is recognized and, since there are few immediate prospects of therapeutic alternatives, strategies for maintaining the efficacy of existing anthelmintic families and containing the spread of resistance are urgently needed.

For the BZDs, one approach has been to characterize the pharmacokinetic (PK) behaviour of the parent compounds and their metabolites (figure 1), and to examine ways of modifying that behaviour. To this end, using albendazole (ABZ)

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or fenbendazole (FBZ) and their metabolites as representative BZD molecules, it has been demonstrated that in both ruminants and monogastrics, their absorption and distribution can be modified by either food withdrawal or dietary manipulation (McKellar *et al.* 1993, Hennessy *et al.* 1995, Sánchez *et al.* 1997, 1999, 2000). Moreover, it has also been shown that the metabolism of some BZD compounds is modified in undernourished cattle (Sánchez *et al.* 1996) and can also be altered in ruminants and horses by the co-administration of metabolic inhibitors such as methimazole (Lanusse *et al.* 1993) and piperonyl butoxide (Benchaoui and McKellar 1996, Gokbulut *et al.* 2002, Sánchez *et al.* 2002).

In human medicine, ABZ is considered by many as the drug of choice for neurocysticercosis and it is frequently used in combination with dexamethasone (DXM). DXM is required primarily to treat the frequently severe inflammatory reaction resulting from cysticercal death. Interestingly, however, DXM treatment also results in increased plasma ABZ-sulphoxide concentrations (Jung *et al.* 1990, Takayanagui *et al.* 1997, Sotelo and Jung 1998). The reason(s) for this is unclear since, in rats, DXM induces cytochrome P4503A (Monostory and Vereczkey 1994, Lin *et al.* 1999) but, in the same species, sulphonation of ABZ-sulphoxide depends on P4501A (Souhaili-El Amri *et al.* 1988). Other studies carried out in cattle suggest that DXM, either alone or following starvation for 48 h, resulted in increased serum haptoglobin concentrations, which is a marker for hepatic lipidosis (Yoshino *et al.* 1993). More recently, raised serum concentrations of β -hydroxybutyrate and free fatty acids have been correlated with a decreased rate of biotransformation of ABZ, and delayed elimination of the parent drug and its metabolites in undernourished cattle (Sánchez *et al.* 1996).

In an effort to confirm and extend the previous observations, the present study compared and contrasted the effects of co-administration of two formulations of DXM, with different longevities of action, on hepatic function (using a panel of plasma biochemical markers) and plasma PK behaviour of FBZ and its chiral (racemic oxfendazole, OFZ and its enantiomers (–)OFZ1 and (+)OFZ2) and non-chiral (FBZ sulphone, FBZSO₂) metabolites (figure 1) in sheep.

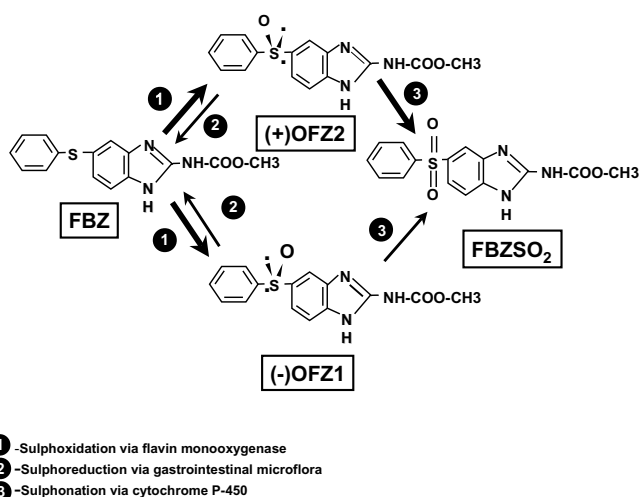


Figure 1. Proposed structural and metabolic relationships between fenbendazole (FBZ), oxfendazole enantiomers ((–)OFZ1 and (+) OFZ2) and fenbendazole sulphone (FBZSO₂).

Materials and methods

Experimental design

Experimental animals and treatment. Twelve 6–8-month-old, parasite-free, female Dorset sheep (30–40 kg) were used in a two-phase crossover experiment, with a 6-week interval between phases. Animals were housed in individual pens within an open span barn and fed standard maintenance concentrate and hay rations. Water was available *ad libitum*. Treatments were as follows:

- Phase I: groups ($n = 2$ for all groups) A and B: 5 mg kg⁻¹ body weight FBZ (Panacur[®] 5% suspension, Hoechst, Roussel Vet, Milton Keynes, Berks, UK) orally; C and D: single intramuscular (i.m.) injection of a short-acting DXM solution (0.2 mg kg⁻¹ body weight Azium[®], Schering Plough Animal Health Ltd, Welwyn Garden City, Herts, UK), then 5 mg kg⁻¹ body weight FBZ orally; E and F: single intramuscular injection of a long-acting DXM suspension (0.2 mg kg⁻¹ body weight Voren[®], Boehringer Ingelheim Ltd, Bracknell, Berks, UK), and immediately 5 mg kg⁻¹ body weight FBZ orally.
- Phase II: groups C and E: oral FBZ alone; A and F: i.m. DXM solution, then oral FBZ; B and D: i.m. DXM suspension, then oral FBZ. All dosages were as for Phase I. Heparinized whole blood was taken by jugular venepuncture for PK analysis at time 0 (pretreatment blank sample) and 15, 30, 45 min, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, 72, 96 and 120 h post-treatment. Subsamples taken at 0, 4, 8, 12, 24, 48 and 72 h post-treatment were analysed for β -hydroxybutyrate (β -HOB), L-aspartate aminotransferase (AST), γ -glutamyltransferase (γ -GT), L-glutamic dehydrogenase (GLDH) and non-esterified fatty acids (NEFA), as indices of hepatic dysfunction. Plasma was stored frozen in capped plastic vials at approximately -20°C until HPLC analysis. All animal procedures and management protocols were approved by the Moredun Research Institute Experiments and Ethics Committee and carried out under approved British Home Office licences in accordance with the Animals (Scientific Procedures) Act 1986.

Analytical procedures

Plasma drug analysis. FBZ, oxfendazole (OFZ) and FBZ-sulphone (FBZSO₂) were isolated from plasma by solid-phase extraction using C₁₈ (Bond Elut[®], Varian UK Ltd, Walton-on-Thames, Surrey, UK) cartridges. Achiral analysis was carried out using a 150 $\times$ 4.6 mm, C₁₈ column (Nemesis[®], Phenomenex Ltd, Macclesfield, Cheshire, UK) and UV detection at 296 nm as described by Sánchez *et al.* (2002). Authentic standards (FBZ, batch# D383; OFZ, batch# A 011; FBZSO₂ batch# MR 12972), all of >99.5% purity, for the method validation were obtained from Hoechst, AG (Frankfurt, Germany): the internal standard, oxibendazole (OXB), was purchased from Sigma UK Ltd, Poole, Dorset, UK (cat No. O3132). Retention times of the authentic standards were approximately 3.1, 3.8, 5.6 and 7.5 min, respectively, for OXB, OFZ, FBZSO₂ and FBZ. Calibration curves for normal ovine plasma, fortified with authentic standards, were constructed over a concentration range of 0.025–1.0 $\mu\text{g ml}^{-1}$ for the test analytes FBZ, OFZ and FBZSO₂ and a fixed concentration (1.0 $\mu\text{g ml}^{-1}$) of OXB internal standard. Regression analysis of concentration versus mean ($n = 3$) test analyte:internal standard peak area ratios showed good linearity with Pearson correlation coefficients >0.999 for all three test analytes. At the lowest concentration tested (0.025 $\mu\text{g ml}^{-1}$), the accuracy – expressed as the mean, $n = 3$, percentage relative recovery where the latter is defined as: (test analyte:internal standard peak area ratio in fortified plasma)/(test analyte:internal standard peak area ratio of authentic standards) $\times 100$ – was 95.5, 98.9 and 95.6%, respectively, for FBZ, OFZ and FBZSO₂ with an associated precision (expressed as the percentage coefficient of variation, CV) of 3.8, 2.7 and 5.2%, respectively. These accuracy and intraday precisions are similar to the relative recoveries and intra- and interassay variations obtained at higher concentrations (0.25–1.0 $\mu\text{g ml}^{-1}$) of the test analytes. CVs for the latter ranged from 1.2 to 8.5% and mean relative recoveries were 95.6, 91.4 and 97.6%, respectively, for FBZ, OFZ and FBZSO₂. Mean ($n = 3$) absolute peak areas (CVs) at 0.025 $\mu\text{g ml}^{-1}$ were 3523 (7.3%), 4090 (6.6%) and 2393 (4.2%) compared with mean ($n = 6$) blank background values (signal:noise ratios) of 111 (31.7), 519 (7.9) and 173 (13.8), respectively, for FBZ, OFZ and FBZSO₂. On the basis of these results, which fall well within the minimum requirements (precision: 20%; accuracy: 80–120%; signal:noise ratio >5) recommended by the US Food and Drug Administration (FDA) (see Guidance for Industry – Bioanalytical Method Validation, May 2001), the limit of quantitation (LOQ) was nominally set at 0.025 $\mu\text{g ml}^{-1}$ for each of the three test analytes. The limit of detection (LOD) was calculated, according to guidelines (see CVMP/VICH/591/98 – FINAL) from the EMEA (European Agency for the Evaluation of Medicinal Products), as $3.3\sigma/S$, where σ is the residual standard deviation of the regression line and S is the slope of the calibration curve. This resulted in estimated LODs of 0.016, 0.014 and 0.012 $\mu\text{g ml}^{-1}$ for FBZ, OFZ and FBZSO₂, respectively. For clarity of presentation, experimental values between LOD and LOQ are plotted in figures 1–3, but values below the assigned LOQ were not included in the pharmacokinetic analysis.

Chiral analysis of OFZ. Physicochemical extraction of the chiral analytes was identical to that detailed above for the achiral analysis, except that concentrated extracts were reconstituted in water/acetonitrile

(96.5:3.5). Percentages of the peak areas of OFZ enantiomers [(-)OFZ1 and (+)OFZ2] were estimated using a 100×4.0 mm, 5μ , α -glycoprotein analytical column (Chiral-AGP, Chrom-Tech Ltd, Congleton, Cheshire, UK) in an isocratic HPLC system (Waters® 625 pump/controller; Waters® 717 plus autosampler, Waters UK Ltd, Elstree, Herts, UK) using water/acetonitrile, 93:7, as mobile phase. (-)OFZ1 and (+)OFZ2 were detected by UV (Waters® 486 UV detector) at 296 nm. Retention times were approximately 6.1 and 7.8 min, respectively, for the (-)OFZ1 and (+)OFZ2 enantiomers. LOQs for the chiral metabolites were established at $0.025 \mu\text{g ml}^{-1}$, and LODs estimated as 0.015 g ml^{-1} for each enantiomer based on signal:baseline noise ratios.

Biochemical studies. Biochemical analyses were performed on an automated microcentrifugal analyser (Instrumentation Laboratory (IL) Monarch 2000, Instrumentation Laboratory, Warrington, Cheshire, UK) using commercial kit reagents (all from Randox Laboratories Ltd, Crumlin, Co. Antrim, UK) as follows: β -HOB (Ranbut®, Cat. No. RB1007), AST (AS407), γ -GT (GT433), GLDH (GL441) and NEFA (FA115).

PK analysis. PK analysis of plasma concentration–time data for FBZ and its metabolites was performed using the computer software package PK Solution® 2.0 (Summit Research Services, Ashland, OH, USA). The concentration–time curves for FBZ parent drug and metabolites in plasma were fitted by a bi-exponential curve (FBZ: $r^2 = \text{about } 0.99$, OFZ and FBZSO₂ $r^2 = 0.98\text{--}0.99$) supported by the following equation (Gibaldi and Perrier 1982):

$$C_p = Be^{\beta t} + Be^{-Kt}.$$

The oral plasma clearance (total clearance, CL) confounded with the dose fraction (F) absorbed of the FBZ in plasma (CL/F) was calculated using the conventional equation described by Gibaldi and Perrier 1982:

$$CL/F = \text{dose/area under the concentration–time curve (AUC)}.$$

The maximum concentrations ($C_{\max}$) and time to $C_{\max}$ ($T_{\max}$) for FBZ and its metabolites obtained in plasma following intravenous administration of OFZ were derived from the plotted concentration–time curve of each animal. Trapezoidal AUCs were calculated according to Gibaldi and Perrier (1982). Mean residence times (MRT) were calculated as $\text{MRT} = \text{AUMC}/\text{AUC}$, where AUC is the area under the concentration–time curve from 0 to infinity, and AUMC is the area under the first moment drug concentration–time curve.

Statistical analysis

Mean plasma PK variables for FBZ obtained for all groups were statistically compared by non-parametric analysis using the Friedman's repeated measures test. Means were considered significantly different at $p < 0.05$. All PK variables are mean $\pm$ SD. A Student's t -test was used for statistical comparison of enantiomeric antipode ((+)OFZ1/(-)OFZ2) concentrations.

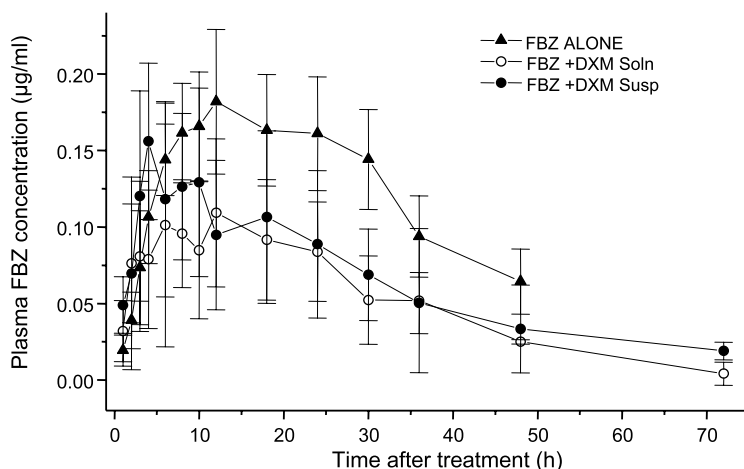


Figure 2. Comparative plasma concentrations of fenbendazole (FBZ) in sheep ($n = 8$) given FBZ (5 mg kg^{-1} body weight, orally) either alone or co-administered with dexamethasone (DXM; 0.2 mg kg^{-1} body weight, intramuscularly) formulated either as a solution (DXM Soln) or suspension (DXM Susp).

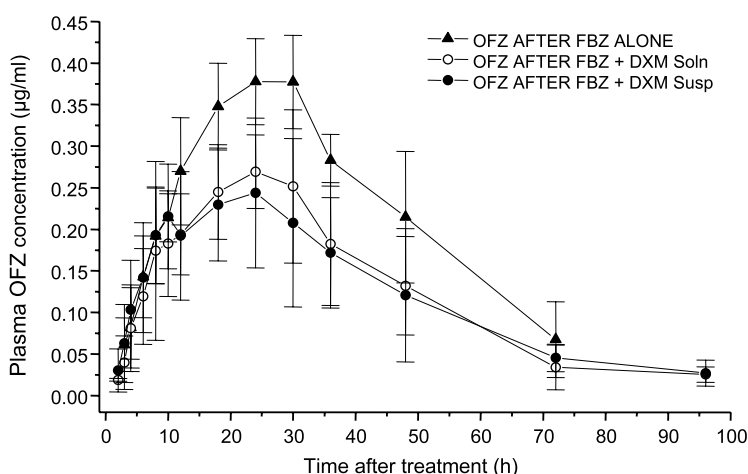


Figure 3. Comparative plasma concentrations of racemic oxfendazole (OFZ) in sheep ($n = 8$) given fenbendazole (FBZ; 5 mg kg^{-1} body weight, orally) either alone or co-administered with dexamethasone (DXM; 0.2 mg kg^{-1} body weight, intramuscularly) formulated either as a solution (DXM Soln) or suspension (DXM Susp).

Results

Clinical biochemistry

There was no indication that either of the DXM treatments, in either phase of the study, consistently affected any of the biochemical markers (data not shown). All values, at all sampling points, for each of the parameters were within the laboratory-defined reference ranges for young sheep (i.e. $\text{AST} < 100 \text{ IU l}^{-1}$; $\text{GLDH} < 10 \text{ IU l}^{-1}$; $\gamma\text{GT} < 44 \text{ IU l}^{-1}$ [all at 30°C : $\beta\text{-HOB} < 1 \text{ mmol l}^{-1}$; $\text{NEFA} < 1 \text{ mmol l}^{-1}$).

Achiral PK analysis

Both formulations of DXM significantly modified the plasma PK behaviour of FBZ and its OFZ metabolite (tables 1 and 2). Plasma FBZ concentrations and

Table 1. Comparative plasma pharmacokinetic (PK) parameters $\pm$ SD ($n = 8$) for fenbendazole (FBZ) in sheep given FBZ (5 mg kg^{-1} body weight orally) alone or in combination with dexamethasone (DXM; 0.2 mg kg^{-1} body weight intramuscularly) formulated either as a short-acting solution (DXM Soln) or long-acting suspension (DXM Susp).

PK parameter	FBZ alone	FBZ + DXM Soln	FBZ + DXM Susp
$T_{1/2ab}$ (h)	3.47 ± 1.09	3.97 ± 2.13	5.06 ± 4.52
$C_{\max}$ ($\mu\text{g ml}^{-1}$)	0.20 ± 0.01	0.14 ± 0.05	0.22 ± 0.10
$T_{\max}$ (h)	13.0 ± 5.6	13.3 ± 6.4	10.0 ± 8.8
$\text{AUC}_{0-\text{LOQ}}$ ($\mu\text{g h ml}^{-1}$)	$6.4 \pm 1.6^a, b$	3.3 ± 1.2^a	4.7 ± 1.0^b
AUMC ($\mu\text{g h}^2 \text{ ml}^{-1}$)	265 ± 120	111 ± 51	177 ± 129
$T_{1/2el}$ (h)	20.5 ± 4.34	15.4 ± 5.28	17.4 ± 6.60
MRT (h)	33.6 ± 7.41	27.6 ± 6.91	29.6 ± 12.9
$V_{d_{\text{area}}}$ (lk g^{-1})	21.7 ± 4.84	23.9 ± 9.34	31.0 ± 13.5
Cl_B (ml h^{-1})	762 ± 256^a	1003 ± 290	1570 ± 5^a
AUC FBZ/OFZ	0.40 ± 0.16	0.48 ± 0.16	0.32 ± 0.10

Superscripts indicate significant ($p < 0.05$ by Friedman test) differences between groups.

Table 2. Plasma pharmacokinetic (PK) parameters $\pm$ SD ($n = 8$) for oxfendazole (OFZ) in sheep given FBZ (5 mg kg^{-1} body weight orally) alone or in combination with either dexamethasone (DXM; 0.2 mg kg^{-1} body weight intramuscularly) formulated either as a short-acting solution (DXM Soln) or long-acting suspension (DXM Susp).

PK parameter	FBZ alone	FBZ + DXM Soln	FBZ + DXM Susp
$T_{1/2\text{for}}$ (h)	6.60 ± 0.96	5.34 ± 0.84	5.79 ± 1.34
C_{max} ($\mu\text{g ml}^{-1}$)	$0.42 \pm 0.12^{\text{a,b}}$	$0.32 \pm 0.08^{\text{a}}$	$0.30 \pm 0.00^{\text{b}}$
T_{max} (h)	25.1 ± 5.74	20.3 ± 6.50	19.6 ± 5.57
$\text{AUC}_{0-\text{LOQ}}$ ($\mu\text{g h ml}^{-1}$)	$17.0 \pm 4.9^{\text{a,b}}$	$10.6 \pm 3.5^{\text{a}}$	$10.5 \pm 3.5^{\text{b}}$
AUMC ($\mu\text{g h}^2 \text{ ml}^{-1}$)	$971 \pm 298^{\text{a,b}}$	$337 \pm 163^{\text{a}}$	$378 \pm 182^{\text{b}}$
$T_{1/2\text{el}}$ (h)	18.7 ± 11.3	12.5 ± 6.20	15.7 ± 5.52
MRT (h)	$41.3 \pm 12.8^{\text{a}}$	$28.1 \pm 8.96^{\text{a}}$	32.2 ± 10.2
AUC OFZ/FBZSO ₂	2.50 ± 0.97	2.70 ± 1.10	2.50 ± 0.45

Superscripts indicate significant ($p < 0.05$ by Friedman test) differences between groups.

Table 3. Plasma pharmacokinetic (PK) parameters $\pm$ SD ($n = 8$) for fenbendazole sulphone (FBZSO₂) in sheep given FBZ (5 mg kg^{-1} body weight orally) alone or in combination with either dexamethasone (DXM; 0.2 mg kg^{-1} body weight intramuscularly) formulated either as a short-acting solution (DXM Soln) or long-acting suspension (DXM Susp).

PK parameter	FBZ alone	FBZ + DXM Soln	FBZ + DXM Susp
$T_{1/2\text{for}}$ (h)	10.8 ± 3.75	13.6 ± 4.28	10.8 ± 4.09
C_{max} ($\mu\text{g ml}^{-1}$)	0.11 ± 0.02	0.09 ± 0.02	0.10 ± 0.05
T_{max} (h)	39.0 ± 18.1	39.0 ± 10.6	27.0 ± 8.27
$\text{AUC}_{0-\text{LOQ}}$ ($\mu\text{g h ml}^{-1}$)	$7.20 \pm 2.10^{\text{a}}$	$3.70 \pm 1.00^{\text{a}}$	4.20 ± 1.60
AUMC ($\mu\text{g h}^2 \text{ ml}^{-1}$)	527 ± 241	251 ± 89	352 ± 328
$T_{1/2\text{el}}$ (h)	29.6 ± 9.11	24.6 ± 7.92	33.1 ± 21.1
MRT (h)	64.1 ± 13.1	57.3 ± 12.7	63.2 ± 28.3

Superscripts indicate significant ($p < 0.05$ by Friedman test) differences between groups.

AUCs were significantly lower (table 1), although the plasma detection period was longer (72 versus 48 h) (figure 2), in the DXM-treated animals compared with those given FBZ alone. Both formulations of DXM markedly altered the PK distribution of FBZ (figure 2). PK profiles for oxfendazole (OFZ) and FBZ sulphone (FBZSO₂) with and without DXM co-administration (figures 3 and 4, and tables 2 and 3) were similar to those for FBZ.

Chiral analysis

Since in the achiral analysis there was no difference in the effects of the two DXM preparations, the chiral analysis was performed on only one of the DXM-treated groups. The comparative plasma concentrations of the OFZ enantiomers, (–)OFZ1 and (+)OFZ2, following treatment with FBZ alone or in combination with DXM solution are shown in figures 5 and 6. Concentrations of (+)OFZ2 were significantly reduced in DXM-pretreated animals (figure 6). Although the trend was generally similar for (–)OFZ1, the magnitude of the decrease was much smaller and not statistically significant. The areas under the concentration–time curves for the (+)OFZ2 enantiomer, like the racemate, were reduced following DXM treatment (table 4), although the effect was greater for (+)OFZ2, which was also present as a greater proportion. The AUC ratio for the (+) and (–) antipodes was similar in both treatment groups. Changes in the enantiomeric proportions and

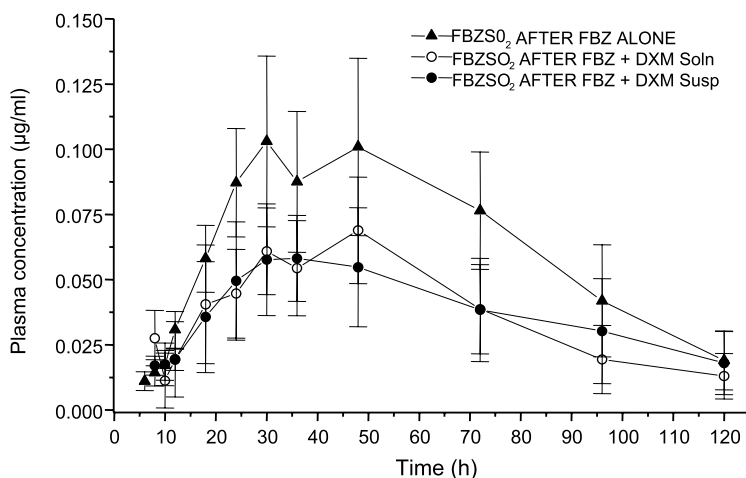


Figure 4. Comparative plasma concentrations of fenbendazole sulphone (FBZSO₂) in sheep ($n = 8$) given fenbendazole (FBZ; 5 mg kg^{-1} body weight, orally) either alone or co-administered with dexamethasone (DXM; 0.2 mg kg^{-1} body weight, intramuscularly) formulated either as a solution (DXM Soln) or suspension (DXM Susp).

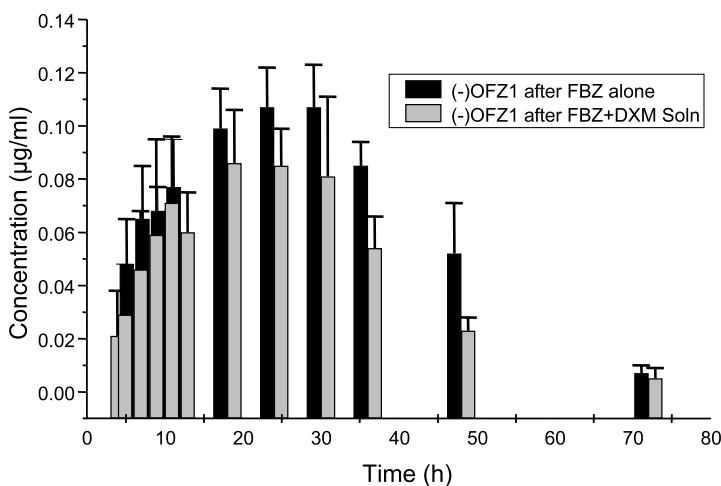


Figure 5. Comparative pharmacokinetic profile of the (-)-OFZ1 enantiomeric form of the chiral metabolite oxfendazole (OFZ), in sheep ($n = 8$) given FBZ (5 mg kg^{-1} body weight, orally) either alone or co-administered with dexamethasone (DXM; 0.2 mg kg^{-1} body weight, intramuscularly) formulated as a solution (DXM Soln).

percentage distributions of the OFZ enantiomers observed, after both treatments, are shown in figure 7. During the 72-h post-treatment period, the mean plasma enantiomeric ratio ((+)-OFZ2:(-)-OFZ1) increased from 1.86 to 15.4 in the animals given FBZ alone, but only from 0.29 to 4.83 in those pretreated with DXM.

Discussion

Over the last decade, a number of new perspectives towards improving and/or maintaining antiparasitic drug efficacy in the face of the increasing prevalence of

Table 4. Area under the concentration–time curve (AUC) (+SD, *n* = 8) obtained for racemic oxfendazole (OFZ) and its enantiomers (–)OFZ1 and (+)OFZ2, after the oral administration of FBZ (5 mg kg^{–1} body weight) either alone or in combination with dexamethasone (DXM; 0.2 mg kg^{–1} body weight intramuscularly) formulated as a short-acting solution (DXM Soln).

AUC (µg h ml ^{–1})	Treatment	
	FBZ alone	FBZ + DXM Soln
OFZ racemate	17.0 ± 4.90*	10.6 ± 3.50
(–)OFZ1	4.50 ± 1.30 (26.5%)	3.04 ± 1.00 (28.7%)
(+)OFZ2	12.5 ± 3.60 (73.5%)*	7.56 ± 2.50 (71.3%)
AUC ratio (+/–)	2.77	2.48

* Values significantly (*p* < 0.05) different to DXM-treated group by a Student’s *t*-test.

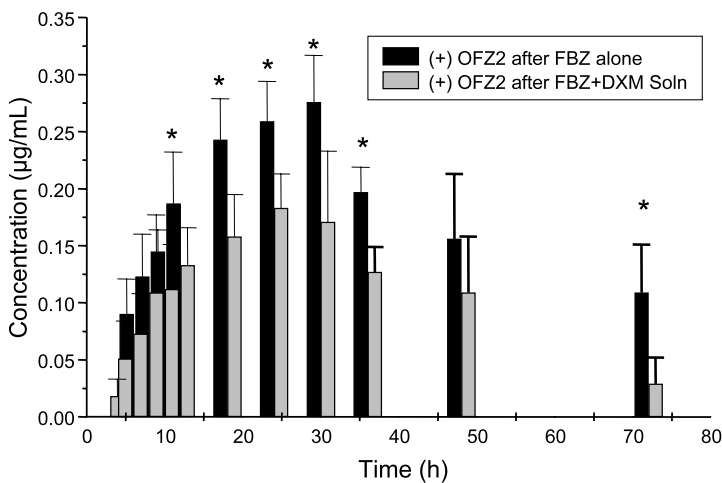


Figure 6. Comparative pharmacokinetic profile of the (+)OFZ2 enantiomeric form of the chiral metabolite oxfendazole (OFZ), in sheep (*n* = 8) given FBZ (5 mg kg^{–1} body weight, orally) either alone or co-administered with dexamethasone (DXM; 0.2 mg kg^{–1} body weight, intramuscularly) formulated as a solution (DXM Soln) **p* < 0.05.

parasite resistance have emerged. Among these, modification of the PKs and metabolism of anthelmintic compounds, particularly those of the BZD family, has attracted significant attention (Sotelo and Jung 1998, Gokbulut *et al.* 2002, McKellar *et al.* 2002).

The combination ABZ and DXM is widely used in human medicine to treat neurocysticercosis, and several authors have reported that treatment results in augmented plasma concentrations of the active metabolite ABZ-sulphoxide and a resultant improvement in antiparasitic efficacy (Jung *et al.* 1990, Takayanagui *et al.* 1997, Sotelo and Jung 1998). In contrast, the present results indicate that in sheep dosed orally with FBZ, pretreatment with DXM formulated either in solution (as DXM alcohol) or in a long-acting (14 days) suspension form (as DXM-21-isonicotinate), decreased the PK profile of FBZ, OFZ (the pharmacologically active sulphoxide of FBZ) and its inactive sulphone metabolite (FBZSO₂). Interestingly, the area under the concentration–time curve obtained when the parent drug (FBZ) was given alone was significantly higher than when it was co-administered with DXM, formulated either as solution (short acting) and suspension

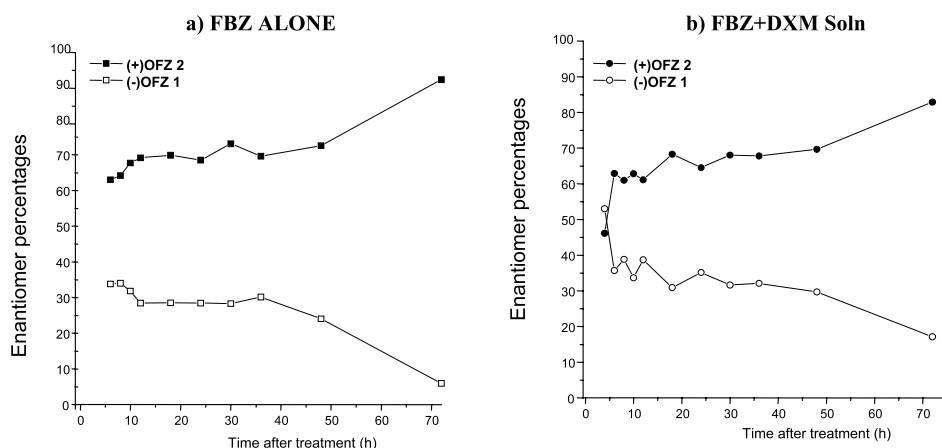


Figure 7. Comparative plasma chiral distribution of oxfendazole (OFZ) in sheep ($n = 8$) given FBZ (5 mg kg^{-1} body weight, orally) either alone or co-administered with dexamethasone (DXM; 0.2 mg kg^{-1} body weight, intramuscularly) formulated as a solution (DXM Soln).

(long acting) (table 1 and figure 2). In addition, the clearance (CL; table 1) of FBZ was markedly higher after DXM treatment, particularly in suspension form ($p < 0.05$). This may reflect alterations in the dissolution of the parent drug in the gastrointestinal tract and its consequent absorption.

After oral administration, drug absorption occurs predominantly across the wall of the small intestine. This process can be mediated by either or both trans- or paracellular transport mechanisms, with the former involving passage into and through epithelial cells and the latter passage via the tight junctions between epithelial cells (Lin *et al.* 1999). Many biological and physicochemical factors can influence the rate and extent of drug absorption by these mechanisms. These include gastric/intestinal transit time, membrane permeability, lumen pH, blood flow rate and first-pass metabolism, as well as the drug's intrinsic properties such as pK_a , molecular size, lipophilicity and solubility (Lin *et al.* 1999). DXM is known to induce numerous physiological changes in both animals and man and effects on the vasculature, gastrointestinal tract, and nervous and endocrine systems have been reported (Campbell and Halushka 1996). The same authors described effects on contractility and relaxation of longitudinal and circular intestinal smooth muscle, and on intestinal prostaglandin (PG) E12 and F receptors modulating acid secretion, and hence pH, in the stomach and duodenum. Inhibition of these PG receptors will decrease pH in the stomach and consequently alter ion-trapping phenomena. In sheep infected with *Haemonchus contortus*, it has been shown that increased abomasal pHs are associated with lower concentrations of ABZ and its metabolites in the abomasum, and an augmented PK profile of both drug and metabolites in plasma (Alvarez *et al.* 1997). The latter might partially explain the results obtained in the present study where similar pH changes could have influenced BZD dissolution and the subsequent distribution of the active metabolites. This hypothesis is supported by the present findings that, following DXM treatment, depletion rates for OFZ (FBZ-sulphoxide) and FBZSO₂ were both markedly lower than for animals given FBZ alone (tables 1–3, and figures 2–4). Interestingly, the depletion results for OFZ, but not the sulphone metabolite,

were in direct contrast to the corresponding ABZ metabolites in man, in which increased concentrations were associated with DXM treatment (Jung *et al.* 1990, Takayanagui *et al.* 1997, Sotelo and Jung 1998). The latter were attributed to previously described properties of DXM as a potent inducer of the cytochrome P450 (CYP) complex (Lin *et al.* 1999). The reasons for these differences are unclear, but may depend on factors other than CYP induction since, in rats, DXM induces CYP3A (Monostory and Vereczkey 1994, Lin *et al.* 1999), whereas in the same species sulphonation of ABZ-sulphoxide depends on CYP1A (Souhaili-El Amri *et al.* 1988). The current results, in which the depletion of FBZ and both its sulphoxide and sulphone metabolites were accelerated by DXM co-administration, are unlikely to have involved hepatic CYP induction alone, since induction usually requires pretreatment with the inducer for several days.

The complexity of BZD metabolism is well established (Ziegler 1989, Delatour *et al.* 1990, Benoit *et al.* 1992, Murray 1992, Lanusse and Prichard 1993). A further aim of the present study was to assess the effects of DXM treatment on the biotransformation of the enantiomeric forms of the chiral methyl carbamate metabolite, OFZ, following oral administration of the prochiral parent drug, FBZ. Previous reports indicate that after oral dosing with FBZ or ABZ, phase I-reversible sulfoxidation and sulphonation reactions occur mainly in liver (Galtier *et al.* 1986, Souhaili-El-Amri *et al.* 1988, Lanusse and Prichard 1993, Virkel 2001), with first-pass microsomal oxidation mediated by the FMO and CYP complexes (Lanusse and Prichard 1993, Lanusse *et al.* 1993). The chiral metabolite OFZ generated by sulfoxidation, also in the liver, is redistributed back to the gastrointestinal tract where it is transformed by sulphoreduction by commensal microbes back to FBZ (Beretta *et al.* 1987, Virkel *et al.* 2002).

It has also been suggested (Lanusse *et al.* 1993) that the sulphone metabolite may be generated from the (–)OFZ1 enantiomer as a CYP substrate, whereas the (+) antipode is derived from the FMO system (Delatour *et al.* 1990, Benoit *et al.* 1992). Based on *in vitro* studies using sheep liver microsomes and ABZ, Virkel (2001) confirmed that 90% of the (+) antipode was formed by the FMO complex and only 10% via CYP. However, only 38% of the (–) enantiomer was generated via FMO, whereas 28% was mediated by CYP3A and the remainder by other CYP enzymes. The observed predominance (71.3–73.5%) of (+)OFZ2 over (–)OFZ1 (table 4) is in accordance with the findings of Cristofol *et al.* (2001) and supports the hypothesis that the sulphone metabolite (FBZSO₂) is generated predominantly from the (–) antipode. Moreover, DXM had no significant effect on the depletion of the (–) enantiomer from blood, as judged by the similar AUCs and (±) enantiomeric ratios (table 4 and figure 5), and this correlates with the comparable mean residence times calculated for FBZSO₂ (table 3).

In contrast, DXM treatment markedly altered the disposition kinetics of the (+)OFZ1 enantiomer (figure 6). These differences were endorsed by the finding that the plasma enantiomeric percentage distribution ratio ((+)OFZ2:(–)OFZ1) increased from 1.86 to 15.4 over the 72 h following treatment with FBZ alone, but only from 0.29 to 4.83 following combined FBZ/DXM treatment (figure 7).

The precise mechanisms underlying the current observations are unclear. However, differences in the PK behaviour of the parent drug FBZ may be due to the inhibition of gut prostaglandin receptors by DXM, which could result in changes in gut pH and hence to alterations in the dissolution and absorption of FBZ. The observed modification of AUCs for racemic OFZ may have depended

on the preferentially decreased generation of the (+) enantiomer due to, as yet undefined, allosteric effects of DXM on the hepatic biotransformation of FBZ.

Whatever the mechanisms, the effects of DXM on plasma PK behaviour of FBZ and its metabolites reported herein may have important implications for the efficacy of this combination against parasites in livestock. Clearly, further studies, particularly in relation to events in the gastrointestinal tract, are required.

Acknowledgements

Part of this work was supported by the Scottish Executive Environment and Rural Affairs Department (SEERAD). S. S. gratefully acknowledges the financial support of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) and SECyT, UNCPBA (Argentina). The authors also thank members of the MRI Clinical Division for expert help with the animal work.

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Bioavailability of dietary doses of ^3H -labelled tea antioxidants (+)-catechin and (–)-epicatechin in rat

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Received 6 January 2003

1. The bioavailability and pharmacokinetic characteristics of the tea antioxidants (+)-catechin and (–)-epicatechin were investigated in the rat following intake of dietary doses.

2. To achieve this objective, tritiated derivatives (tritium was incorporated at the 3-position of the heterocyclic ring) of these compounds were administered to rats orally and intravenously at dose levels equivalent to human dietary levels of intake.

3. Following intravenous administration of both compounds, about one-third of the dose was excreted in the urine and two-thirds in the faeces, indicating extensive biliary excretion. When the same doses were administered orally, only about 5% of the dose of each compound was recovered in the urine.

4. Comparison of the areas under the curve following oral and intravenous administration revealed that the bioavailability of both compounds was less than 5%.

5. Exchange of tritium with water in the blood occurred 3 h after oral, but not after intravenous, administration of the flavanols to rat. This is believed to represent microbial degradation of the compounds by the gut flora.

6. It was established that the bioavailability of the tea antioxidants (+)-catechin and (–)-epicatechin in the rat following intake of dietary doses was poor.

Introduction

It is now widely accepted that phytochemicals contribute to the lower incidence of high morbidity diseases, such as cancer and cardiovascular disease, observed in people consuming diets with a high vegetable and fruit content (Hertog *et al.* 1995). Increasingly, a number of bioactive compounds are being isolated from plant foods that include organosulphates (Mori and Nishikawa 1998), isothiocyanates (Talalay and Fahey 2001), indoles (Xu and Dashwood 1999) and polyphenols (Birt *et al.* 2001, Yang *et al.* 2001a). These anticarcinogens can act through a multitude of mechanisms including modulation of the enzyme systems responsible for the bioactivation of chemical carcinogens (Ioannides 1999). Polyphenols are the most extensively studied group because of their wide distribution in plant products, and their documented potent antioxidant activity is believed to contribute to the beneficial effects ascribed to them (Vinson *et al.* 1995).

Green tea, a beverage with a high polyphenol content (Bu-Abbas *et al.* 1999a), repeatedly affords protection against the carcinogenicity of chemicals in a number of animal models (Ahmad *et al.* 1998, Yang *et al.* 2001b). The major

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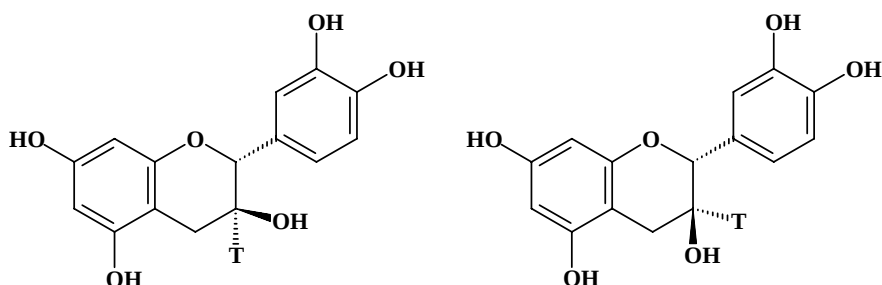


Figure 1. Structure of (+)-catechin and (-)-epicatechin. T, position of tritium.

polyphenols in tea are the flavanols, the principal compounds being (-)-epicatechin and (-)-epigallocatechin, which are present primarily as their gallate esters (Harbowy and Balentine 1997, Bu-Abbas *et al.* 1999a). (+)-Catechin, the isomer of tea (-)-epicatechin, is one of the most widely distributed flavonoids in edible plants, being present, in addition to tea, in red wine and some fruits. The flavanols are believed to mediate many of the beneficial effects of green tea, including its chemopreventive effects in many animal models where it affords protection against spontaneous tumours (Landau *et al.* 1998) as well as radiation- and chemical-induced tumours (Wang *et al.* 1992, Pingzhang *et al.* 1994, Xu *et al.* 1996).

Epidemiological studies on the anticarcinogenic activity of tea have proved inconclusive. In some studies, an inverse correlation between tea consumption and tumorigenesis has been observed, while in others no effect was seen, and in some cases even a direct relationship was reported (Yang *et al.* 2001b). These discrepancies cannot be clarified until quantitative data on the bioavailability of tea flavanols, at dietary levels of intake, are available. Bioavailability studies in the rat have been reported for (+)-catechin, but these employed much higher pharmacological doses, as this flavanol was used in the treatment of acute viral hepatitis (Shaw *et al.* 1982). More recently, a number of pharmacokinetic studies have been undertaken with both flavanols, but once again high doses were used (Chen *et al.* 1997, Hollman *et al.* 1997, Harada *et al.* 1999, Baba *et al.* 2001).

In the present study, (+)-[3-³H]-catechin and (-)-[3-³H]-epicatechin (figure 1) were used to determine the bioavailability and pharmacokinetic characteristics of these tea flavanol in rats following low, dietary levels of intake.

Materials and methods

(+)-[3-³H]-Catechin and (-)-[3-³H]-epicatechin were a generous gift from Stephan Buffnoir and Christian Rolando, Université des Sciences et Techniques de Lille, Lille, France. On HPLC analysis coupled to radiodetection, both compounds gave single peaks with the same retention time as the corresponding unlabelled polyphenol. ³H was introduced at the 3-position, as it was expected to be resistant to metabolism by mammalian enzymes.

Male Wistar albino rats (230–250 g) were purchased from B&K Universal Ltd (Hull, UK). Animals were kept under a constant temperature of 22 ± 1°C, 50% humidity and a 12-h light cycle; they had free access to standard rodent diet (B&K Universal) and water. For urine and faeces collection, rats were housed individually in metabolic cages (Arrowmigh Biosciences, Hereford, UK). The doses employed were equivalent to four 350-ml cups of tea. The dose was based on a 2.5% (w/v) brew containing 29 µg ml⁻¹ (+)-catechin and 225 µg ml⁻¹ (-)-epicatechin (Bu-Abbas *et al.* 1999a). The final doses administered to rats were 0.58 mg kg⁻¹ for (+)-[3-³H]-catechin and 4.5 mg kg⁻¹ for (-)-[3-³H]-epicatechin. These levels corresponded to 15–30 µCi/animal. For the oral studies, animals received the above doses as a single intragastric administration, and the same doses were administered via the

caudal vein for the intravenous dose; in the latter case, the animals were anaesthetized with Sagatal (pentobarbital sodium, 60 mg kg^{–1} injected intraperitoneally).

Urine and faeces were collected daily for 4 days. Aliquots of urine (250 µl) were mixed with scintillation cocktail (SafefluorTM, Packard Chemical Co., Inc., Meriden, CT, USA) and counted. Faecal homogenates (10% in water) were prepared, and aliquots (250 µl) were placed in scintillation vials and digested using SolvableTM 100 µl (Packard) at 50°C for 1 h. After cooling, 0.1 M EDTA (20 µl) and 30% (v/v) hydrogen peroxide were added and incubated for a further 1 h. Following cooling, the decolourized samples were mixed with scintillant and radioactivity was counted. Each analysis was carried out in triplicate.

For the pharmacokinetic analysis, approximately 250 µl blood were withdrawn from the rat tail, at regular time intervals for 8 h, and placed into lithium-heparinized centrifuge tubes. A sample was also obtained 24 h after administration. Aliquots of blood (25 µl) were digested and decolourized as described for the faecal homogenates. Pharmacokinetic analysis was carried out using the PK solutionsTM software package (v.2.0, Summit Research Services, Montrose, CO, USA). Animal data were analysed individually and are presented as mean ± SD. To correct for tritium exchange with water, blood samples were dried, resuspended in the original volume of water and the radioactivity counted.

Results

Following intravenous administration of (+)-[3-³H]-catechin, about 80% of the dose was excreted within 24 h, but low levels were still excreted 4 days after administration. About two-thirds of the dose was present in the faeces and one-third was excreted in the urine (table 1). A very similar picture emerged when (–)-[3-³H]-epicatechin was given intravenously (table 2). When the same dose of (+)-[3-³H]-catechin was administered orally, about 85% of the dose was excreted during the first 24 h, but in this case only 5% of the dose was recovered in the urine (table 1). Similarly, oral administration of (–)-[3-³H]-epicatechin resulted in only 3% of the dose being excreted in the urine (table 2).

Blood levels of radioactivity after intravenous administration of tritiated (+)-catechin and (–)-epicatechin are shown in figure 2, and following oral administration in figure 3. The pharmacokinetic parameters following intravenous administration of the polyphenols were calculated using a two-compartment model and a one-compartment model following the oral administrations as these models gave the best fit for the experimental data. Following oral, but not intravenous,

Table 1. Excretion of (+)-catechin material following single intravenous and oral administration to rats.

Time after administration (h)	Excretion in urine (% of dose)	Excretion in faeces (% of dose)	Total (% of dose)
Intravenous administration			
0–24	32.8	48.0	80.8
> 24–48	2.0	8.1	10.1
> 48–72	0.9	2.8	2.8
> 72–96	0.7	1.8	2.5
Total (% of dose)	36.4 ± 15.4	60.7 ± 32.0	97.1 ± 23.6
Oral administration			
0–24	4.6	80.6	85.2
> 24–48	0.4	5.5	5.9
> 48–72	0.2	0.4	0.4
> 72–96	0.1	2.2	2.2
Total (% of dose)	5.2 ± 6.2	88.8 ± 21.4	94.0 ± 13.8

(+)-[3-³H]-catechin was administered intravenously or by gastric intubation to rats as a single dose (0.58 mg kg^{–1}). Levels in the urine and faeces represent (+)-catechin and/or metabolites. Results are the mean ± SD for four animals.

Table 2. Excretion of (–)-epicatechin material following single intravenous and oral administration to rats.

Time after administration (h)	Excretion in urine (% of dose)	Excretion in faeces (% of dose)	Total (% of dose)
Intravenous administration			
0–24	34.4	47.8	82.2
> 24–48	1.2	7.6	8.8
> 48–72	0.7	1.9	2.6
> 72–96	0.5	1.1	1.6
Total (% of dose)	36.8 ± 17.2	58.3 ± 19.2	95.1 ± 18.2
Oral administration			
0–24	2.5	84.0	86.5
> 24–48	0.2	5.8	6.0
> 48–72	0.1	0.3	0.4
> 72–96	0.1	0.2	0.3
Total (% of dose)	2.9 ± 4.4	90.3 ± 21.6	93.2 ± 13.0

(–)-[3-³H]-epicatechin was administered intravenously or by gastric intubation to rats as a single dose (4.50 mg kg^{−1}). Levels in the urine and faeces represent (–)-epicatechin and/or metabolites. Results are the mean ± SD for four animals.

administration of the flavanols, significant tritium exchange with water took place; this became evident only 3 h after the intragastric administration and increased with time (figure 3).

The pharmacokinetic parameters following oral and intravenous administration of (+)-[3-³H]-catechin are shown in table 3, while those for (–)-[3-³H]-epicatechin are shown in table 4. The values are based on the total radioactivity in plasma after subtraction of that due to ³H₂O. The oral bioavailability of radioactivity of (+)-[3-³H]-catechin was about 3%, and that for (–)-[3-³H]-epicatechin was 4%.

Discussion

Following epidemiological studies, polyphenol consumption has been inversely related to the incidence of cancer and cardiovascular disease (Hertog *et al.* 1995). Tea, especially green tea, contains high concentrations of flavanols, and since this beverage enjoys world-wide consumption, it is a major dietary source of polyphenols. Similarly, high levels of flavanols are also present in chocolate, which also appears to be an important source, particularly among the young (Arts *et al.* 2001). However, in order to elucidate the mechanisms of action of flavanols, it is essential to determine their bioavailability and pharmacokinetic properties at normal dietary levels. Although a number of studies have been carried out to define the pharmacokinetic characteristic of flavanols, these have employed very large doses (about 50 mg kg^{−1}) that are very unlikely to be achieved through dietary intake (Hollman *et al.* 1997, Baba *et al.* 2001). In the present study, radiolabelled (+)-[3-³H]-catechin and (–)-[3-³H]-epicatechin were used to investigate their bioavailability and pharmacokinetics in rat at dietary levels of intake. Note that in these studies pharmacokinetic parameters were determined for the whole radioactivity and not the parent flavanol. It was not possible to monitor the levels of the parent flavanol since very low doses were employed to mimic dietary intake and consequently plasma levels were low, particularly following oral administration, beyond the limit of detection of the radiodetector. In rats,

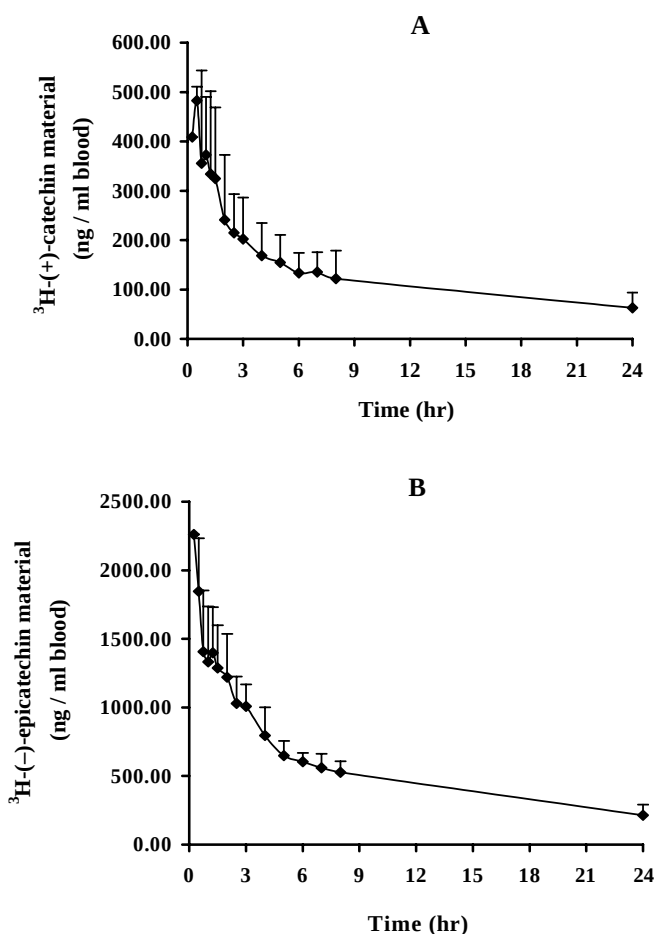


Figure 2. Blood levels of (+)-[3-³H]-catechin and (-)-[3-³H]-epicatechin and/or their metabolites following single intravenous administration. Rats received a single intravenous dose of (+)-[3-³H]-catechin (0.58 mg kg⁻¹) or (-)-[3-³H]-epicatechin (4.5 mg kg⁻¹). Results are the mean $\pm$ SD for four animals. A, (+)-catechin; B, (-)-epicatechin.

(+)-catechin and (-)-epicatechin are present in the plasma, urine and bile in the form of metabolites, principally as glucuronide, and to a lesser extent as sulphate, conjugates of the parent compounds as well as of the *O*-methylated derivatives, and the aglycone is present at very low levels in the plasma and bile and urine (Harada *et al.* 1999, Manach *et al.* 1999, Okushio *et al.* 1999, Baba *et al.* 2001). These metabolites are generated in both the liver and small intestine (Donovan *et al.* 2001).

Following oral administration of dietary doses, blood peak levels of radioactivity in rat were attained in just over 1 h, which is similar to that reported in man (Hollman *et al.* 1997). A comparison of the areas under the curve (AUCs) for radioactivity following oral administration of (+)-[3-³H]-catechin and (-)-[3-³H]-epicatechin, corrected for the difference in dose levels, showed that these were higher for (+)-[3-³H]-catechin, suggesting more efficient absorption. This is further supported by the observation that more of the radioactivity was excreted in the urine for (+)-[3-³H]-catechin compared with (-)-[3-³H]-epicatechin, and

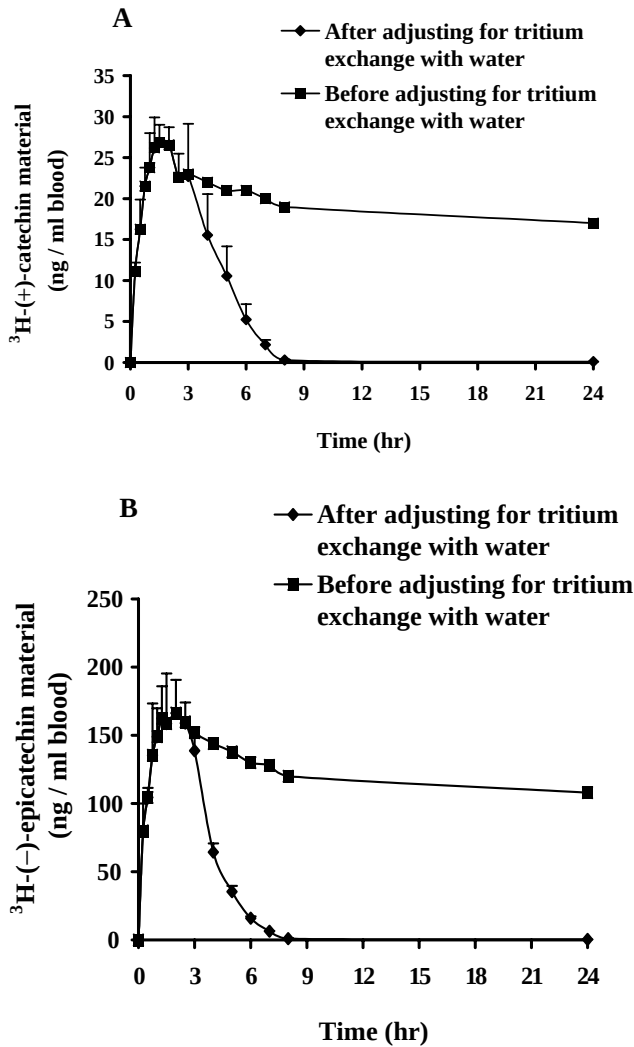


Figure 3. Blood levels of (+)-[3-³H]-catechin and (-)-[3-³H]-epicatechin and/or their metabolites following single oral administration. Rats received a single oral dose of (+)-[3-³H]-catechin (0.58 mg kg⁻¹) or (-)-[3-³H]-epicatechin (4.5 mg kg⁻¹). Results are the mean $\pm$ SD for four animals. A, (+)-catechin; B, (-)-epicatechin.

the absorption rate constant was greater for (+)-[3-³H]-catechin. When the AUCs of radioactivity for the two routes are compared, the oral bioavailability of radioactivity of (+)-[3-³H]-catechin was about 3% and for (-)-[3-³H]-epicatechin was 4%. In concordance with these findings, when the two polyphenols were given to the animals via the intravenous route, more than one-third of the dose was recovered in the urine, whereas when the same dose was administered orally, only 5% of the dose of (+)-[3-³H]-catechin and 3% of (-)-[3-³H]-epicatechin was recovered in the urine.

The present study clearly shows that at dietary levels of intake, the bioavailability of these tea flavanols in rats is poor and consequently blood levels are low. Bearing in mind that only a fraction of the radioactivity in the blood represents

Table 3. Pharmacokinetic parameters of (+)-catechin in the rat based on radioactivity from (+)-[3-³H]-catechin.

Intravenous administration		Oral administration	
Pharmacokinetic parameter	Mean $\pm$ SD	Pharmacokinetic parameter	Mean $\pm$ SD
k_d (h ⁻¹)	1.21 $\pm$ 0.26	k_e (h ⁻¹)	0.43 $\pm$ 0.06
k_{12} (h ⁻¹)	1.26 $\pm$ 0.29	k_a (h ⁻¹)	1.43 $\pm$ 0.46
k_{21} (h ⁻¹)	1.27 $\pm$ 0.51	$t_{1/2}$ (h)	1.63 $\pm$ 0.22
k_{10} (h ⁻¹)	0.42 $\pm$ 0.20	t_{max} (h)	1.25 $\pm$ 0.24
$t_{1/2\alpha}$ (h)	0.26 $\pm$ 0.06	C_{max} (ng ml ⁻¹)	30.40 $\pm$ 1.80
$t_{1/2\beta}$ (h)	4.09 $\pm$ 0.51	AUC (μ g h ml ⁻¹)	0.113 $\pm$ 0.015
Cl (l h ⁻¹)	0.043 $\pm$ 0.008		
Vd (l kg ⁻¹)	1.01 $\pm$ 0.23		
AUC (μ g h ml ⁻¹)	3.62 $\pm$ 0.88		

(+)-[3-³H]-catechin was administered to rats as a single oral or intravenous dose (0.58 mg kg⁻¹). Intravenous data were analysed using a two-compartment model and oral data using a one-compartment model. Results are the mean $\pm$ SD for four animals.

k_d , rate constant for distribution; k_{12} , rate constant for distribution from the central to the peripheral compartment; k_{21} , rate constant for distribution from the peripheral to the central compartment; k_{10} , rate constant for elimination from the central compartment; $t_{1/2\alpha}$, blood half-life of α phase; $t_{1/2\beta}$, blood half-life (half-life of the terminal phase β); Cl, clearance; Vd, apparent volume of distribution; AUC, area under the curve; k_e , rate constant for elimination; k_a , rate constant for absorption; $t_{1/2}$, blood half-life; t_{max} , time to reach maximum blood concentration; C_{max} , maximum blood concentration.

 Table 4. Pharmacokinetic parameters of (-)-epicatechin in the rat based on radioactivity from (-)-[3-³H]-epicatechin.

Intravenous administration		Oral administration	
Pharmacokinetic parameter	Mean $\pm$ SD	Pharmacokinetic parameter	Mean $\pm$ SD
k_d (h ⁻¹)	1.71 $\pm$ 0.48	k_e (h ⁻¹)	0.65 $\pm$ 0.05
k_{12} (h ⁻¹)	1.63 $\pm$ 1.36	k_a (h ⁻¹)	1.24 $\pm$ 0.12
k_{21} (h ⁻¹)	2.12 $\pm$ 0.78	$t_{1/2}$ (h)	1.08 $\pm$ 0.05
k_{10} (h ⁻¹)	3.80 $\pm$ 0.14	t_{max} (h)	1.10 $\pm$ 0.08
$t_{1/2\alpha}$ (h)	0.19 $\pm$ 0.04	C_{max} (ng ml ⁻¹)	196.5 $\pm$ 18.1
$t_{1/2\beta}$ (h)	3.65 $\pm$ 0.49	AUC (μ g h ml ⁻¹)	0.617 $\pm$ 0.038
Cl (l h ⁻¹)	0.078 $\pm$ 0.015		
Vd (l kg ⁻¹)	1.67 $\pm$ 0.51		
AUC (μ g h ml ⁻¹)	14.79 $\pm$ 3.08		

(-)-[3-³H]-epicatechin was administered to rats as a single oral or intravenous dose (4.50 mg kg⁻¹). Intravenous data were analysed using a two-compartment model and oral data using a one-compartment model. Results are the mean $\pm$ SD for four animals.

For abbreviations, see table 3.

the unmetabolized compound (Baba *et al.* 2001), and that polyphenols have high affinity for proteins (Siebert *et al.* 1996) and thus are likely to be substantially protein bound, then it can be concluded that the tissue levels of flavanols following dietary intake are likely to be very low. In previous studies, however, employing oral doses of (+)-catechin orders of magnitude larger than those used in the current study (about 40 mg kg⁻¹) it was reported that nearly 60% of the dose was excreted in the urine (Gott and Griffiths 1987). At high doses, presystemic metabolism of these flavanols may be saturated, allowing more of the dose to survive passage to the systemic circulation (Piskula and Terao 1998). Under such

conditions, bioavailability may not be dose dependent; *in vitro* studies using Caco-2 cells, a human intestinal cell line, indicated the presence of an efflux mechanism, possibly involving the MRP2 transporter (Vaidyanathan and Walle 2001), which may be saturated at high doses, such as those employed in most previously reported pharmacokinetic investigations, thus allowing better absorption at high doses. Note that the absorption of these flavanols from tea may be influenced by other tea components or milk, which is commonly added to tea in the UK. Work so far indicates that addition of milk to tea does not influence the plasma concentration of flavanols (van Het Hof *et al.* 1998). However, the possibility that the pharmacokinetic characteristics of a flavanol may be influenced by the presence of the other flavanols in tea cannot be ignored (Baba *et al.* 2001).

The ^3H -label was introduced at the 3-position of the polyphenols as this was envisaged to be resistant to metabolism by mammalian enzymes and is stable at low pH, and thus can survive the conditions in the stomach. However, a significant tritium exchange with water occurred, following oral administration, implying metabolism at this position. The fact that such an exchange took place only when the polyphenols were administered orally clearly indicates that such metabolism arose presystemically. It is suggested that metabolism in the 3-position was the consequence of microbial attack, and this is in concordance with the observation that tritium exchange was detectable only following a lag of 3 h after the intragastric administration, suggesting that the compounds were broken down in the colon. Intestinal mammalian enzymes would be expected to catalyse the metabolism of these flavanols by conjugation with glucuronide and sulphate and by *O*-methylation (Hollman *et al.* 1997). Such metabolism would leave the 3-position intact. However, a number of studies both *in vivo* and *in vitro* have established that flavonoids, including (+)-catechin and (–)-epicatechin, are also subject to extensive microbial metabolism by ring fission of the heterocyclic ring to produce valerolactones, which are further metabolized to other products (Das and Griffiths 1968, Hollman *et al.* 1997, Okushio *et al.* 1999, Wang *et al.* 2001). In human studies, γ -valerolactones formed by reductive cleavage of the heterocyclic ring catalysed by intestinal microbes are major urinary metabolites (Li *et al.* 2000).

Maximum plasma concentrations were attained in about 1–2 h for both polyphenols (Piskula and Terao 1998). Both flavanols are characterized by short half-lives, $t_{1/2\beta}$ of about 4 and 3.6 h, respectively, for (+)-[3- ^3H]-catechin and (–)-[3- ^3H]-epicatechin, calculated following intravenous administration. Indeed, more than 80% of the dose was excreted within 24 h after administration. As these flavanols have hydroxyl groups available, without requirement for prior oxidation/reduction to generate these, rapid conjugation may explain their fast clearance. Such conjugates, because of their large molecular weight and hydrophilicity, would be expected to undergo excretion through the biliary route, and this fact is borne out by the experimental findings where after intravenous administration two-thirds of the dose was found in the faeces. The tritium exchange is not believed to have influenced the excretion data, since the half-life of $^3\text{H}_2\text{O}$ in the rat is about 6 days, and since some 85% of the oral polyphenol dose was excreted within 24 h. Since the tritium exchange is very likely a consequence of microbial degradation in the gut, the plasma levels of radioactivity, following oral administration, represent the unchanged flavanol and/or metabolites resulting from mammalian metabolism. Consequently, the AUC_{oral} , calculated

after correction of plasma levels for tritium exchange, does not underestimate the levels of the flavanol and/or metabolites.

In conclusion, the present studies show that (+)-catechin and its isomer, (–)-epicatechin, have similar pharmacokinetic characteristics in the rat. When orally administered at dietary levels of intake, their bioavailability is poor. This observation argues against these polyphenols playing a major role in the chemopreventive effect of tea (Ahmad *et al.* 1998, Young *et al.* 2001a). However, when the anticarcinogenic activity of tea is evaluated, it is administered as the only source of drinking fluid, and it is possible that under such conditions high steady-state tissue levels are achieved and sustained. However, following repeated intake, the plasma concentration of (–)-epicatechin decreases in both mice and rats, although the mechanism of this phenomenon is unclear (Kim *et al.* 2000). Alternatively, the metabolic products of these flavanols, mammalian and/or microbial, may be responsible for their chemopreventive effect. Finally, the possibility that other components of tea may contribute to its anticarcinogenic activity should not be ignored. Caffeine, for example, has been shown to mediate some of the biological activities of tea (Bu-Abbas *et al.* 1999b, McArdle *et al.* 1999). In extrapolating the present findings to man, it is important to emphasize that species differences between rat and man have been reported in the conjugation of (–)-epicatechin with sulphate and glucuronide (Vaidyanathan and Walle 2002).

Acknowledgements

The authors thank Stephan Buffnoir and Christian Rolando, Université des Sciences et Techniques de Lille, for the generous gift of (+)-[3-³H]-catechin and (–)-[3-³H]-epicatechin, and they gratefully acknowledge the financial support for this study from the European Union, Project No. PL0653.

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Absorption, distribution, metabolism and excretion of telmesteine, a mucolytic agent, in rat

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Received 5 December 2002

1. The metabolism and disposition of telmesteine, a muco-active agent, have been investigated following single oral or intravenous administration of ^{14}C -telmesteine in the Sprague–Dawley rat.

2. ^{14}C -telmesteine was rapidly absorbed after oral dosing (20 and 50 mg kg^{-1}) with an oral bioavailability of $>90\%$ both in male and female rats. The C_{max} and area under the curve of the radioactivity in plasma increased proportionally to the administered dose and those values in female rats were 30% higher than in male rats.

3. Telmesteine was distributed over all organs except for brain and the tissue/plasma ratio of the radioactivity 30 min after dosing was relatively low with a range of 0.1–0.8 except for excretory organs.

4. Excretion of the radioactivity was 86% of the dose in the urine and 0.6% in the faeces up to 7 days after oral administration. Biliary excretion of the radioactivity in bile duct-cannulated rats was about 3% for the first 24 h. The unchanged compound mainly accounted for the radioactivity in the urine and plasma.

5. Telmesteine was hardly metabolized in microsomal incubations. A glucuronide conjugate was detected in the urine and bile, but the amount of glucuronide was less than 6% of excreted radioactivity.

Introduction

Thiazolidine-4-carboxylic acid is a sulfur-containing analogue of proline, which can be attached to prolyl-tRNA and incorporated into proteins (Papavasiliou and Mehler 1970, Budisa *et al.* 1998). It has been investigated as an anti-tumour agent (Brugarolas and Gosalvaz 1980), as an antioxidant stimulating the immune system in ageing mice (Weber *et al.* 1982) and as an antibacterial agent for urinary tract infection (Reese *et al.* 1996).

Telmesteine (3-ethoxy-carbonyl 4-carboxyl-1 thia-3 aza-cyclopentane), an analogue of L-thiazolidine-4-carboxylic acid, is a muco-active drug with anti-protease activity used in the management of both acute and chronic bronchitis and in obstructive airways disease. In a 6-month double-blind clinical trial in patients with severe chronic obstructive pulmonary emphysema, the number of bronchitis episodes and their severity were reduced by treatment with telmesteine (Mancini *et al.* 1993). Telmesteine in combination with antibiotics also reduced objective and subjective symptoms in acute and chronic bronchitis patients (Grassi *et al.* 1993).

The present paper describes the absorption, distribution, metabolism and excretion of telmesteine after single administration in rat.

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Materials and methods

Chemicals

^{14}C -telmesteine (specific activity $10.97\ \mu\text{Ci mg}^{-1}$) was synthesized at the Institute of Radiosynthesis (Suwon, Korea) with a radiochemical purity $\geq 98\%$, as judged by HPLC-radiochromatography. The chemical structure and ^{14}C -labelled position are shown in figure 1. Unlabelled telmesteine (Lot No. 06942) was provided by Korong Pharmaceutical Co. (Suwon, Korea) with a chemical purity of 99%. All other chemicals were the highest grade commercially available.

Animals

Male and female Sprague–Dawley rats weighing 180–250 g (7–8 weeks old) were purchased from DaeHan Laboratory Animal Research Center Co. (Taejeon, Korea). They were housed in a temperature- ($23 \pm 2^\circ\text{C}$) and humidity- ($55 \pm 10^\circ\text{C}$) controlled room and exposed to a controlled 12-h cycle of light/dark, and allowed free access to standard laboratory chow (DaeHan Laboratory Animal, Taejeon, Korea) and water.

Preparation of dosing solution

Appropriate quantities of ^{14}C -labelled telmesteine were diluted with cold telmesteine to adjust the specific activity required for dose preparation ($4.5\ \mu\text{Ci mg}^{-1}$). Telmesteine was dissolved in normal saline to a final concentration of $10\ \text{mg ml}^{-1}$.

Study design and sample collection

Pharmacokinetics. Two days before dosing, the femoral artery and vein (intravenous [i.v.] only) were cannulated using PE-50 and PE-10 tubing (Becton Dickinson, Lincoln Park, NJ, USA). The cannulae were fixed to the back of the neck. The rats were fasted overnight before drug administration and until 6 h after dosing. For oral experiments, rats (four in each group) were given a single dose of either 20 or $50\ \text{mg kg}^{-1}$ ^{14}C -telmesteine. Heparinized samples of blood (0.4 ml) were collected at 0, 5, 15, 30 min, 1, 1.5, 2, 3, 5 and 8 h post-dose. For the i.v. experiment, rats (four in each group) were given a single $20\ \text{mg kg}^{-1}$ bolus of ^{14}C -telmesteine, and blood samples were collected at 0, 1, 5, 10, 20, 30 min, 1, 1.5, 2, 3, 5 and 8 h post-dose. Plasma was harvested after centrifugation and stored frozen at -20°C until analysed.

Biliary excretion. The bile duct was cannulated with PE10 tubing 1 h before dosing under pentobarbital anaesthesia ($40\ \text{mg kg}^{-1}$ intraperitoneally). The male rats (four animals) were dosed with ^{14}C -telmesteine orally at $20\ \text{mg kg}^{-1}$. Bile was collected at 1-h intervals for the first 10 h and then from 10 to 24 h.

Tissue distribution. Rats (four animals per group) were dosed with ^{14}C -telmesteine orally at $20\ \text{mg kg}^{-1}$. At 0.5 and 6 h after dosing, each animal was lightly anaesthetized with diethyl ether, blood was collected by heart puncture and the rats were then killed by cervical dislocation. The representative tissues or organs were rapidly dissected, weighed and patted dry on a combustion pad in preparation for sample oxidation.

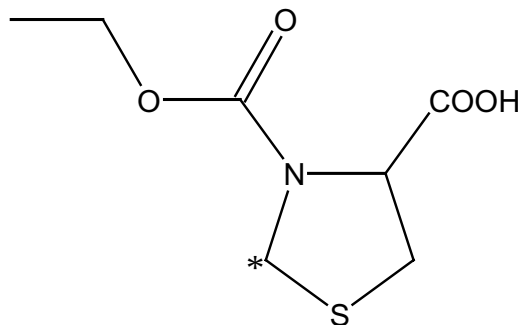


Figure 1. Chemical structure of telmesteine. *Position of ^{14}C -label.

Whole-body autoradiography. Following oral administration of ^{14}C -telmesteine 20 mg kg^{-1} in male rats, the animals were killed by deep anaesthesia with diethyl ether at 2 h after dosing and frozen with liquid nitrogen. The body sections were prepared as described by Motoji *et al.* (1995). The autoradiograms were obtained using a BAS 2000 Image analyser (Fuji Film Co., Kanagawa, Japan).

Excretion. Following an overnight fast, four rats were dosed with ^{14}C -telmesteine orally at 20 mg kg^{-1} and housed individually in metabolic cages equipped with a urine and faeces separator. Urine, faeces and washing samples were collected and weighed at the following intervals: predose, 0–12, > 12–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h. Urine and faecal samples were stored separately at -20°C until analysis. Aliquots of urine from the first 24 h were used for metabolic profile characterization.

Metabolism studies

In vitro metabolism. Microsomal incubations (final volume 1.0 ml) consisted of 1.0 mg ml^{-1} microsomal protein in 100 mM phosphate buffer ($\text{pH } 7.4$) with final concentrations of 10 mM glucose 6-phosphate, 0.67 mM $\beta\text{-NADP}^+$ and 1 U ml^{-1} glucose 6-phosphate dehydrogenase. ^{14}C -telmesteine (0.1 mM final concentration), buffer and microsomes were mixed and kept at 37°C for 3 min, and the reaction was started by adding an NADPH-generating system. After 2-h incubation at 37°C , the reaction was quenched by adding $0.1\text{ ml } 1\text{ N HCl}$, extracted twice with the same volume of methylene chloride and dried under N_2 gas. The residue was dissolved in $100\text{ }\mu\text{l}$ 20% acetonitrile in 0.1% acetic acid and then analysed by HPLC. Microsomal testosterone 2α -, 6β -, 7α - and 16β -hydroxylase activities were measured as positive control activities of the microsomes.

In vivo metabolic profiling. For the characterization of urinary and biliary metabolites, 1 ml urine and bile was passed through activated Sep-Pak C_{18} cartridges and washed twice with 1 ml distilled water. Radioactivity was eluted with 1 ml methanol. The eluate was dried under nitrogen gas and the residue dissolved in $100\text{ }\mu\text{l}$ HPLC initial mobile phase. For the analysis of plasma radioactivity, 2 vols acetonitrile were added to 0.3 ml pooled plasma. The supernatant was taken after centrifugation at $1000g$ for 10 min and dried under nitrogen gas. The residue was dissolved in $50\text{ }\mu\text{l}$ HPLC initial mobile phase and $30\text{ }\mu\text{l}$ were injected to HPLC column for analysis.

CYP induction and inhibition

Rats were treated with daily oral administration of telmesteine for 7 consecutive days at doses of 20, 100 and 500 mg kg^{-1} . Microsomal fractions were prepared from the liver homogenates by differential ultracentrifugation as described by Lee *et al.* (1998). Immunoblot analysis was performed to measure the level of CYP proteins. Briefly, microsomal protein ($10\text{ }\mu\text{g}$ protein) from each rat liver was resolved by 10 or 12% SDS-polyacrylamide gel electrophoresis. The fractionated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane, probed with specific polyclonal antibodies raised against rat CYP1A2, CYP2B1/2, CYP2C11 and CYP2E1 (GenTest, Woburn, MA, USA), and then incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG antibody. Immune complexes were detected by chemiluminescence with SuperSignal Kit (Pierce, Rockford, IL, USA).

In CYP inhibition experiments, testosterone 2α -, 6β -, 7α - and 16β -hydroxylase activities were measured as enzyme-selective marker activities for CYP2C11, CYP3A, CYP2A1 and CYP2B, respectively. Briefly, testosterone ($40\text{ }\mu\text{M}$) was incubated with microsomes supplemented with an NADPH-generating system in the presence of telmesteine (0.25 – $50\text{ }\mu\text{M}$) for 20 min. The metabolites were extracted with methylene chloride followed by analysis using reverse-phased HPLC, as described by Wong and Bandiera (1996).

Analytical methods

Radioactivity measurements. Plasma, urine and bile were mixed with 10 ml scintillation fluid (Insta Gel XF, Packard) and counted directly for radioactivity. Aliquots of solid samples (blood, organs, faeces) were weighed and combusted in a sample oxidizer (Tri-Carb Model 307, Packard, Meriden, CT, USA). The resulting $^{14}\text{CO}_2$ was absorbed on Carbosorb and then mixed with Permafluor V scintillation fluid. The radioactivity of samples was counted using a liquid scintillation counter (Tri-Carb).

HPLC analysis. Chromatographic separation of telmesteine and metabolites was achieved on a YMC-Pak C8 column ($4.6\text{ mm} \times 15\text{ cm}$, $5\text{ }\mu\text{m}$, YMC) using a linear gradient of 20% acetonitrile in 0.1% acetic acid to 40% acetonitrile over 25 min followed by 5-min isocratic elution at a flow rate of 1.0 ml min^{-1} .

By using a 1/10 splitter, one-tenth of the eluate was transferred to ion-trap mass spectrometer (LC-MSD Trap, Agilent, Palo Alto, CA, USA) for mass monitoring, while the other was transferred to a β -RAM radioactivity flow detector (IN/US Corp., Tampa, FL, USA) for radioactivity monitoring. The retention times of telmestine and its metabolite were 21.1 and 15.8 min, respectively.

Data analysis. Pharmacokinetic parameters were calculated based on the assumption that total radioactivity in plasma represented only unchanged drug (table 3). The area under the plasma concentration–time curve (AUC), total plasma clearance (Cl_t), terminal half-life ($t_{1/2}$) and volume of distribution at steady-state ($V_{d_{ss}}$) were determined by a non-compartmental method using WinNonlin (Scientific Consulting, Inc., Lexington, KY, USA). Maximum plasma concentration (C_{max}) and the corresponding T_{max} were reported as observed. Oral bioavailability was calculated using:

$$\text{Bioavailability} = (\text{AUC}_{\text{oral}}/\text{dose}_{\text{oral}}) \times (\text{dose}_{\text{iv}}/\text{AUC}_{\text{iv}} \times 100)$$

Results

Plasma concentrations of radioactivity

The plasma concentrations of the radioactivity after single oral and intravenous administration of ^{14}C -telmestine in male and female rats are shown in figure 2 and the pharmacokinetic parameters listed in table 1. After i.v. administration (20 mg kg^{-1}) to male rats, the radioactivity was eliminated with a half-life of 0.05 h. The volume of distribution (1297 ml kg^{-1}) was higher than the volume of total body water and clearance occurred rapidly ($2085 \pm 206 \text{ ml h}^{-1}$). A marked sex difference was noted for AUC. The AUC of the radioactivity in the female was 30% higher than that in the male rat, and these results might be due to the difference in the rate of urinary excretion. Following oral administration of ^{14}C -telmestine 20 and 50 mg kg^{-1} , the drug was absorbed rapidly with a peak concentration of the radioactivity occurring within 30 min. Increases in AUC of the radioactivity were almost proportional to dose. The maximum concentration and AUC of the radioactivity in female rats were 30% higher than those in male rats, whereas total clearance was lower in females. The maximum concentration time did not differ between males and females. Oral bioavailability of the radioactivity was estimated at 91–100% for doses administered regardless of sex.

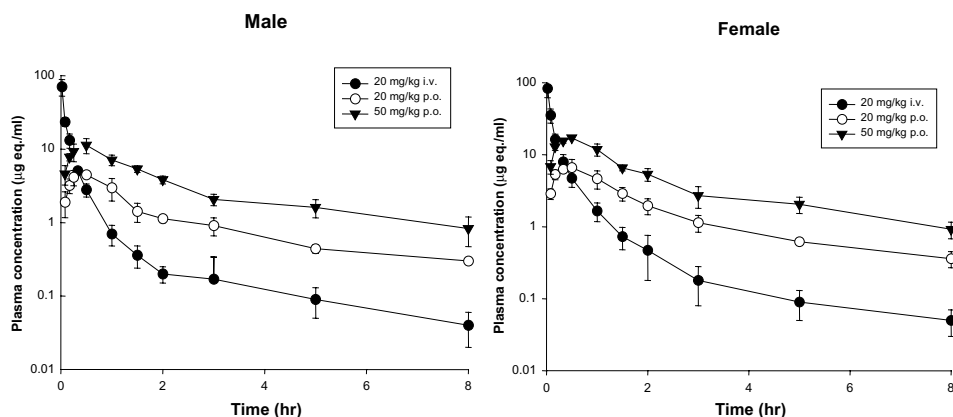


Figure 2. Mean plasma concentrations of radioactivity in male and female rats following either a single oral dose (20 and 50 mg kg^{-1}) or a single intravenous dose (20 mg kg^{-1}) of ^{14}C -telmestine. Data are the mean $\pm$ SD of four animals.

Table 1. Pharmacokinetic parameters of radioactivity in rats after single oral and intravenous administration of ¹⁴C-telmestine.

Sex	Dose (mg kg ⁻¹)	Dosing route	AUC (µg h ml ⁻¹)	Cl _t (ml h ⁻¹ kg ⁻¹)	Vd _{ss} (ml kg ⁻¹)	t _{1/2} (h)	C _{max} (µg ml ⁻¹)	T _{max} (h)	F (%)
Male	20	i.v.	9.95 ± 1.42	2085 ± 206	1297 ± 464	0.05 ± 0.03	–	–	
	20	p.o.	9.04 ± 0.94	2057 ± 202	–	2.42 ± 0.31	4.6805 ± 0.52	0.42 ± 0.14	90.8
	50	p.o.	24.32 ± 2.60	1843 ± 287	–	2.41 ± 0.62	11.29 ± 2.61	0.50 ± 0.10	97.8
Female	20	i.v.	13.83 ± 2.79	1529 ± 285	930 ± 372	0.08 ± 0.01	–	–	
	20	p.o.	13.39 ± 1.22	1343 ± 84	–	2.23 ± 0.33	7.26 ± 1.47	0.33 ± 0.14	96.8
	50	p.o.	34.50 ± 2.71	1340 ± 96	–	2.09 ± 0.17	17.19 ± 0.85	0.42 ± 0.10	99.8

Data are the mean ± SD of four animals.

Tissue distribution of radioactivity

The tissue distribution of the radioactivity after a single oral administration of ^{14}C -telmesteine 20 mg kg^{-1} is described in table 2. Radioactivity was widely distributed into all tissues except for brain. The concentration of the radioactivity in the kidney at 30 min after dosing was higher than in plasma as renal clearance accounted for most of the total clearance. The concentrations of the radioactivity in other tissues were lower than in plasma, ranging from 1.08 to $7.43\text{ }\mu\text{g eq. ml}^{-1}$. The radioactivity in all tissues appeared to decline at a lower rate and tissue plasma ratios at 6 h after dosing were higher compared with the corresponding values at 2 h after dosing, except for brain and muscle. The whole-body autoradiograms after oral administration of ^{14}C -telmesteine 20 mg kg^{-1} in male rat are shown in figure 3. At 30 min after dosing, high levels of the radioactivity were observed in the kidney and gastrointestinal contents. The radioactivity in liver, spleen and heart were relatively high compared with other tissues.

Excretion of radioactivity

Excretion of the radioactivity in urine and faeces after single oral administration of ^{14}C -telmesteine 20 mg kg^{-1} is shown in figure 4. Urinary and faecal recoveries of the radioactivity for the first 24 h were $84.99 \pm 4.63\%$, and $0.42 \pm 0.23\%$ of administered dose, respectively. The radioactivity recovered within 7 days was $87.50 \pm 4.63\%$, with $86.90 \pm 4.64\%$ appearing in the urine and $0.60 \pm 0.11\%$ in the faeces. Biliary excretion of the radioactivity after single oral administration of ^{14}C -telmesteine 20 mg kg^{-1} is shown in figure 4. Radioactivity excreted in bile over 24 h was $2.87 \pm 0.55\%$ of administered dose.

Table 2. Tissue concentrations of radioactivity after single oral administration of ^{14}C -telmesteine 20 mg kg^{-1} in the male rat.

Organ	$\mu\text{g-eq. g}^{-1}$ or ml^{-1} tissue	
	0.5 h	6 h
Adrenal	1.84 ± 0.70	1.52 ± 0.65
Bladder	27.73 ± 13.71	4.71 ± 1.54
Blood cell	3.48 ± 1.67	< 0.05
Brain	0.20 ± 0.06	0.10 ± 0.00
Heart	2.68 ± 0.83	0.21 ± 0.07
Kidney	45.77 ± 15.33	2.16 ± 1.22
Large intestine	3.36 ± 1.27	0.58 ± 0.22
Liver	7.43 ± 3.30	0.84 ± 0.41
Lung	3.81 ± 1.38	1.00 ± 0.95
Muscle	1.41 ± 0.36	0.07 ± 0.01
Plasma	10.38 ± 3.07	0.36 ± 0.28
Seminal vesicle	3.89 ± 1.76	1.70 ± 0.90
Small intestine	53.46 ± 31.49	0.99 ± 0.52
Spleen	2.01 ± 0.63	0.62 ± 0.06
Stomach	47.26 ± 23.58	6.28 ± 2.16
Testis	1.08 ± 0.26	1.10 ± 0.41
Thymus	1.60 ± 0.42	0.31 ± 0.07
Thyroid	2.37 ± 0.64	0.27 ± 0.08
Whole blood	4.66 ± 1.85	0.14 ± 0.06

Data are the mean $\pm$ SD of four animals.

kidney line section

midline section

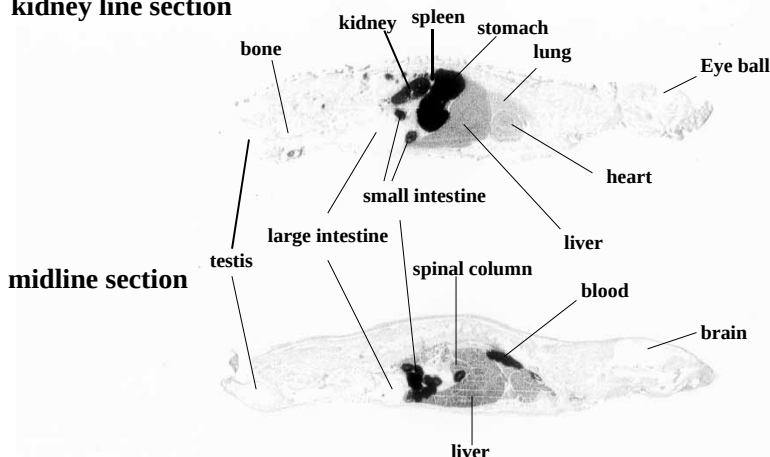


Figure 3. Whole-body autoradiograms showing the distribution of radioactivity at 30 min after oral administration of ^{14}C -telmestaine 20 mg kg^{-1} in male rats.

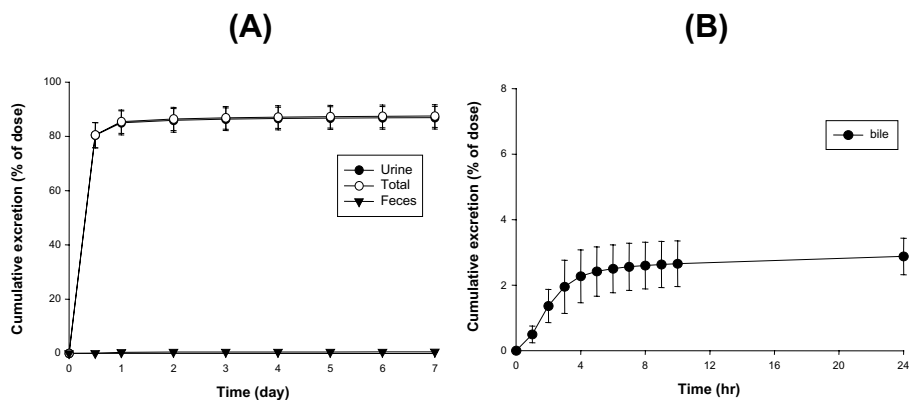


Figure 4. Excretion of radioactivity after single oral administration of ^{14}C -telmestaine 20 mg kg^{-1} in the male rat. (A) Urinary and faecal excretion of radioactivity; (B) biliary excretion of radioactivity in bile duct-cannulated rat. Data are the mean $\pm$ SD of four animals.

Metabolic profiling

HPLC radiochromatograms of 0–12 h urine and 0–10 h bile collected after oral administration of ^{14}C -telmestaine (figure 5) demonstrated two radioactive peaks. The radioactive peaks were characterized by ion-trap mass spectrometry, and the fragmentation (MS/MS) spectra of both telmestaine and metabolite are shown in figure 6. Telmestaine eluting at 21.1 min yielded a molecular ion $[\text{M} - \text{H}]^-$ at m/z 204. In the product ion mass spectrum, loss of the ethoxy moiety from the deprotonated molecule of telmestaine (m/z 204) yielded a fragment at m/z 160. Loss of the carbethoxy moiety and an H_2O molecule and subsequent loss of CO resulted in fragment ions at m/z 114 and 86, respectively. The telmestaine

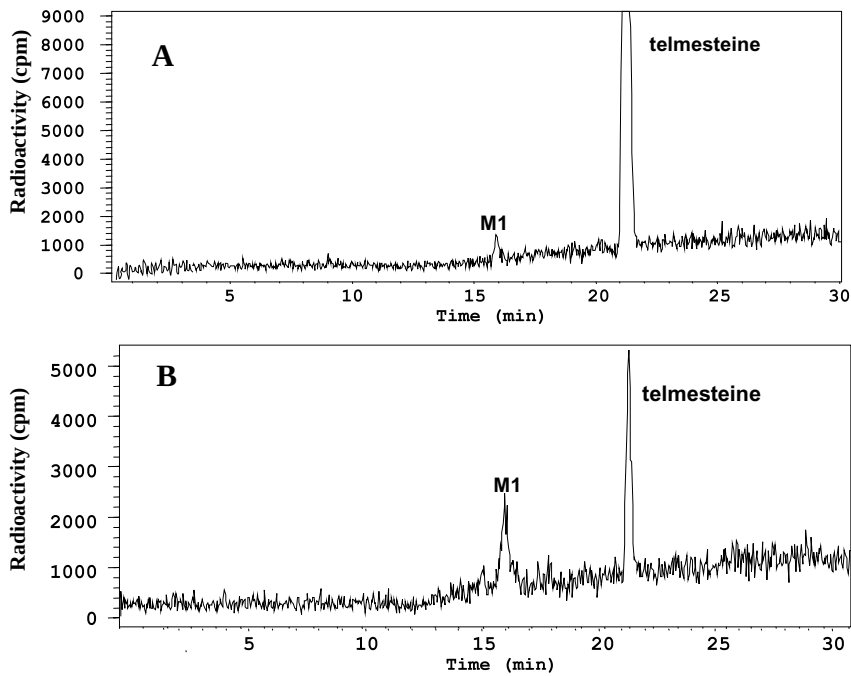


Figure 5. Radiochromatograms of urine collected at 0–12 h (A) and bile collected at 0–10 h (B) after oral administration of ^{14}C -telmestine 20 mg kg^{-1} in the male rat.

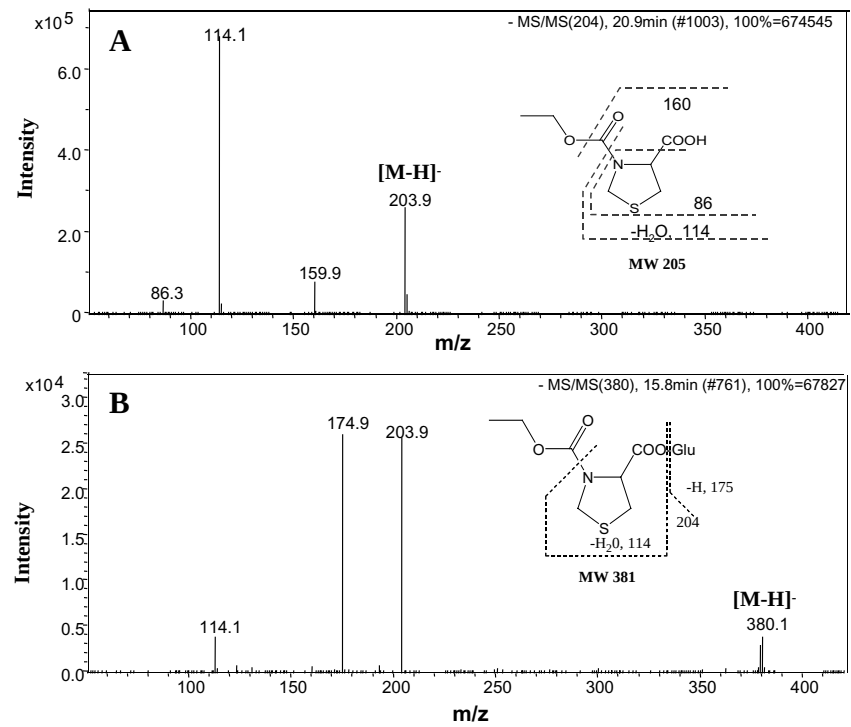


Figure 6. Negative mode product ion mass spectra of telmestine (A) and metabolite (B).

Table 3. Relative proportion of telmesteine and its glucuronide in plasma, urine and bile.

	Relative percentage of radioactivity	
	Telmesteine glucuronide	Telmesteine
Urine	5.1	94.9
Bile	44.1	55.9
Plasma	—	100

Urine was pooled for the first 24 h; bile was pooled for 10 h. Plasma was collected at 30 min after dosing.

—, Not detected.

Data were derived from three animals.

metabolite eluting at 15.8 min yielded a molecular ion $[M - H]^-$ at m/z 380, suggesting that the molecular weight of this metabolite was 381. In a product ion mass spectrum, the major fragment ion was at m/z 204 (glucuronide moiety), 175 (glucuronide moiety) and 114. Therefore, this metabolite was tentatively assigned as telmesteine glucuronide.

The relative percentage of the unchanged compound and telmesteine glucuronide in the urine and bile is described in table 3. The unchanged compound accounted for 94.9% of urinary radioactivity and 55.9% of biliary radioactivity. No metabolites were detected in plasma at 30 min after dosing in the analytical system. The same results were observed in plasma collected at later time points (data not shown). Total amount of telmesteine glucuronide is assumed to be less than 6% of the dose because more than 86% of the administered dose is excreted into the urine.

Discussion

After oral administration of ^{14}C -telmesteine 20 and 50 mg kg^{-1} in rats, maximal concentration of radioactivity was achieved within 30 min, indicating that absorption of the radiolabelled dose from the gastrointestinal tract was rapid. The maximum plasma concentration (C_{max}) of total radioactivity and the AUC increased almost proportionally to the dose and other kinetic parameters ($t_{1/2}$, T_{max}) were not different between the 20- and 50 mg kg^{-1} doses, indicating that telmesteine has linear kinetics over the dose range $20\text{--}50\text{ mg kg}^{-1}$. Plasma pharmacokinetic profiles of the radioactivity showed a significant gender difference in rats. In female rats, the AUC and C_{max} of the radioactivity were 30% higher than in male rats. On the contrary, total clearance in females was significantly lower than in males. Telmesteine is metabolically stable and the majority of the drug was excreted as intact parent drug, suggesting that the sex difference is due mainly to the rate of excretion. Oral bioavailability of the total radioactivity (F) was calculated by comparison of the AUC between oral administration and i.v. injection. F values were higher than 90% regardless of dose and sex.

After oral administration of ^{14}C -telmesteine 20 mg kg^{-1} , the excretion of the radioactivity in the male rat was 86% of the dose in the urine and 0.7% in the faeces. Approximately 3% of the radioactivity was excreted in bile for the first 24 h in bile-duct cannulated rats, which was much larger than the amount of radioactivity detected in the faeces. These results demonstrated that most of the radioactivity

was excreted into the urine and less than 1% of the radioactivity was excreted into the faeces via the bile. The results also suggested that orally administered telmesteine was completely absorbed in the gastrointestinal tract. Extensive urinary excretion of unchanged telmesteine is consistent with high oral bioavailability.

^{14}C -telmesteine was well distributed into all tissues examined, except for brain and eyeball. Lower levels in the brain tissue indicate that penetration of telmesteine across the blood-brain barrier is limited. The concentration of radioactivity in the kidney and bladder was higher than in the plasma, and the tissue/plasma concentration ratio in other tissues was in the range 0.1–0.8, suggesting that telmesteine may have a relatively low tissue affinity in the rat. Tissue distribution results correlated well with whole-body autoradiograms.

Incubation of ^{14}C -telmesteine with rat liver microsomes did not generate any metabolites, suggesting that telmesteine was not metabolized via CYP enzyme-mediated oxidation. HPLC/radiochromatogram analysis of urine and bile extracts revealed similar metabolic profiles. More than 90% of the total excreted radioactivity was associated with parent telmesteine, indicating that telmesteine was metabolically stable in the rats. The structure of the metabolite was tentatively characterized as a glucuronide conjugate as judged by negative-ion tandem mass analysis. A structure analogue of telmesteine, 2-oxo-thiazolidine-4-carboxylate, was reported to be metabolized to glutathione and taurine conjugates in rat hepatocytes (Coloso *et al.* 1991) and thiazolidine-4-carboxylate is readily dehydrogenated in *Escherichia coli* (Deutch *et al.* 2001). However, these latter derivatives were not detected in the case of telmesteine. There was no evidence that telmesteine was metabolized via CYP enzyme-mediated oxidation, as evidenced by the result that unchanged compound accounted for most of the radioactivity in the urine and faeces. Telmesteine did not induce the levels of CYP enzymes after daily oral administration for 7 days and no marked inhibitory effect of telmesteine was noted for any CYP enzyme in human liver microsomes (data not shown), suggesting that telmesteine appears to have a low potential for interactions with CYP enzymes.

In conclusion, the present study demonstrated that administered telmesteine was absorbed in the gastrointestinal tract very rapidly and showed linear pharmacokinetics over the investigated dose range. Telmesteine was eliminated, principally as the parent compound, in urine. There was little evidence of CYP-mediated metabolism.

Acknowledgements

The authors thank Dr Young-Sik Kim for the synthesizing ^{14}C -telmesteine. This work was supported by the National Research Laboratory Program from the Korean Ministry of Science and Technology and by grants from Korong Pharmaceutical Co.

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Species and gender differences in the formation of an active metabolite of a substituted 2,4-thiazolidinedione insulin sensitizer

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Received 6 January 2003

1. The metabolism of a substituted 2,4-thiazolidinedione (P1) with dual PPAR α/γ activity was evaluated in male and female rats, dogs and monkeys. A *para*-hydroxylated metabolite (M1) with potent PPAR γ -selective agonist, was a major circulating drug-related component in female rats, dogs and monkeys, but not in male rats (M1-to-P1 exposure ratio of <1, 3–5, 5 and 5–11 in male rat, monkey, female rat, and dog, respectively).

2. M1 (%) formed *in vitro* (5, 53, 57–65, 67 and 67% in male rat, monkey, female rat, dog, and human liver microsomes, respectively), rank ordered with M1 (%) formed *in vivo* (24–45, 53–57, 78, 75–85%, for male rat, monkey, female rat and dog, respectively, after oral administration of P1).

3. The plasma clearance of M1 was higher in male rats (32 ml min⁻¹ kg⁻¹ compared with 6, 7 and 2 ml min⁻¹ kg⁻¹ in female rat, male monkey and male dogs, respectively).

4. The low amounts of M1 observed in male rats, with the appearance of products of the cleavage of the propyl group between the phenyl groups was probably due to the presence of the sex-specific CYP2C11, which cleaves P1 at the propyl bridge. None of the CYPs present in female rats cleaved P1 at this site and M1 was only produced by CYP2C6. In humans, only CYP2C8 and the polymorphic CYP2C19 produced M1.

Introduction

Several potent antihyperglycemic agents of the substituted thiazolidinedione (TZDs) class are being investigated, developed or used for the treatment and prevention of type 2 diabetes. Among these oral (p.o.) antidiabetic agents, rosiglitazone and pioglitazone have been approved for human use by the US Food and Drug Administration (FDA). Others, such as ciglitazone, have been extensively studied (Yoshida *et al.* 1983, Torii *et al.* 1984), whereas troglitazone was withdrawn from the market due to its association with rare, but severe, hepatic toxicity (Watkins and Whitcomb 1998, *Physicians' Desk Reference* 1999).

The pharmacological mechanism of action of these antidiabetic drugs involves activation of a specific nuclear receptor, PPAR γ , a ligand-activated transcription factor, that regulates the expression of genes leading to enhanced insulin sensitivity (Berger *et al.* 1996). PPAR α is a related receptor that serves to regulate fatty acid oxidation and circulating lipid levels (Berger and Moller 2002) and PPAR α

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agonists may potentially improve lipid profiles and reduce cardiovascular risk. Substitutions in the side chains of these TZDs are thought to be responsible for differences in their disposition, metabolism and antidiabetic efficacy (Sohda *et al.* 1990; Lehmann *et al.* 1995, Berger *et al.* 1996). Rosiglitazone contains an aminopyridyl side chain (Henry 1997, Young *et al.* 1998), whereas pioglitazone contains an ethylpyridyl side chain (Krieter *et al.* 1994). In the case of ciglitazone, the side chain consists of a methylcyclohexylmethoxy group (Yoshida *et al.* 1983), and for troglitazone it is a trimethylbenzoquinone or vitamin E moiety (Kawai *et al.* 1997). Major metabolic pathways for these TZDs involve production of mono- and di-hydroxylated metabolites of the side chains, with further glucuronidation or sulfate conjugation (Yoshida *et al.* 1983, Torii *et al.* 1984, Bolton *et al.* 1996, Kawai *et al.* 1997). Oxidative cleavage of the TZD ring is also a possible metabolic route, as observed for troglitazone, for which reactive intermediate formation with subsequent covalent adduct formation has been detected in rats (Kassahun *et al.* 2001). Quantitative and qualitative differences in metabolism have been observed for troglitazone between male and female rats, including the formation of a rat-specific hydroxylated metabolite (Odaka *et al.* 1995, Kawai *et al.* 1997). It is known that gender differences in rat metabolism can be attributed to gender-specific expression of certain cytochrome (CYP) P450s (Waxman 1985, Shapiro *et al.* 1995). In addition, differences between rat and human metabolism have been reported for troglitazone, with humans producing a quinone-type metabolite not observed systemically in rats (*Physicians' Desk Reference* 1999). These latter metabolites have been implicated as reactive intermediates in drug-induced hepatic toxicity linked to acetaminophen and diclofenac (Pumford and Halmes 1997, Bort *et al.* 1999). Gender and species differences in drug disposition, such as those observed in the metabolism of substituted TZDs in preclinical species, may be further compounded if the metabolites are pharmacologically or toxicologically active.

In the case of 5-[3-(3-(4-phenoxy-2-propylphenoxy)propoxy-phenyl] 2,4-thiazolidinedione (P1), a PPAR α/γ agonist with demonstrated efficacy in animal models of type 2 diabetes, a dose-dependent increase in heart weight was observed in rats, dogs and monkeys following multiple doses once a day, for 14 consecutive weeks (Merck Research Laboratories [MRL], unpublished data). These observations were consistent with observations of heart weight increase with all marketed TZDs. In rats, the heart weight increase was significantly greater in females than in males. In dogs, no gender differences were observed in findings associated with myocardial oedema; however, these effects were much greater than in the male rat and the monkey (MRL, unpublished data). The differences in toxicity between species, however, could not be explained by exposure to parent drug P1, which was similar between species when the same dose level was administered. To evaluate if any of the above findings were related to differences in metabolism, a series of experiments was conducted in rats, dogs and monkeys. In these studies, the role of specific dog, rat and human CYPs involved in the metabolism of P1 were evaluated.

Materials and methods

Test materials

P1 (5-[3-(3-(4-phenoxy-2-propylphenoxy)propoxy)-phenyl]-2,4-thiazolidinedione, 99% pure) (figure 1) was supplied by Process Research at MRL. Radiolabelled compounds were synthesized by

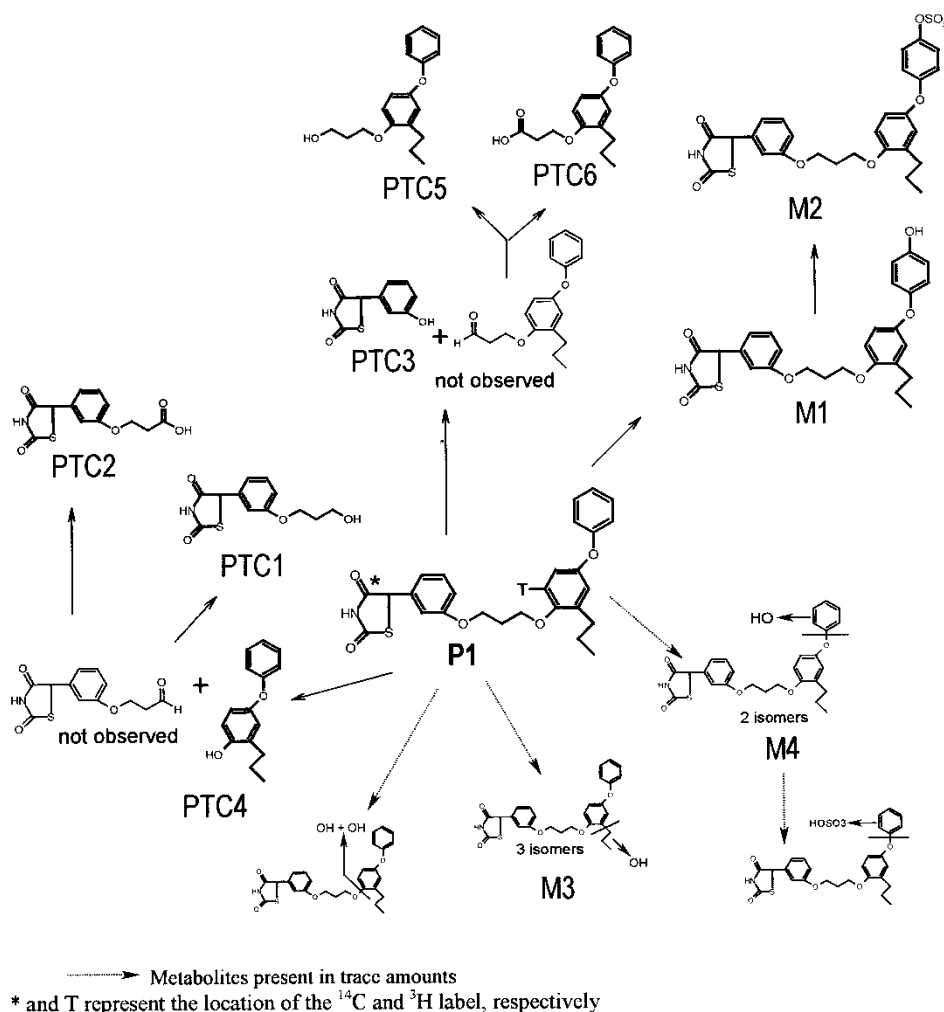


Figure 1. Metabolism of P1 in male and female rats and male dogs.

the Labeled Compound Synthesis Group, MRL. [Thiazolidinedione-4- ^{14}C] P1 (^{14}C P1) was provided as a solid, with a radiochemical purity $>99\%$, as determined by high-performance liquid chromatography (HPLC), and a specific activity of $41 \mu\text{Ci mg}^{-1}$. [Phenoxy-6- ^3H] P1 (^3H P1) was provided as a solution in ethanol. The radiochemical purity was determined by HPLC to be $>98\%$. The specific activity was 28 mCi mg^{-1} . [^{14}C] P1 and/or [^3H] P1 were diluted with unlabelled parent to achieve the final specific activities used in the animal and *in vitro* studies described below. Metabolite 1 (M1; figure 1), supplied by Medicinal Chemistry, MRL, was 98% pure and was used to compare its HPLC retention time and mass spectrum (MS) fragmentation pattern to that of radiolabelled metabolites. All other reagents were obtained from commercial sources.

Animal studies

All animal studies were conducted with approval of the Institutional Animal Care and Use Committee.

Pharmacokinetic studies

Animal phase. Male Sprague-Dawley rats (about 350–450 g) were dosed with P1 intravenously (i.v.) at 0.1, 0.5, 2 and 5 mg kg^{-1} or p.o. at 0.2, 1, 2, 10 and 100 mg kg^{-1} . Female Sprague-Dawley rats (about 250–350 g) were dosed with P1 i.v. at 1 and 5 mg kg^{-1} or p.o. at 1, 10 and 100 mg kg^{-1} . Male rhesus

macaques (about 10–12 kg) and male beagle dogs (12–15 kg) were dosed i.v. at 0.1, 0.5 or 2 mg kg⁻¹ and p.o. at 0.2 or 2 mg kg⁻¹. The oral pharmacokinetics of P1 were also evaluated in male db/db mice at 2 mg kg⁻¹ and in male Syrian hamsters at 2 and 3 mg kg⁻¹. Pharmacokinetics following multiple dose administrations were evaluated in male and female beagle dogs following single daily administrations of P1 at 0.5, 3 and 10 mg kg⁻¹ day⁻¹ for 14 consecutive weeks. To evaluate the pharmacokinetics of M1, male and female Sprague–Dawley rats, beagle dogs and Rhesus monkeys were dosed with M1 i.v. (0.5 mg kg⁻¹). All studies were conducted using three animals per dose level, except for monkey studies, in which two animals were used per dose level, and for dog multiple dose studies, in which six animals were used per gender per dose level. For i.v. dosing, P1 and M1 were dissolved in ethanol:PEG400:saline (10/40/50 v/v/v). For p.o. dosing, they were suspended in 0.02% SDS and 0.5% methylcellulose. Heparinized blood was collected at 0, 0.083, 0.25, 0.50, 1, 2, 4, 6, 8, 12 and 24 h post-dose. For studies with db/db mice and Syrian hamsters, blood samples were obtained by cardiac puncture ($n=3$ per time point). Plasma was obtained by centrifugation and stored at -80°C until analysis.

Bioanalytical methods. Concentrations of P1 and M1 in rat plasma were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a PE Sciex API 3000 mass spectrometer interfaced via turbo ion spray in negative ionization mode to an HPLC system. The HPLC system consisted of two Perkin Elmer Series 200 micro pumps and a Perkin Elmer Series 200 autosampler. Plasma (0.1 ml) aliquots were pipetted into disposable 16×125 mm culture test tubes and a monofluorinated analogue of P1 was added as an internal standard (IS) (20 ng), followed by 0.05 ml HPLC-grade ethanol and 0.5 ml distilled H₂O. Test tubes were gently shaken and ethyl acetate (2.5 ml) was added. Tubes were vortexed (3 min) and centrifuged at room temperature (15 min at 1850g). The organic phase was transferred into disposable 16×100 mm culture tubes and evaporated to dryness under N₂ at room temperature. The dry extracts were reconstituted in 0.15 ml water: methanol:acetonitrile (10:30:60 v/v/v) and submitted to LC-MS/MS analysis. Analytes were chromatographed on a Zorbax SB C8 column (4.6×75 mm) and eluted at 1 ml min⁻¹ using acetonitrile:methanol:water (60:30:10 v/v/v) containing 5 mM ammonium acetate. The HPLC flow was split and only one-fifth was directed to the mass spectrometer. Under these conditions, P1, M1 and the IS eluted at 1.05, 0.89 and 1.03 min, respectively (chromatograms not shown). Quantitation was achieved by negative ion LC-MS/MS multiple reaction monitoring (MRM) of ions produced by collision-induced dissociation (nitrogen was used as the collision gas). The MRM transitions were m/z 476 $\rightarrow$ 405 for P1, 492 $\rightarrow$ 449 for M1 and 494 $\rightarrow$ 451 for IS. Calibration standards were prepared by adding P1 and M1 (usually triplicates of eight to 12 concentrations ranging from 0.1 to 2500 ng ml⁻¹) and IS (20 ng) to rat plasma. The lower limit of quantitation for P1 and M1 was 2 ng ml⁻¹.

Pharmacokinetic calculations. Pharmacokinetic parameters were calculated by established non-compartmental methods. The area under the plasma concentration versus time curve from 0 to 24 h (AUC_(0–24)) and the area under the first moment of the plasma concentration versus time curve (AUMC) were determined using Watson software with linear trapezoidal interpolation in the ascending slope and logarithmic trapezoidal interpolation in the descending slope. The portion of the AUC from the last measurable concentration to infinity (AUC_(0–inf)) was estimated by C_t/k_{el} , where C_t is the last measurable concentration and k_{el} is the elimination rate constant. The latter was determined from the concentration versus time curve at the terminal phase by linear regression of the semilogarithmic plot.

In vivo metabolism studies

Metabolism in male and female rats following administration of a single dose. Eight male (about 350 g) and eight female (about 300 g) Sprague–Dawley adult rats received a single p.o. administration of [¹⁴C] P1 at 20 mg kg⁻¹ (about 250 μCi per rat). Two males and two females were euthanized at 1, 4, 8 and 24 h. Blood (cardiac puncture) was collected in heparinized tubes. Plasma, liver and kidney were collected at each time point. In addition, for the 24-h group, plasma samples were obtained at 0.25, 1, 4, 8 and 24 h, and urine and faeces were collected for the 0–24-h interval.

Metabolism in male and female rats following administration of multiple doses. From an ongoing 14-week study (once daily dosing) in Sprague–Dawley rats, two males and two females from each of the 1- and 100-mg kg⁻¹ dosing groups were administered [¹⁴C]/[³H] P1 as the last two doses (dose-days 97 and 98). Each animal received approximately 20 μCi ¹⁴C and 85 μCi ³H. Blood was collected at 1, 4, 8 and 24 h after each of the last two doses, and centrifuged to yield plasma. Animals were euthanized 24 h after the last dose, and livers and kidneys were collected. Urine and faeces were collected through 24 h following the last administration.

Metabolism in bile duct cannulated male and female rats. Two male and two female bile duct-cannulated Sprague-Dawley rats (males about 330 g, females about 290 g) were dosed p.o. with 20 mg [$^3\text{H}/^{14}\text{C}$] P1 kg^{-1} (approximately 40 $\mu\text{Ci } ^{14}\text{C}$ and 120 $\mu\text{Ci } ^3\text{H}$ per rat). Bile was collected in pre-tared containers at 0–4, 4–8, 8–24, 24–48, 48–72, 72–96 and 96–120 h post-dose. Urine and faeces were collected at 24-h intervals through 120 h post-dose.

Metabolism in bile duct cannulated male dogs. Two bile duct-cannulated male beagle dogs (about 10–12 kg) were dosed i.v. (2 mg kg^{-1}) and two dogs were dosed p.o. (5 mg kg^{-1}) with [$^3\text{H}/^{14}\text{C}$] P1. Bile was collected in pre-tared containers at predose, 0–4, 4–8, 8–24 and 24-h intervals through 120 h post-dose. Urine and faeces were collected at 24-h intervals through 120 h post-dose. Plasma samples were obtained at 1, 4, 8, 24, 32 and 48 h post-dose.

In vitro metabolism studies

Solutions and reagents for *in vitro* incubations. A cofactor solution was prepared by mixing 4:2:1 (v/v/v) of stock solutions of 50 mM glucose 6-phosphate (0.705 g in 50 ml water), 10 mM NADP $^+$ (0.383 g in 50 ml H $_2$ O) and 14 U ml^{-1} glucose 6-phosphate dehydrogenase. The incubation buffer was prepared by mixing 20:2:12 (v/v/v) of stock solutions of 1 M KH $_2$ PO $_4$ (pH 7.4), 0.1 M EDTA (pH 7.4) and 0.1 M MgCl $_2$ with 8 ml microsomal protein, diluted accordingly to yield a final concentration of 2 mg microsomal protein ml^{-1} incubation.

Microsomal incubations. Male and female human, rat, dog and monkey liver microsomes were prepared following procedures described in the literature (Raucy and Lasker 1991). [^{14}C]P1 was incubated, in duplicate, at 0, 1, 10 and 100 μM with and without NADPH (controls) for 30 and 60 min. Reactions were initiated by the addition of cofactor solution after pre-incubation for 2 min at 37°C. Each incubation vial contained 70 μl cofactor solution, 128 μl incubation buffer (described above) and 2 μl substrate for a total volume of 200 μl . Reactions were terminated with 400 μl 50:50 (v/v) ethanol:acetonitrile, cooled over wet ice and centrifuged. Aliquots (200 μl) of each incubation vial were transferred to HPLC vials for analysis.

Recombinant CYP incubations. Membranes containing recombinant human, rat and dog CYP enzymes were obtained from baculovirus-infected insect cells and prepared by Dr Tom Rushmore, Department of Drug Metabolism, MRL. Duplicate P1 concentrations of 0 and 1 μM were incubated with and without NADPH (controls) for 1 h. Reactions were initiated by the addition of cofactor solution after pre-incubation for 2 min at 37°C. Each incubation vial contained 40 pmol enzyme, 70 μl cofactor solution, 128 μl incubation buffer (described above) and 2 μl substrate in a total volume of 200 μl . Reactions were terminated with 400 μl 50:50 (v/v) ethanol:acetonitrile, cooled over wet ice and centrifuged. Aliquots (200 μl) of each incubation vial were transferred to HPLC vials for analysis.

Hepatocyte incubations. Human, rat, dog and monkey hepatocytes (male and female) were isolated based on a two-step procedure (Pang *et al.* 1997). Suspensions contained approximately 10^6 cells ml^{-1} . Duplicate substrate concentrations of 0, 1, 10 and 100 μM were incubated for 0, 1 and 4 h. Reactions were terminated by addition of 2 vols 1:1 (v/v) ethanol:acetonitrile, cooled over wet ice and centrifuged. HPLC analysis followed as described for microsomal incubations.

Determination of total radioactivity

Total radioactivity amounts in blood, plasma and homogenates of faeces and tissues were determined by combusting triplicate aliquots of the specimen (Packard Model 307 Oxidizer), followed by determination of radioactivity (Packard Tri-Carb Liquid Scintillation Analyzer). Total radioactivity amounts in bile, urine and extracts of *in vitro* and *in vivo* samples for HPLC analysis were determined by mixing duplicate sample aliquots with 5 ml Ultima-M Scintillation cocktail and counting directly in a Beckman Liquid Scintillation Counter (Model LS 3801).

Extraction of radioactivity for HPLC analysis

Rat faeces and tissues were homogenized with 3 vols H $_2$ O and frozen at -20°C until analysis. Aliquots of rat kidney and liver homogenates, and rat and dog plasma were treated with an equal volume of acetonitrile:ethanol (1:1 v/v), vortexed and centrifuged (15 min at 7000 g). Supernatants were transferred into clean tubes, dried under nitrogen at room temperature and reconstituted in HPLC mobile phase for LC-MS/MS analysis.

Liquid chromatography-tandem mass spectrometry analysis

Metabolite identification was conducted by LC-MS/MS. The system consisted of a Perkin-Elmer Sciex API-3000 triple quadrupole mass spectrometer (Concord, Ontario, Canada) interfaced to a Perkin-Elmer HPLC system (Norwalk, CT, USA) equipped with two Series 200 micropumps and a

Perkin-Elmer Series 200 autosampler. Analyte separation was achieved using an Eclipse XDB-C8 column (3.0 mm i.d. $\times$ 15 cm, 3.5 μ m, Hewlett-Packard). Mobile phases were: (A) 5 mM ammonium acetate in 9:1 (v/v) water:acetonitrile and (B) 5 mM ammonium acetate in 9:1 (v/v) acetonitrile:water. The gradient increased linearly from 30 to 70% B in 35 min, and from 70 to 90% B in 2 min, followed by a hold at 90% B for 5 min. The column was equilibrated with the initial conditions for 10 min before the next injection. For analysis of male and female plasma samples, the gradient used was as follows: linear increase from 40 to 70% B in 35 min, and from 70 to 90% B in 2 min, followed by a hold for 5 min at 90% B. The flow was 0.5 ml min⁻¹. The LC effluent was split with one-fifth directed to the mass spectrometer and the remaining directed to a Packard Flow Scintillation Analyzer (Downers Grove, IL, USA). The scintillant (Packard, Ultima Flo-M) flow rate was 1.2 ml min⁻¹ (three times the LC flow) for radioactivity detection. LC-MS and LC-MS/MS experiments were conducted using a Turbo-ion spray interface scanning in the negative-ion mode. The turbo-ion spray interface was operated at source temperature of 350°C and voltage of 5000 V. A declustering potential of 28 V, focusing potential of 350 V and entrance potential of -10 V were used. Nitrogen was used for all the nebulizer, auxiliary and curtain gases.

Determination of M1 formation

In vitro. The percentage of M1 formed *in vitro* was determined from the ratio of AUC of M1 formation over the AUC of P1 metabolized in hepatocytes over 1 h. Studies were conducted at 1 and 2 μ M substrate concentration with sampling every 10 min.

In vivo. The overall fraction of an p.o. dose of P1 that is converted to M1 by both presystemic and systemic mechanisms (f_{po}) was estimated as follows (Pang and Kwan 1983):

$$f_{po} = \frac{AUC_{M1,D_{po}}}{D_{po}} * Cl_{M1},$$

where $AUC_{M1,D_{po}}$ is the area under the M1 plasma concentration-time curve after p.o. administration of the parent drug, P1, and D_{po} is the p.o. dose of P1. The systemic clearance of M1 (Cl_{M1}) was determined experimentally.

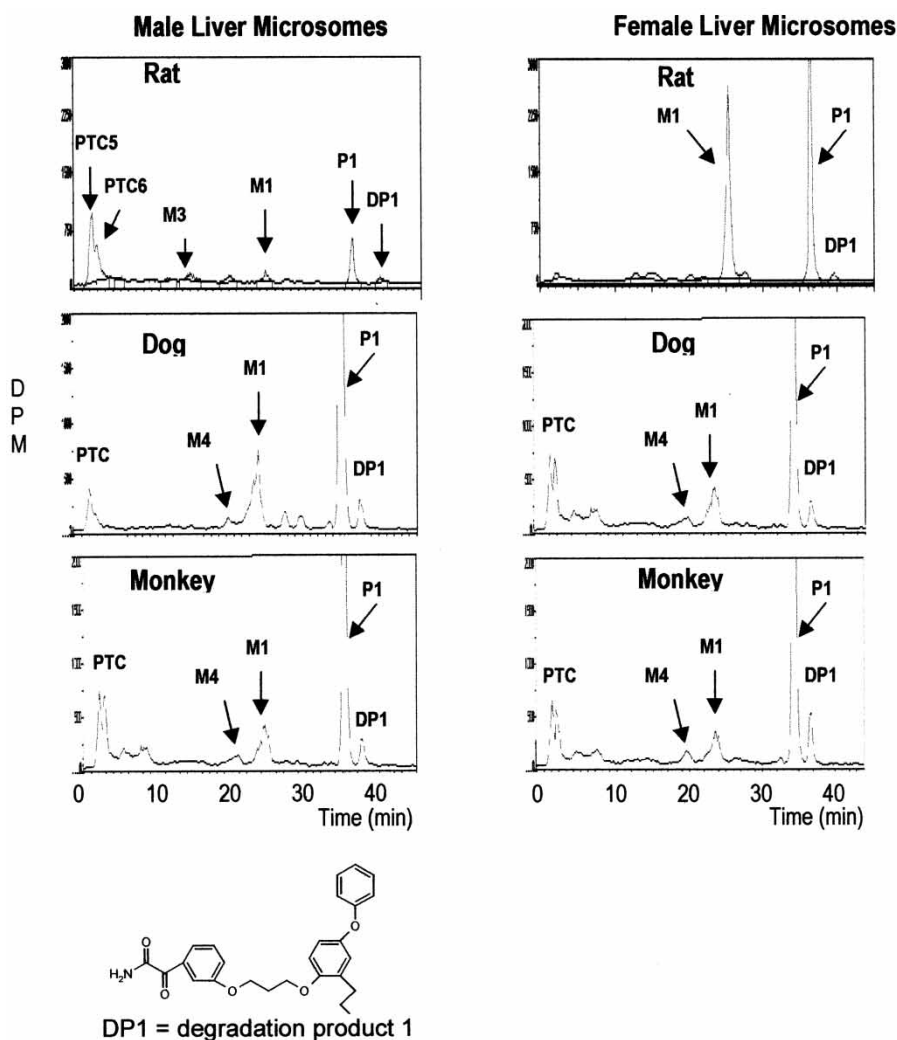
Results

In vitro metabolism

Significant gender differences in the metabolism of P1 were observed in rats, but not in dogs, monkeys or humans (figures 2 and 3). Products of oxidative metabolism of P1 in rat, dog and monkey liver microsomes and hepatocytes included hydroxylation in the *para*-position of the terminal phenyl group (M1) and cleavage at the propyl bridge between phenyl rings (PTC), leading to a series of products, designated as PTC1-6 (figure 1). M1 was a major product of P1 metabolism in female rat, and male and female dog, monkey and human, whereas cleavage at the propyl bridge appeared to be the predominant biotransformation pathway in the male rat (figures 2 and 3). A sulfate conjugate of M1, M2, was observed in hepatocyte incubations with hepatocytes from female rats, and male and female dogs and humans (figure 3).

The amount of M1 formed after incubation of P1 (1 and 2 μ M) with male rat hepatocytes was low and approximately 5% of the total parent metabolized, compared with 57-65% in female rat hepatocytes (table 1). The overall metabolism of P1 in female rat hepatocytes was approximately 50% lower than that observed in male rat hepatocytes at the same substrate concentration. Similarly to what was observed with P1 incubations in female rat hepatocytes, a significant amount of M1 was formed in incubations of P1 with dog (67%), monkey (53%) and human (67%) hepatocytes.

On incubation of M1 (1 and 10 μ M, 60 min) with the male and female rat, dog, monkey and human liver microsomes, less than 5% turnover was observed, indicating that M1 was not a good substrate for oxidative metabolism (data not shown).



PTC1, PTC2 and PTC3 were observed by LC-MS/MS only

DP1 was present in the $-NADPH$ controls, and is probably a degradation product

Figure 2. Metabolism of $[^3H]P1$ in liver microsomes.

P1 was incubated with recombinant rat liver CYPs to determine which enzymes were responsible for its metabolism. The CYP isozymes examined included CYP2C11, 2C12, 2C6, 2C13, 3A2, 3A1, 2A2, 2D1 and 2B1. A summary of the results is shown in table 2. Metabolites generated by the male-specific CYP2C11 corresponded to the products of the cleavage at the propyl bridge between the phenyl rings PTC1–6. Minimal turnover of P1 was observed with the female-specific CYP2C12. CYP2C6 was the only rat CYP to metabolize P1 to M1. CYP3A2 generated mostly PTC4, and turnover by CYP3A1 and CYP2A2 was low. No metabolism was observed in incubations with CYP2D1, 2B1 and 2C13.

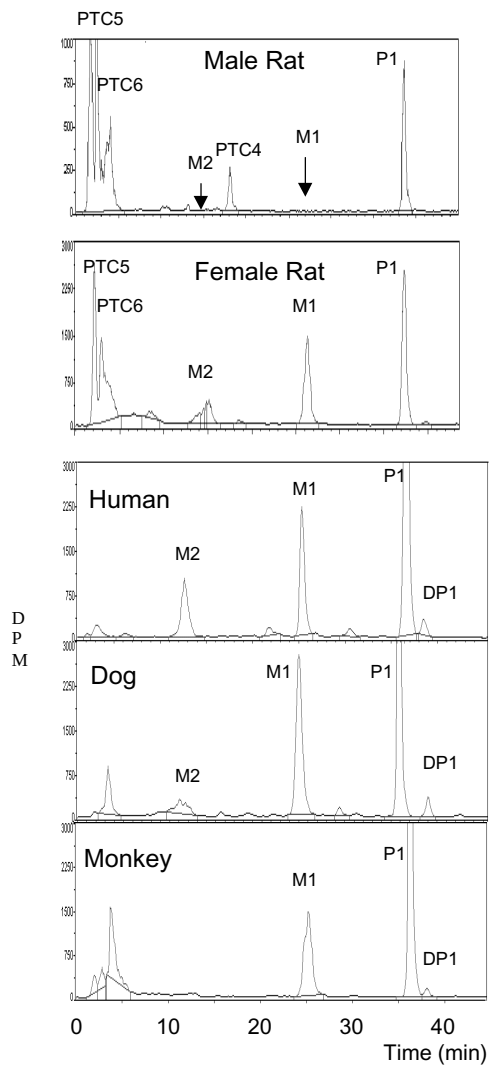


Figure 3. Metabolism of [³H]-P1 in hepatocytes.

Table 1. Estimation of the formation of M1 following incubations in hepatocytes.

Species	P1 concentration (μM)	AUC _(0–60 min) P1 (nM h ^{–1})	AUC _(0–60 min) M1 (nM h ^{–1})	M1 formed (%)
Male rat	1	108	5	4.6
	2	206	9	4.4
Female rat	1	46	26	57
	2	125	76	65
Male dog	1	306	206	67
	2	514	344	67
Male monkey	1	362	194	53
	2	705	367	52
Human	1	103	69	66
	2	195	130	67

Data are means from triplicate determinations.

Table 2. Metabolism of P1 by rat recombinant CYPs.

Isozyme	P1 turnover (%)	M1 formed (pmol)	Other metabolites (pmol formed)	CYP (%) in total microsomal Protein ^a	
				Females	Males
CYP2C11	55	n.d.	PTC1-6 (99)	n.d.	40
CYP3A2	43	n.d.	PTC4 (80), DP1 (16)	< 1	5
CYP3A1	11	n.d.	PTC4 (11), DP1 (11)	< 1	< 1
CYP2C12	9	n.d.	PTC4 (9), DP1 (9)	30	n.d.
CYP2C6	8	16		23	14
CYP2A2	6	n.d.	PTC4 (11)	< 1	2
CYP2D1	0	n.d.	n.d.	unknown	unknown
CYP2B1	0	n.d.	n.d.	< 1	< 2
CYP2C13	0	n.d.	n.d.	n.d.	17

Incubations were conducted with 1 μ M initial concentration of P1 and 200 pmol ml⁻¹ of recombinant CYP for 60 min.

^aCompiled from Thomas *et al.* (1981), Kamataki *et al.* (1983), (1985), Waxman *et al.* (1985), Bandiera *et al.* (1986), Thummel *et al.* (1988), Yamazoe *et al.* (1988), Cooper *et al.* (1993).

n.d., Not detected.

Table 3. Metabolism of P1 by dog and human recombinant CYPs.

Human			Dog		
Isozyme	P1 turnover (%)	M1 formed (pmol)	Isozyme	P1 turnover (%)	M1 formed (pmol)
CYP3A4	47	n.d.	CYP3A26	56	n.d.
CYP2C19	44	80	CYP1A1	40	50
CYP2C8	24	14	CYP3A12	16	n.d.
CYP2D6	4	2	CYP2B11	3	n.d.
CYP1A2	6	n.d.	CYP2C21	3	n.d.
CYP2A6	5	n.d.	CYP2C41	3	n.d.
CYP2B6	7	n.d.			
CYP2C9	6	n.d.			
CYP2E1	6	n.d.			

Incubations were conducted with 1 μ M initial concentration of P1 and 200 pmol ml⁻¹ of recombinant CYP for 60 min.

n.d., Not detected.

Further examination of P1 metabolism was conducted with recombinant dog (CYP3A26, 1A1, 3A12, 2B11, 2C21 and 2C41) and human (CYP3A4, 2C19, 2C8, 2D6, 1A2, 2A6, 2B6, 2C9 and 2E1) liver CYP450s at a substrate concentration of 1 μ M. A summary of results is given in table 3. Significant turnover was observed with dog liver recombinant CYP3A26 (56%), 1A1 (40%) and 3A12 (16%). M1 was only observed as a metabolism product of CYP1A1. Significant turnover of P1 was observed with the human recombinant CYP3A4 (47%), 2C19 (44%) and 2C8 (24%). The enzymes responsible for the formation of M1 were CYP2C8 and 2C19 (figure 4). M1 was a significant component of the metabolism by CYP2C8, and the major metabolite formed by CYP2C19.

In vivo metabolism

No differences in radioactivity levels in blood, plasma, kidney and liver were apparent between male and female rat in the tissues examined following single

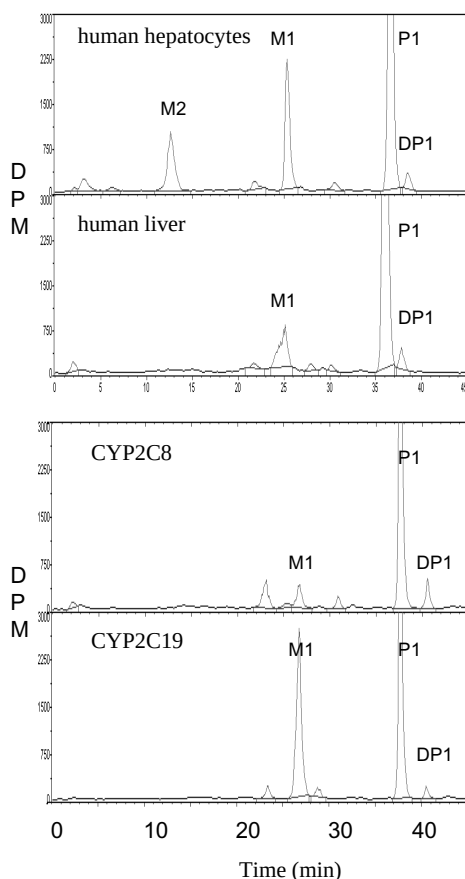


Figure 4. Metabolism of P1 (1 μ M) in human liver microsomes, hepatocytes and recombinant CYP450C8 and 2C19.

(20 mg kg⁻¹) or multiple (1 mg kg⁻¹) dose administration (table 4). Following multiple dose administration at 100 mg kg⁻¹ total radioactivity, amounts were higher in female liver and kidney and could be explained by the presence of M1 in these tissues, a metabolite with a longer elimination half-life than that of P1 (see below). Radioactivity levels were higher in the liver than in kidney and plasma in all treatment groups. Multiple administration did not affect levels at 24 h post-dose.

There were no differences in the elimination of radioactivity between males and females following single dose administration to rats (table 5). Only a small percentage of the radioactivity was eliminated in urine ($\leq 10\%$), and most of the radioactivity was recovered as metabolites in bile, where P1 and M1 were not detected, indicating that the drug was well absorbed and had undergone extensive hepatic metabolism.

The quantitative gender differences in M1 formation observed in rats *in vitro* were consistent with gender differences in levels of M1 found circulating in plasma. Following administration of P1, M1 was a major circulating metabolite in female rat plasma, as indicated by the high proportion of radioactivity attributed to M1 in plasma radiochromatograms, 40–50% at different time points (figures 5 and 6A).

Table 4. Total radioactivity levels in selected specimens following oral administration of P1 to male and female rats.

Dose	Sex	Time (h) postdose	Liver ($\mu\text{g eq. g}^{-1}$)	Kidney ($\mu\text{g eq. g}^{-1}$)	Blood ($\mu\text{g eq. g}^{-1}$)	Plasma ($\mu\text{g eq. g}^{-1}$)
20 mg kg ⁻¹	f	1	92.0	21.6	10.1	25.9
	f	4	45.3	7.8	5.60	10.9
	f	8	34.7	6.1	3.12	5.8
	f	24	5.1	0.6	0.33	0.5
	m	1	86.5	22.3	12.8	26.8
	m	4	43.7	7.1	7.12	7.8
	m	8	30.0	4.2	2.44	4.7
	m	24	7.2	0.6	0.72	1.0
1 mg kg ⁻¹ day ⁻¹ (m.d.)	f	24	0.9	0.2		
	m	24	0.9	0.2		
100 mg kg ⁻¹ day ⁻¹ (m.d.)	f	24	34.9	8.0		
	m	24	68.3	15.8		

m.d., Multiple doses, animals were dosed once daily with P1 at either 1 or 10 mg kg⁻¹ for 14 weeks, the last two days of dosing (dose-days 97 and 98) animals were dosed with radiolabelled compound.

Means of $n = 3$ animals/gender for the 20 mg kg⁻¹ group and $n = 2$ per gender and dose level for the 1 and 100 mg kg⁻¹ groups.

Table 5. Recovery (%) of dose in bile, urine and faeces after administration of P1 to male and female rats.

Specimen	Dose (%)			
	5 mg kg ⁻¹ p.o.		2 mg kg ⁻¹ i.v	
	Male	Female	Male	Female
Bile	69	76	98	105
Urine	1	3	3	1
Faeces	24	26	< 1	< 1
Total	94	105	102	107

$n = 2$.

The proportion of M1 circulating in male rat plasma was significantly less (< 5% of the total radioactivity in plasma). Products of the bridge cleavage between the phenyl rings were the major metabolites of P1 found in the systemic circulation of male rats (60–70% of the total plasma radioactivity). No gender differences were observed in rats in the proportion of parent drug present in systemic circulation (20–40% of the total radioactivity). Similar gender differences were observed in the liver (figure 6B) and kidney (figure 6C) metabolite profiles. Gender differences were not as pronounced for the 100 mg kg⁻¹ group, consistent with pharmacokinetic observations of saturation of first-pass metabolism (data not shown).

Following a single p.o. dose administration of P1 to dogs, approximately 50% of the compound was absorbed and eliminated in bile and only a small fraction of the dose (< 1%) was recovered in urine. The remaining radioactivity was recovered in faeces. P1 and M1 were not detected in bile, indicating that the drug had been extensively metabolized in the liver. Similar to what was observed in female rats, M1 was a major metabolite in dog plasma at all time points evaluated (figure 7). Negligible amounts of PTC were detected.

(Obtained using HPLC Method 1)

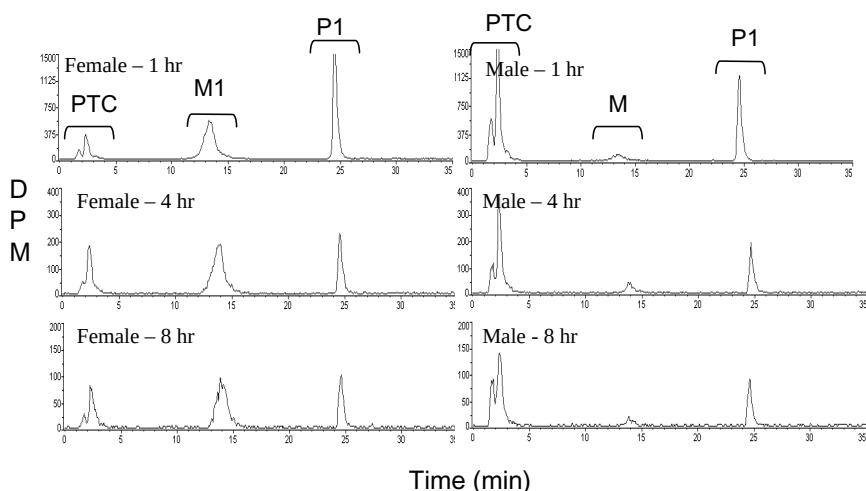


Figure 5. HPLC- ^{14}C -radiochromatograms of plasma extracts after a single oral administration of $[^{14}\text{C}]\text{P1}$ to male and female rats.

Pharmacokinetics

The pharmacokinetics of P1 were similar in all preclinical species examined and were characterized by moderate clearance, low-to-moderate volume of distribution and relatively short half-life, with good p.o. bioavailability ($> 50\%$) (table 6). Plasma AUCs increased proportionally to the dose levels administered following i.v. administration ($1\text{--}5\text{ mg kg}^{-1}$ for female rats, $0.1\text{--}2\text{ mg kg}^{-1}$ for the other species) and greater than dose proportional following p.o. administration ($1\text{--}10\text{ mg kg}^{-1}$ for female rats, $0.2\text{--}2\text{ mg kg}^{-1}$ for the other species).

No significant gender differences were observed in the i.v. and p.o. pharmacokinetics of P1 in rats. Plasma clearance (Cl_p), terminal half-life ($t_{1/2}$) and the volume of distribution (Vd_{ss}) were similar in the two genders (table 6). A summary of the p.o. data is shown in tables 7 and 8. After single dose administration to rats, average AUCs were approximately 25 times greater for the 10-mg kg^{-1} dose than for the 1 mg kg^{-1} dose in both genders. Maximum concentrations of P1 in plasma (C_{max}) were similar between males and females at each dose level. Averages increased approximately 35 times between the 1- and 10-mg kg^{-1} dose. Between 10 and 100 mg kg^{-1} , the increase in C_{max} was less than dose proportional. Averages were approximately four times greater for the 100-mg kg^{-1} dose when compared with the 1-mg kg^{-1} dose, suggestive of slower absorption at the high dose, as indicated by the protracted plasma-time curve (not shown). Bioavailability at 10 mg kg^{-1} was estimated at about 50%.

Significant gender differences were observed in the production of M1 in rats. $\text{AUC}_{(0\text{--}24\text{h})}$ values for M1 after P1 administration were about three to six times higher in female than in male rats at all dose levels (table 7). M1 $\text{AUC}_{(0\text{--}24\text{h})}$ values were approximately dose proportional in male and female rats between 1 and 10 mg kg^{-1} . Evaluations between 10 and 100 mg kg^{-1} could not be made, since the parent drug was still undergoing absorption. $\text{AUC}_{(0\text{--}24\text{h})}$ values for M1 were highest in dogs, being about two, about six and about 20 times higher than in female rat, male monkey and male rat, respectively (table 7). As in the rat,

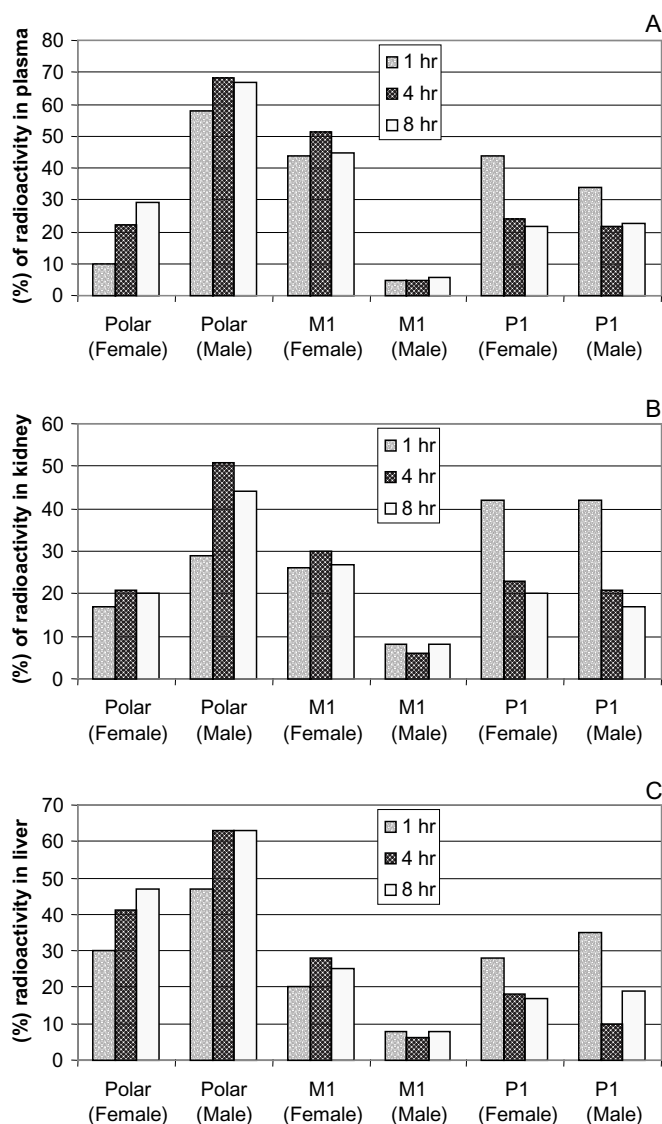


Figure 6. Distribution of radioactivity (% total) between P1 and the major metabolites in plasma (a), liver (b) and kidney (c) versus time after a single oral administration of [^{14}C] P1 at 20 mg kg^{-1} in male and female rats. Data are the means from three animals per gender/time point.

$\text{AUC}_{(0-24\text{h})}$ values of M1 in dog and monkey were approximately dose proportional. A summary of the species differences in systemic exposure to P1 and M1 following p.o. administration at 2 mg kg^{-1} is shown in figure 8. In dogs, multiple administration of P1 for 14 weeks did not result in significant changes in the plasma AUCs of P1 and M1 (table 8). The average exposure ratio of M1:P1 was dose dependent and ranged between 5 and 11 for dogs, between 3 and 5 for monkeys, between 1 and 5 for female rats, and between 0.4 and 0.8 for male rats (table 7). Similar to what was observed in male rats, the amount of M1 in circulation was a small percentage of that of parent for db/db mice and hamsters, species used as *in vivo* efficacy models.

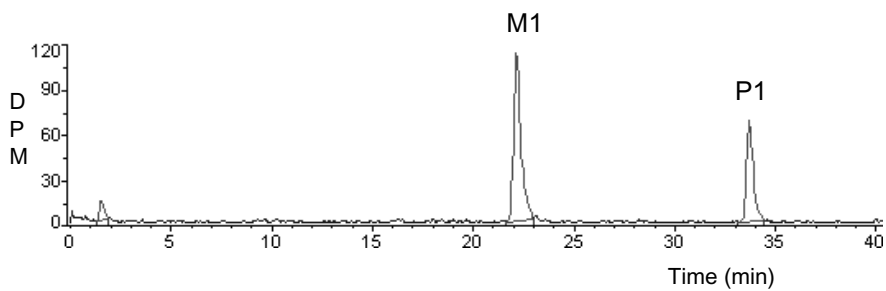


Figure 7. HPLC-¹⁴C-radiochromatograms of pooled plasma extracts after a single oral administration of [¹⁴C]P1 to male dogs (0–24 hr pooled sample).

Table 6. Pharmacokinetics of P1 in preclinical species following i.v. administration.

Species	Dose (mg kg ⁻¹)	Dose adjusted AUC _{inf} (μg h ml ⁻¹)	Cl (ml min kg ⁻¹)	Vd _{ss} (l kg ⁻¹)	t _{1/2} (h)
Male rat	0.1	1.57 (0.12)	11 (1)	1.1 (0.0)	2.5 (0.1)
	0.5	1.44 (0.23)	12 (3)	1.5 (0.1)	2.5 (0.5)
	2	1.67 (0.28)	10 (2)	1.3 (0.2)	2.3 (0.2)
	5	1.85 (0.20)	9 (1)	0.7 (0.1)	1.3 (0.2)
Female rat	1	2.82 (0.18)	6 (0)	0.6 (0.1)	1.3 (0.1)
	5	2.45 (0.10)	7 (0)	0.8 (0.1)	1.7 (0.3)
Dog	0.1	2.9 (0.57)	6 (1)	0.6 (0.1)	2.5 (0.4)
	0.5	2.68 (0.42)	6 (1)	0.6 (0.2)	1.7 (0.3)
	2	3.4 (0.50)	5 (1)	0.5 (0.1)	2.5 (0.9)
Monkey	0.1	2.08–2.55	7–8	0.5–0.5	2.5–2.8
	0.5	1.61–1.92	9–10	0.4–0.6	2.1–2.3
	2	1.72–1.92	9–10	0.5–0.5	1.9–2.7

Data are mean ± SD (*n* = 3) for rats and dogs, and individual values (*n* = 2) for monkeys.

Table 7. C_{max}, Dose-adjusted AUC and M1/P1 AUC ratio in preclinical species following a single oral administration of P1.

Species	Dose (mg kg ⁻¹)	t _{max} (h) P1	C _{max} (μg ml ⁻¹)		Dose-adjusted AUC _(0–24hr) (μg h ml ⁻¹ mg ⁻¹ kg)		AUC ratio M1/P1
			P1	M1	P1	M1	
Male dog	0.2	1	0.07 (0.03)	0.25 (0.07)	0.60 (0.07)	6.50 (0.91)	11.0
	2	1	2.04 (0.58)	3.57 (0.38)	1.40 (0.21)	7.30 (0.24)	5.2
Female rat	1	1	0.12 (0.03)	0.56 (0.18)	0.61 (0.05)	2.99 (0.51)	4.9
	10	1	5.16 (0.94)	7.83 (0.70)	1.52 (0.06)	3.33 (0.21)	2.2
	100	1–4	12.89 (4.90)	33.30 (2.05)	1.30 (0.28)	1.36 (0.33)	1.1
Male rat	0.2	1	0.02 (0.07)	0.01 (0.00)	0.34 (0.08)	0.14 (0.02)	0.4
	1	2	0.15 (0.02)	0.12 (0.01)	0.62 (0.09)	0.47 (0.03)	0.8
	2	1	0.56 (0.14)	0.23 (0.02)	0.75 (0.20)	0.32 (0.04)	0.4
	10	1	4.07 (1.58)	1.82 (0.70)	1.28 (0.25)	0.55 (0.04)	0.4
	100	2	13.60 (1.88)	4.35 (0.70)	1.12 (0.13)	0.46 (0.09)	0.4
Male monkey	0.2	0.5	0.04–0.06	0.08–0.11	0.26–0.26	1.11–1.45	5.0
	2	0.5	0.71–0.91	0.83–1.55	0.47–0.42	1.14–1.70	3.2
Male db/db mice	2	1	1.57	0.39	5.8	1.4	0.2
Male hamster	2	0.5	0.55	0.22	1.53	0.74	0.5
	3	1	0.74	0.38	1.57	0.75	0.5

Data are mean ± SD (*n* = 3) for rats and dogs, individual values (*n* = 2) for monkeys, and serial bleeds (*n* = 3) for db/db mice and hamsters.

Table 8. Dose-adjusted AUC and M1/P1 AUC ratio following multiple oral administrations of P1 to male and female dogs.

Species	Dose level (mg kg ⁻¹)	Dose-adjusted AUC (0–24hr) (µg h ml ⁻¹ mg ⁻¹ kg)		AUC ratio M1/P1
		P1	M1	
Female dog	0.5	1.0 (0.4)	7.4 (4.8)	7.4
	3	1.2 (0.3)	6.3 (1.6)	5.3
	10	1.6 (0.6)	5.8 (1.6)	3.6
Male dog	0.5	1.0 (0.2)	8.2 (0.8)	8.2
	3	1.4 (0.6)	8.2 (3.1)	5.8
	10	1.7 (0.25)	5.8 (1.1)	3.4

Data are mean ± SD (*n* = 6/gender). Animals were dose once daily for 14 consecutive weeks.

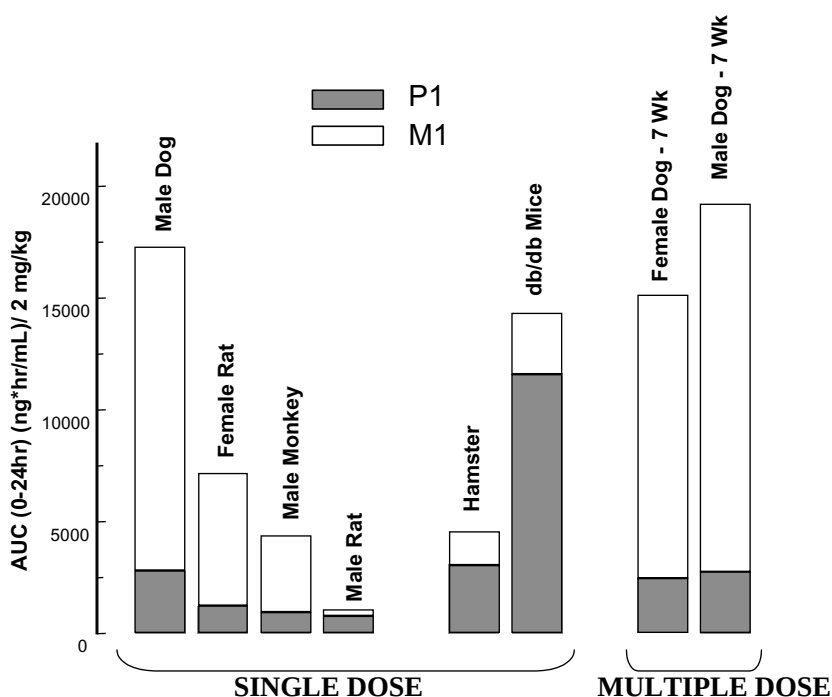


Figure 8. Species differences in systemic exposure to P1 and M1 following oral administration of P1 (dose adjusted to 2 mg kg⁻¹).

The i.v. pharmacokinetics of M1 are shown in table 9. The plasma clearance of M1 was high in male rats (32 ml min⁻¹ kg⁻¹), moderate in female rats and male monkeys (6 and 7 ml min⁻¹ kg⁻¹, respectively), and low in the male dog (2 ml min⁻¹ kg⁻¹). The terminal half-life of M1 was shorter in male rats (4.2 h) than in female rats and male dogs (8 and 10 h, respectively).

The overall fraction of a p.o. dose of P1 that was converted to M1 by both presystemic and systemic mechanisms was calculated as 24–45% in male rats, which was lower than that in female rats (78%) and male monkeys and male dogs (53–57 and 75–85%, respectively) (table 10).

Table 9. Pharmacokinetic parameters for M1 following i.v. administration of M1 to rat, dog and monkey (0.5 mg kg⁻¹).

Species/gender	Compound	AUC _(0-inf) (µg h ml ⁻¹)	Cl _P (ml min ⁻¹ kg ⁻¹)	Vd _{ss} (l/kg ⁻¹)	t _{1/2} (h)
rat/female	M1	1.5 (0.46)	6 (2)	2.6 (0.4)	8.0 (1.5)
rat/male	M1	0.3 (0.05)	32 (6)	5.4 (1.0)	4.2 (0.2)
dog/male	M1	4.3 (0.87)	2 (1)	0.6 (0.2)	10.0 (3.6)
monkey/male	M1	1.1–1.4	7	n.d.	n.d.

Data are mean ± SD (*n* = 3) for rats and dogs, individual values (*n* = 2) for monkeys.
n.d., Not determined.

Table 10. Estimation of the dose (%) converted to M1 after oral administration.

Species	Gender	Dose level	
		0.2 mg kg ⁻¹	2 mg kg ⁻¹
Dog	m	75	85
Rat	f	78	78
Monkey	m	53	57
Rat	m	24	45

Discussion

The similar pharmacokinetics and elimination of P1 in male and female rats failed to explain the gender differences in toxicity observed in this species. Systemic exposure to parent drug following either p.o. or i.v. administration was similar between male and female rats at the dose levels evaluated. As with other glitazones, P1, a Merck-substituted 2,4-TZD containing a phenoxy propyl-phenoxy moiety, underwent extensive hepatic metabolism, followed by biliary excretion of the metabolites, in both male and female rats.

Upon analysis of drug-related components in the systemic circulation, significant gender differences in metabolism of P1 became evident. Though systemic exposure to parent drug was similar in male and female rats, exposure to M1, a *p*-hydroxylated metabolite of P1, was much higher in female than in male rats at the same dose levels. At low dose levels, the exposure ratio of M1:P1 was approximately 0.4 and 5 in male and female rats, respectively. The gender differences in systemic exposure to M1 were not as pronounced at the higher dose levels, probably because of saturation effects of first-pass metabolism.

M1 was also a major component of systemic circulation in dogs, where its exposure was five to 11 times higher than that for P1. As in the rat, exposure to M1 was less than proportional to the dose administered. However, in contrast to rats, no gender differences were observed in dogs. The exposure ratio of M1:P1 observed in monkeys was approximately 3–5, and similar to the values observed for female rats at the same dose levels.

Further evaluation of the pharmacokinetics of M1 revealed significant differences between male and female rats, and between rats, dogs and monkeys. When administered i.v., M1 exhibited a much lower clearance in female than male rats (6 versus 32 ml min⁻¹ kg⁻¹) and a much longer *t*_{1/2} (8 versus 4 h). The clearance of M1 in monkeys (7 ml min⁻¹ kg⁻¹) was similar to that observed in female rats and higher than in dogs (2 ml min⁻¹ kg⁻¹). The longest *t*_{1/2} for M1 was observed in dogs.

The differences in exposure to M1 relative to parent drug observed between male and female rat, and between rat, dog and monkey, are of interest because M1 is a pharmacologically active metabolite. In contrast to parent P1, which is a dual PPAR α/γ agonist (EC₅₀ values of 19 and 5 nM for α and γ , respectively), M1 is PPAR γ -selective with EC₅₀ values of 953 and 30 nM for α and γ , respectively (D. Moller, MRL, personal communication). When the presence of M1 in the systemic circulation is taken into account, then exposure to pharmacologically active material in dogs is much higher than in female rats, followed by monkeys and male rats. If exposure to total pharmacologically active material in the systemic circulation is considered when making inferences on potential drug-related toxicity, then the rank order of exposures to M1 plus P1 in the species evaluated correlated with the severity of the toxicity observed.

The gender differences in exposure to M1 observed in pharmacokinetic studies in rats were confirmed in studies with [¹⁴C]-labelled P1, where it was observed that M1 was the major circulating metabolite in female rats. Interestingly, the total amount of radioactivity and levels of P1 present in systemic circulation were similar in both genders. The balance of the drug-related material in the circulation of male rats was found as products of the cleavage of the bridge linkage between the phenyl groups, these cleavage products being devoid of pharmacological activity (D. Moller, personal communication). Similar gender differences in exposure to drug-related material were observed in liver and kidney. In dogs, M1 was the only metabolite of P1 detected in the systemic circulation.

M1 was not detected in the bile of male rats, female rats or dogs; rather, a sulfate conjugate of M1 and products of cleavage at the propyl linkage between the phenyl rings were the most abundant metabolites.

The gender and species differences in metabolism observed *in vivo* were also observed *in vitro* using liver microsomes and hepatocytes. In male rats, the major products of oxidative metabolism were products of the bridge cleavage between the phenyl rings, whereas in female rats, male and female dogs, and male monkeys the major product of oxidative metabolism was M1. This was reflected in the percentage of M1 formed from P1 in hepatocyte incubations, which was lowest in male rats (approximately 5%), followed by monkeys (approximately 50%), and highest in female rats and dogs (60–75 and 70%, respectively). These values followed the same rank order as the overall fraction of an p.o. dose of P1 that was converted to M1 by both presystemic and systemic mechanisms (24–45, 53–57, 78 and 75–85%, in male rat, male monkey, female rat, and male dog, respectively). The abundance of M1 in the systemic circulation was also determined by its elimination, which appeared to be primarily via sulfation. As previously discussed, the systemic clearance of M1 was much higher in male rats than in other species, with dog having the lowest clearance rate, suggesting that M1 sulfation may be slow in dogs.

From these pharmacokinetic and metabolism studies, it was determined that the ability to extrapolate efficacy and toxicity of P1 between species may be related to differences in metabolism, and specifically formation of M1. Information on the enzymes responsible for the metabolism and sources of potential polymorphism became critical in planning clinical studies, since these events would affect the M1:P1 balance and, thus, the balance of the PPAR α/γ activity and duration of pharmacological activity.

Gender differences in rat metabolism have been known for many years. It is known that some rat CYPs are gender specific (Legrauerend *et al.* 1992, Waxman,

1995) and that the sexually dimorphic, ultradian rhythms in the circulating growth hormone regulate the gender expression of rat hepatic monooxygenases (Agrawal and Shapiro 2000). Using recombinant rat CYPs, it was determined that CYP2C11 was the only major enzyme capable of metabolizing P1 to products of the bridge cleavage between the phenyl rings. Rat CYP2C11 is expressed only in male rat hepatocytes, represents about 40% of the total microsomal protein, and is the major microsomal steroid 16 α -hydroxylase and steroid 2 α -hydroxylase of rat liver (Kamataki *et al.* 1983, 1985). This observation coupled to the low percentage of M1 and abundance of PTC formed *in vitro* and *in vivo* in male rats suggested that CYP2C11 was responsible for the high abundance of PTC and low exposure to M1 in systemic circulation in male rats. None of the liver CYP enzymes present in female rats had significant turnover of P1 to PTC. In addition, from the CYPs present in female rats, only CYP2C6 significantly metabolized M1. Thus, the presence of M1 in female rats was attributed to CYP2C6, an enzyme which is more abundant in female than in male rat liver microsomes (about 23 versus about 14% of the total microsomal protein, respectively; Waxman *et al.* 1985). CYP2C7, an enzyme more predominant in female than in male rat liver microsomes (about 17 versus about 8% of the total microsomal protein; Bandiera *et al.* 1986), and with 70% homology to CYP2C6, was not available for examination. Since CYP2C11 is not present in female rats, it is possible that female-predominant CYP2C6, the major enzyme in the female rat, is responsible for the metabolism of P1 to M1 *in vivo* and *in vitro*.

In addition to CYP2C11, 2D1 and 2D7, other enzymes are either gender specific or gender predominant. CYP2C12, a major female-specific enzyme that constitutes approximately 30% of the total liver microsomal protein (Katamaki *et al.* 1985), elicited a low turnover of P1. CYP3A2, which is more abundant in male than in female rat liver microsomes (about 5 versus 1%, respectively; Yamazoe *et al.* 1988, Cooper *et al.* 1993) generated mostly PTC4. Turnover of P1 by CYP3A1 and 2A2 was low, and given their low abundance in rat liver microsomes (<2% of the total microsomal protein), they are unlikely to be major participants in the metabolism of P1.

In dogs, the recombinant CYP3A26 and 3A12 metabolized P1; however, only CYP1A1 was capable of metabolizing P1 to M1. In humans, the recombinant CYP3A4 metabolized P1, but only CYP2C8 and 2C19 produced M1. The latter is of clinical significance since CYP2C19 is a polymorphic enzyme in humans, with approximately 20% of people in oriental populations lack this enzyme, and some of its substrates are widely used drugs (Hasler *et al.* 1999). If a major route of metabolism of P1 to M1 in humans is via CYP2C19, variable efficacy and safety findings are an expected consequence.

The participation of the CYP2C human enzymes has been demonstrated in the metabolism of other glitazones. CYP2C8 and 3A4 are involved in the metabolism of troglitazone to its quinone-type metabolite (Yamazaki *et al.* 1999), whereas CYP2C8 is the primary enzyme responsible for the hydroxylation and N-demethylation of rosiglitazone, with minor contributions from CYP2C9 (Baldwin *et al.* 1999). Based on the good correlation between the *in vitro* and *in vivo* findings in the rat and the observation that M1 is a major component of the *in vitro* metabolism in humans, it was concluded that this monohydroxylated metabolite would probably be a major circulating species in human plasma. The presence of M1 in the systemic circulation in humans with an exposure ratio of

M1:P1 of about 4 was confirmed in a preliminary clinical study with five healthy volunteers (MRL, unpublished data). Variability in this ratio between patients is likely to cause differences in efficacy at the same dose levels. In addition, the involvement of the specific human enzymes in the formation of M1 and the potential contribution of the polymorphic CYP2C19 will need to be evaluated.

Preliminary estimates of the antihyperglycemic and lipid-altering dose of P1 were based on extensive preclinical animal model studies conducted on its glucose-lowering efficacy, lipid-altering efficacy, and comparison with other TZDs and fibric acid derivatives. Syrian hamsters were used as a model for measuring cholesterol- and triglyceride-lowering effects, and db/db mice were used to determine antihyperglycemic efficacy. In these two animal models, the exposure ratio M1:P1 was < 3 , indicating that the efficacy observed was mostly derived from parent drug. Thus, these models could not be used for predicting the efficacy of P1 in man, since the potential efficacy of P1 in man is affected by the presence of levels of M1 in the systemic circulation, which were four times greater than levels of P1. Moreover, prediction of toxicological effects of P1 in man is challenging because of the varying proportions of M1:P1 in the different preclinical species. It is also unknown if exposures to M1 will have an additive or synergistic effect on the potential toxicity of the compound.

In conclusion, the metabolism of the substituted 2,4- thiazolidine, P1, to M1, a metabolite with biological activity different than that of parent, presents an unusual case for a development candidate drug. The qualitative gender differences observed in the rat and differences between preclinical species create a challenge in understanding and predicting pharmacological and toxicological effects. Management of the differences in exposures coupled to the differential biological activities of M1 and P1 will be a critical issue in the design of clinical studies.

Acknowledgements

The authors thank the following (all MRL): CarolAnn Keohane for assistance with the rat studies; Dr Susan Iliff, Cesare Gai, Bonnie Friscino and Alison Kulick for assistance with the studies in monkeys; Dr William Feeney, Don Hora, Paul Cunningham, Alison Parlapiano and Gina Seeburger for assistance with the studies in dogs; Jianmei Pang and Jiffry Ismail for preparation of the hepatocytes; Dr Tom Rushmore and Dr Magang Shu for providing the recombinant CYPs; Dr Ralph Stearns for outstanding mentorship and stimulating discussions.

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Metabolic fate of pitavastatin, a new inhibitor of HMG-CoA reductase: similarities and difference in the metabolism of pitavastatin in monkeys and humans

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Received 10 January 2003

1. To elucidate any potential species differences, the *in vitro* metabolism of pitavastatin and its lactone was studied with hepatic and renal microsomes from rats, dogs, rabbits, monkeys and humans.

2. With the addition of UDP-glucuronic acid to hepatic microsomes, pitavastatin lactone was identified as the main metabolite in several animals, including humans.

3. Metabolic clearances of pitavastatin and its lactone in monkey hepatic microsome were much greater than in humans.

4. M4, a metabolite of pitavastatin with a 3-dehydroxy structure, was converted to its lactone form in monkey hepatic microsomes in the presence of UDP-glucuronic acid as well as to pitavastatin. These results implied that lactonization is a common pathway for drugs such as 5-hydroxy pentanoic acid derivatives.

5. The acid forms were metabolized to their lactone forms because of their structural characteristics.

6. UDP-glucuronosyltransferase is the key enzyme responsible for the lactonization of pitavastatin, and overall metabolism is different compared with humans owing to the extensive oxidative metabolism of pitavastatin and its lactone in monkey.

Introduction

In general, elevated levels of low-density lipoprotein (LDL)-cholesterol are considered the most important risk factor for coronary artery disease. Lipid-lowering drugs can be divided into 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), bile-acid sequestrants (resins), nicotinic acid, fibrates and probucol. Among these, statins have had a revolutionary impact on the treatment of hyperlipidemia since the discovery of pravastatin. The most effective method of lowering LDL-cholesterol is the administration of an inhibitor of HMG-CoA reductase, a rate-limiting enzyme of the cholesterol synthesis pathway (Brown *et al.* 1993, Havel *et al.* 1995). Pitavastatin is a highly potent inhibitor of HMG-CoA reductase that causes a significant reduction in total serum cholesterol, LDL-cholesterol and triglyceride levels in animals (Suzuki *et al.* 2000, 2001, Aoki *et al.* 2002). In humans, a persistent improvement in serum lipid levels and the safety of pitavastatin has been confirmed in clinical practice, and this

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drug had a successful effect on a patient with familial hypercholesterolemia (Kajinami *et al.* 2000a,b, Saito *et al.* 2002).

Pitavastatin is only minimally metabolized in human hepatic microsomes (Fujino *et al.* 1999a, 2001) and its biotransformation pathways have been identified as lactonization, β -oxidative degradation of the side-chain, hydroxylation of the quinoline ring, and conjugation with β -glucuronic acid and taurine (Kojima *et al.* 1999a). In particular, lactonization is the major metabolic pathway in humans and, interestingly, the lactone form can be converted to an unchanged form non-enzymatically (Fujino *et al.* 1999b, Kojima *et al.* 1999b). Recently, the mechanism of lactonization was clarified in humans, i.e. initial glucuronidation by UDP-glucuronosyltransferase (UGT) and then spontaneous lactonization by an elimination reaction of the glucuronide moiety (Fujino *et al.* 2003).

High bioavailability of pitavastatin was observed in a previous study on several animal species (about 80%), except monkeys (about 20%), indicating that a species difference exists (Fujino *et al.* 1999b). To clarify the species differences in pitavastatin lactone metabolism, *in vitro* phase I and II metabolic reactions were performed in the present study. In addition, the metabolism of pitavastatin metabolites, M3 (5-keto pitavastatin) and M4 (3-dehydroxy pitavastatin), in the presence of UDP-glucuronic acid (UDPGA), was further investigated to clarify the mechanism of lactonization using an LC/MS (LC/MS/MS) method.

Materials and methods

Materials

^{14}C -pitavastatin (2.2 MBq mg^{-1} , 99.8% pure) was synthesized by Amersham Co. Ltd (Little Chalfort, UK). ^{14}C -pitavastatin lactone (2.2 MBq mg^{-1} , 99.0% pure) was generated from the incubation of ^{14}C -pitavastatin with monkey hepatic microsomes by the method described below. Pitavastatin and its metabolites, M3 (5-keto pitavastatin) and M4 (3-dehydroxy pitavastatin), were synthesized at Nissan Chemicals Industries Co. Ltd (Chiba, Japan). Hepatic and renal pooled human and other animal microsomes were purchased from GENTEST (Woburn, MA, USA) and IVT Co. Ltd (Baltimore, MD, USA), respectively. *p*-Nitrophenol and ketoprofen (UGT inhibitors) and UDPGA were purchased from Sigma Chemical Co. (St Louis, MO, USA). β -NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were products of the Oriental Yeast Co. (Tokyo, Japan). All other chemicals and organic solvents were commercially available and of analytical grade from Wako Pure Chemical Industries (Osaka, Japan).

Synthesis of ^{14}C -pitavastatin lactone

The incubation mixture contained ^{14}C -pitavastatin as the substrate ($50\text{ }\mu\text{M}$), monkey hepatic microsomal protein (1 mg ml^{-1}) and 0.5 M UDPGA in 50 mM Tris buffer (pH 7.4) in a final incubation volume of 5 ml , and was incubated at 37°C for 120 min . The reaction was terminated by the addition of methanol and the supernatant fraction was evaporated until dry. ^{14}C -pitavastatin lactone was isolated according to a previous HPLC method (Kimata *et al.* 1998) and was used in the following experiments.

Phase II reaction of ^{14}C -pitavastatin in various microsome in the presence of UDPGA

The incubation mixture in the phase II reaction contained ^{14}C -pitavastatin as the substrate ($2.5\text{ }\mu\text{M}$), hepatic or renal microsomal protein (1 mg ml^{-1}), and 0.5 M UDPGA in a final volume of 0.5 ml 50 mM Tris buffer (pH 7.4), and was incubated at 37°C for 120 min . The reaction was quenched with 0.5 ml methanol, and after evaporation of the supernatant the residue was dissolved in HPLC mobile phase. A sample was injected into a HPLC-radioluminography system (Baba *et al.* 1994, Fujino *et al.* 1999a) and analysed by a bioimaging analyser (BAS-2000, Fuji photo film, Tokyo, Japan) to calculate the ratio of the unchanged form and its metabolites.

Inhibition of pitavastatin lactonization by chemical inhibitors

The inhibition of pitavastatin lactonization was examined using UGT inhibitors. The UGT inhibitors, *p*-nitrophenol (200, 1000 μ M) or ketoprofen (150, 750 μ M), were co-incubated with ^{14} C-pitavastatin (2.5 μ M) in the presence of UDPGA at 37°C for 120 min. Inhibition data were expressed as a percentage of the corresponding controls that were incubated with no inhibitor. All other conditions were the same as described above.

Phase II reaction of pitavastatin, M3 and M4 in monkey microsomes in the presence of UDPGA

To gain a better understanding of the mechanism of lactonization in terms of structural characteristics, the metabolites of pitavastatin, M3 and M4 were determined according to an LC/MS (LC/MS/MS) method. Pitavastatin, M3 or M4 (2.5 μ M) were incubated with monkey hepatic microsomes in the presence of UDPGA at 37°C for 4 h. The reaction was quenched with 1 ml 0.1 M acetate buffer (pH 4)/2-propanol (97/3), and after centrifugation at 10 000 *g* for 2 min the supernatant was injected into the LC/MS (LC/MS/MS) system.

Metabolites were analysed by LC/MS for determination of molecular weights, and by LC/MS/MS for assignment of the structures. A Waters Alliance HPLC system (2690 separation module, 2487 detector, Milford, MA) with a column switching valve (Lab PRO Rheodyne, Western Analytical Products, Murrieta, CA), connected to a Finnigan Mat TSQ-7000 mass spectrometer, San Jose, CA, was used. The operating conditions were: ionization mode, APCI; detected ion, positive; spray voltage, 4.5 kV; sheath gas, N₂; capillary temperature, 250°C; and mass range, *m/z* 200–800. For LC/MS/MS analysis, additional conditions were: collision gas, Ar; collision gas pressure, about 2 mTorr; and collision energy, 25–35 eV. LC separations were performed on a Cosmosil 5C₁₈ column, 4.6 $\times$ 150 mm (Nakalai tesque, Tokyo, Japan), using a gradient elution solvent (A) 0.1 mol l⁻¹ ammonium acetate buffer at pH 4/ acetonitrile (90/10) and (B) (20/80), at a flow rate of 0.6 ml min⁻¹. The linear gradient program was: 20% solvent B for 5 min, changed to 85% solvent B for 40 min, and then held at 85% solvent B for 5 min. Before separation, deprotenation was performed on a Sumi Bio Trap 500 C₁₈ column, 4 $\times$ 30 mm (Sumika Chemical Analysis Service, Osaka, Japan) with 0.1 mol l⁻¹ ammonium acetate buffer at pH 4/isopropanol (97/3), and then introduced into the separation column by a switching valve.

Phase I reactions of ^{14} C-pitavastatin and ^{14} C-pitavastatin lactone in monkey microsomes

The metabolic clearance (Cl) of pitavastatin and its lactone was evaluated using monkey hepatic microsomes in the presence of a NADPH-regenerating system, according to previously described conditions (Fujino *et al.* 1999a). ^{14} C-pitavastatin or ^{14} C-pitavastatin lactone (2.5 μ M) were incubated at 37°C for 120 min. In the determination of the *in vitro* Cl in the phase I reaction, the concentration of the unchanged form in the reaction mixture was calculated from the total radioactivity and the composition of the unchanged form by the HPLC-radioluminography method. The first-order elimination rate constant (*k*) was obtained from the regression line of the substrate, and Cl was calculated using the following equation

$$\text{Cl} = k \times (\text{ml incubation})/(\text{mg microsomes}).$$

In the case of the lactone, pitavastatin spontaneously generated by the hydrolysis of its lactone was calculated as unchanged lactone.

Results

Phase II reaction of ^{14} C-pitavastatin in various microsome in the presence of UDPGA

After the addition of UDPGA, pitavastatin lactone appeared to be the main metabolite (instead of pitavastatin glucuronide) in hepatic microsomes obtained from rats, dogs, rabbits, monkeys and humans (figure 1). Table 1 shows the composition of the unchanged drug and its metabolites. Pitavastatin lactone was produced most (47.4%) in monkeys and in other species (1.8–11.1%). Although the unknown metabolites were negligible in humans, rabbits and rats, they were detected as equal to or more than pitavastatin lactone in rabbits and rats. When pitavastatin was incubated with renal microsomes in the presence of UDPGA, about 20% pitavastatin lactone was detected in human microsomes, but little was detected in monkey and dog microsomes (table 2).

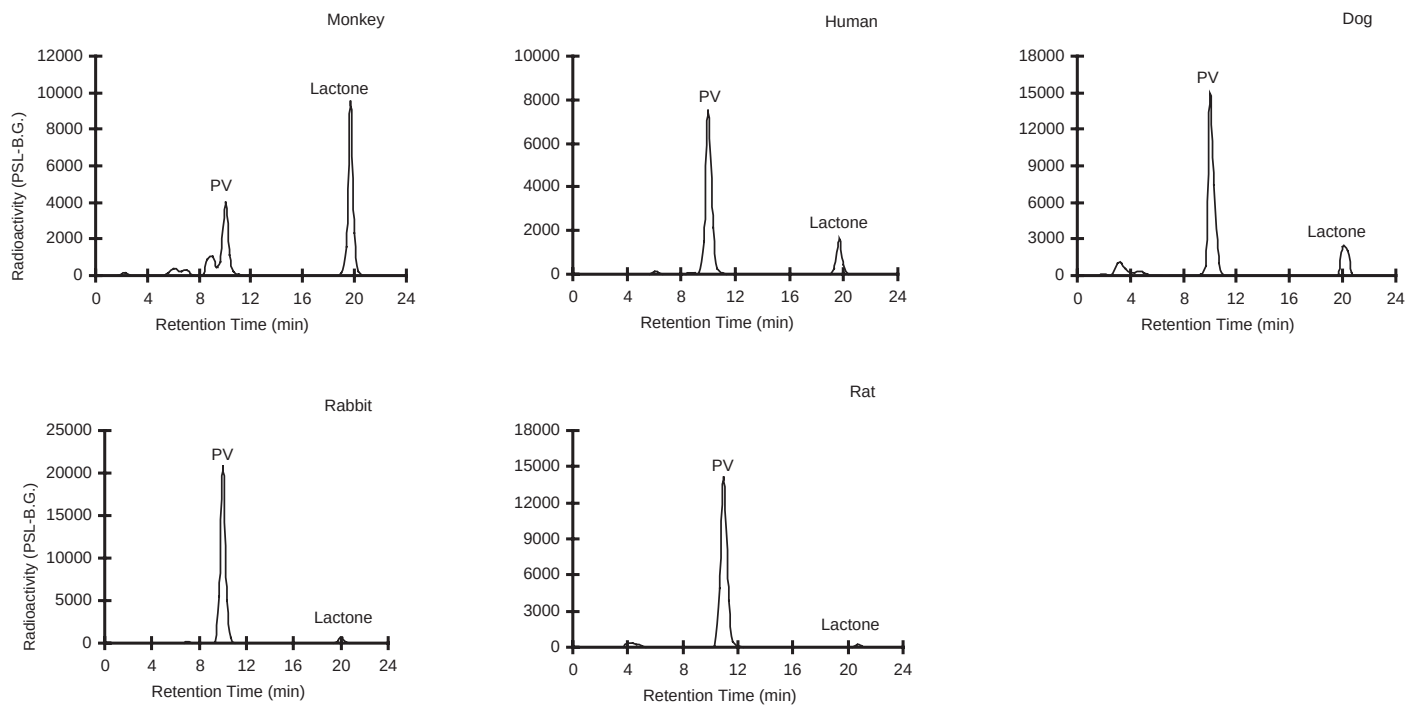


Figure 1. HPLC radiochromatograms of the reaction mixture after a 2-h incubation with pitavastatin with human, monkey, dog, rabbit and rat hepatic microsomes in the presence of UDPGA (phase II reaction). PV, pitavastatin, lactone: pitavastatin lactone.

Table 1. Metabolism of pitavastatin after incubation with hepatic microsomes in the presence of UDPGA.

Species	Percentage composition		
	Pitavastatin	Lactone form	Unknown*
Human	88.6 ± 1.9	9.6 ± 1.5	1.8 ± 0.4
Monkey	32.1 ± 9.0	47.4 ± 2.7	20.5 ± 7.3
Dog	78.8 ± 0.5	11.1 ± 0.2	10.1 ± 0.4
Rabbit	95.7 ± 0.2	2.4 ± 0.1	2.0 ± 0.2
Rat	93.8 ± 0.2	1.8 ± 0.1	4.3 ± 0.2

Values are the mean ± SE of three samples.

*Sum of metabolites excluding lactone.

Table 2. Metabolism of pitavastatin after incubation with renal microsomes in the presence of UDPGA.

Species	Percentage composition		
	Pitavastatin	Lactone form	Unknown*
Human	72.8 ± 0.5	22.1 ± 0.4	4.6 ± 0.4
Monkey	99.2 ± 0.3	0.5 ± 0.1	0.2 ± 0.1
Dog	99.0 ± 0.2	0.5 ± 0.0	0.2 ± 0.1

Values are the mean ± SE of three samples.

*Sum of metabolites excluding lactone.

Inhibition of pitavastatin lactonization by chemical inhibitors

Ketoprofen and *p*-nitrophenol extensively inhibited the lactonization of pitavastatin in human and monkey hepatic microsomes in the presence of UDPGA (figure 2). *p*-Nitrophenol inhibited lactonization in a concentration-dependent manner in monkeys and humans, and ketoprofen modestly inhibited lactonization, particularly in monkeys. These results indicate that pitavastatin lactonization was catalysed by UGT.

Phase II reaction of pitavastatin, M3 and M4 in monkey microsomes in the presence of UDPGA

Chromatographic analysis of incubation mixtures containing pitavastatin revealed three metabolites, and mass spectra gave MH⁺ ions at *m/z* 598, 580 and 404, respectively. The metabolite with a MH⁺ ion at *m/z* 404 was assigned to pitavastatin lactone by LC/MS/MS analysis. LC/MS/MS analysis of the ions at 598 and 580 resulted in the production of daughter ions at *m/z* 422 and 404, indicating the metabolites with MH⁺ ions at *m/z* 598 and 580 were β-D-glucuronides of pitavastatin and pitavastatin lactone, respectively (figure 3 and table 3).

In the case of M3, two metabolites appeared and LC/MS analysis of the metabolites resulted in the same MH⁺ ion detected at *m/z* 596. LC/MS/MS analysis of both ions at *m/z* 596 resulted in the production of the same fragment ions at *m/z* 420, and of the resulting glucuronides of M3, one was the 3-*O*-ether glucuronide and the other the 1-*O*-acyl glucuronide (figure 4 and table 3).

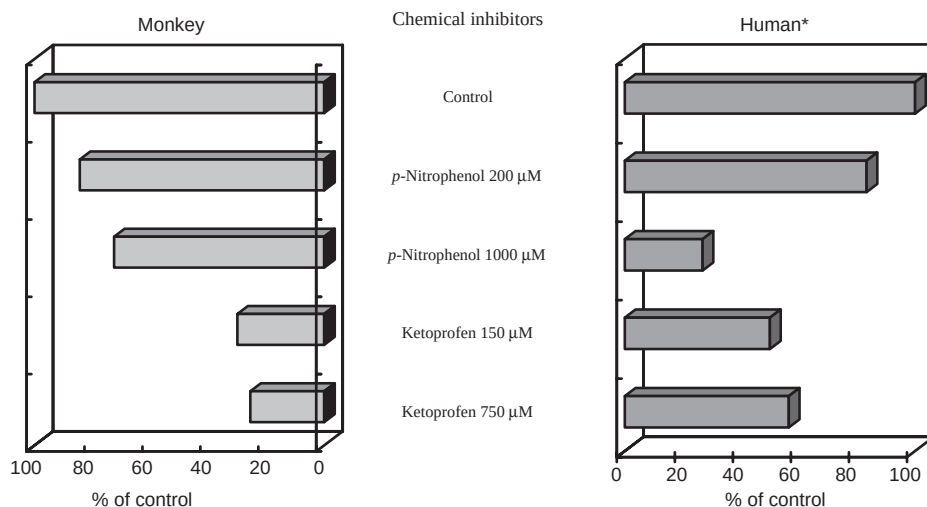


Figure 2. Inhibition of pitavastatin lactonization by *p*-nitrophenol and ketoprofen in monkey and human hepatic microsomes. Data are the average of two determinations. *Quoted from Fujino *et al.* (2003).

LC/MS analysis of the three metabolites of M4 resulted in spectra with MH^+ ions at m/z 582, 582 and 388, respectively. LC/MS/MS analysis of both MH^+ ions at 582 resulted in the detection of fragment ions at m/z 406, indicating glucuronides of M4. The glucuronide linkages of the two metabolites were assumed to be via the ether and ester of M4, respectively. LC/MS/MS analysis of the metabolite with the MH^+ ion at m/z 388 was assigned to the M4 lactone, indicating that lactonization of M4 occurred by the addition of UDPGA (figure 5 and table 3).

Phase I reactions of ^{14}C -pitavastatin and ^{14}C -pitavastatin lactone in monkey microsomes

Phase I reactions of ^{14}C -pitavastatin and ^{14}C -pitavastatin lactone in human and monkey hepatic microsomes were investigated and the representative HPLC-radioluminography is shown in figure 6. After the incubation of pitavastatin in the presence of an NADPH-regenerating system for 2 h, M13 and M14 were formed in monkey hepatic microsomes, and other metabolites were negligible in humans.

In the case of pitavastatin lactone, a number of metabolites were detected in monkey hepatic microsomes. In contrast to monkeys, the lactone was stable in human microsomes. Kinetics studies showed that the metabolic clearances of pitavastatin and its lactone form in monkey hepatic microsomes were 26.8 and 76.1 $\mu\text{l min}^{-1} \text{mg}^{-1}$ protein, respectively (table 4). On the other hand, with the renal microsomes from humans, monkeys and dogs, no metabolites were found (data not shown).

Discussion

Pitavastatin lactone is a major plasma metabolite after intravenous or oral administration of pitavastatin to monkeys, dogs and humans (Fujino *et al.* 1999).

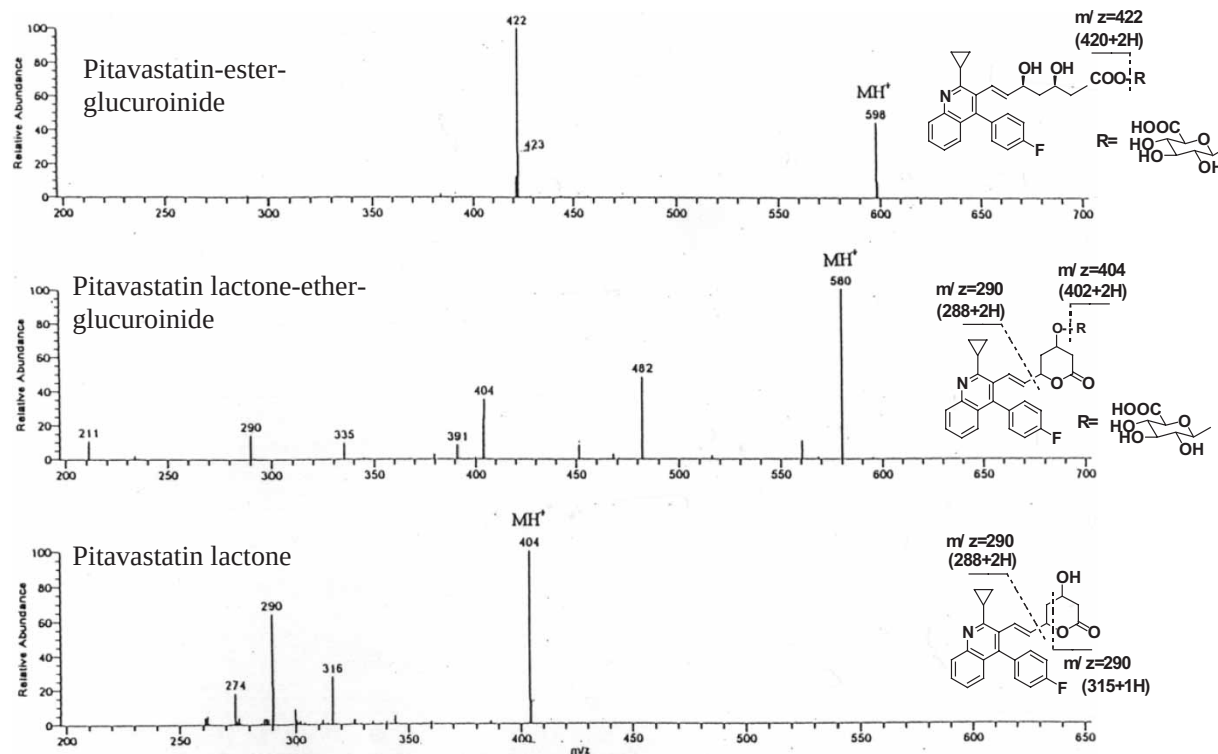


Figure 3. LC/MS/MS spectra of three metabolites derived from pitavastatin after incubation with monkey microsomes in the presence of UDPGA.

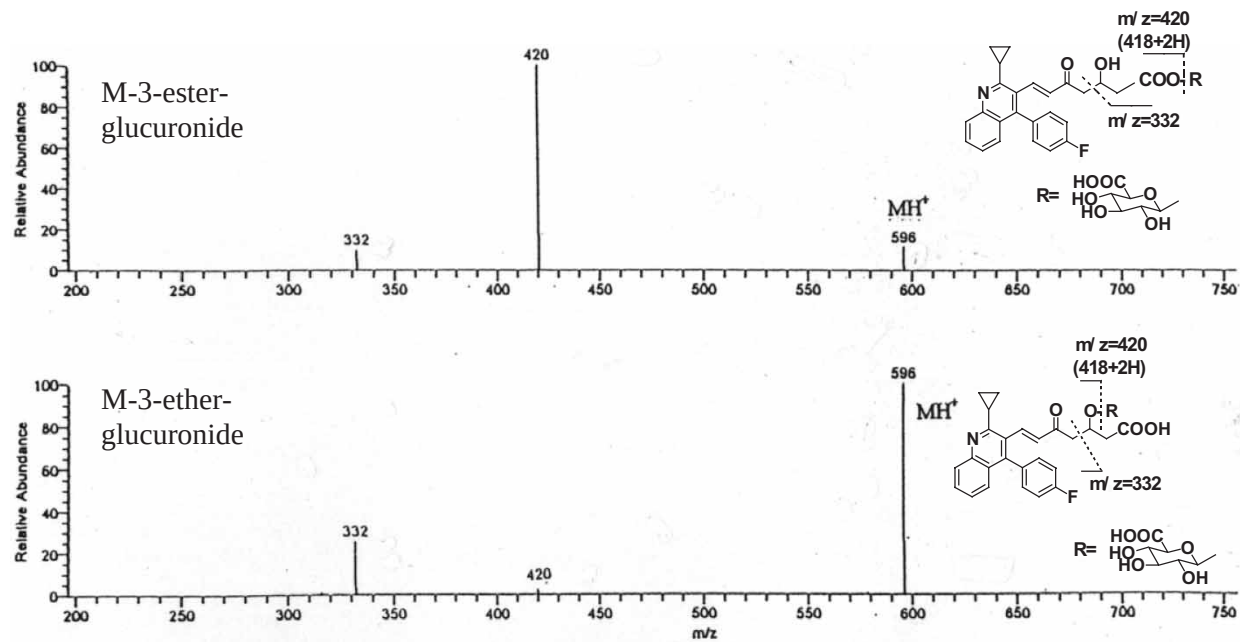


Figure 4. LC/MS/MS spectra of two metabolites derived from M3 after incubation with monkey microsomes in the presence of UDPGA.

Table 3. LC/MS and LC/MS/MS analysis of pitavastatin, M3 and M4.

Substrate	Metabolites	Observed MH ⁺ <i>m/z</i>	MS/MS ions <i>m/z</i>
Pitavastatin	ester-glucuronide	598	422
	lactone ether-glucuronide	580	404
	lactone	404	290
M3	ester-glucuronide	596	420, 332
	ether-glucuronide	596	420, 332
M4	ester-glucuronide	582	406
	ether-glucuronide	582	406
	lactone	388	290

In the case of rats, pitavastatin lactone was detected in bile, but it was not detected in rabbits (Kojima *et al.* 1999a). Furthermore, the hepatic cytochrome P450 system, mainly CYP2C9 in humans, has been reported to be involved in pitavastatin metabolism and the species differences characterized (Fujino *et al.* 2002).

Initially, to obtain information on species difference in lactonization, the metabolism of pitavastatin in hepatic and/or renal microsomes from humans, monkeys, dogs, rabbits and rats, in the presence of UDPGA, was investigated in the current study. Although pitavastatin lactone was detected in hepatic microsomes obtained from all animals, including humans, the formation rates of pitavastatin lactone were different. Additionally, pitavastatin lactone was detected in human renal microsomes, but not in other animal renal microsomes. Lactonization of pitavastatin has recently been regarded as being catalysed by UGT in human hepatic microsomes (Fujino *et al.* 2003). Furthermore, ketoprofen and *p*-nitrophenol, known UGT inhibitors, inhibited lactonization in monkey hepatic microsomes. These results suggest that UGT is responsible for the biotransformation of pitavastatin to pitavastatin lactone, not only in humans, but also in other animal species. Additionally, the activity of pitavastatin lactonization was highest in monkeys, followed by dogs and humans, indicating that the metabolic rates catalysed by UGT differ extensively between monkeys and other animals.

In the present study, the metabolism of pitavastatin and its lactone in human and monkey hepatic microsomes, in the presence of an NADPH-regenerating system, was also investigated. The metabolic clearances of pitavastatin and its lactone in human hepatic microsomes were 3.4 and 4.6 $\mu\text{L min}^{-1} \text{mg}^{-1}$ protein, respectively (Fujino *et al.* 2003). The metabolic clearance of atorvastatin lactone is 83-fold greater than that of its acid form in human hepatic microsomes (Jacobsen *et al.* 2000). Thus, the metabolic characteristics between the acid and lactone forms of pitavastatin are almost similar in human hepatic microsomes, indicating that they are substantially different from atorvastatin. This result suggests that the biotransformation of pitavastatin to pitavastatin lactone is considered not to affect the metabolic clearance of pitavastatin in humans. In the case of monkey hepatic microsomes, the metabolic clearances of pitavastatin and its lactone were eight- and 16-fold greater than in humans, respectively (Fujino *et al.* 2003). Furthermore, the metabolic clearance of pitavastatin lactone in monkeys was threefold greater than that of pitavastatin (Fujino *et al.* 2003), suggesting that the metabolism of pitavastatin in monkeys proceeds mainly via the lactone, which was formed by UGT. These findings indicate that both cytochrome P450 and UGT enzyme systems metabolize pitavastatin and its lactone much more extensively in monkey

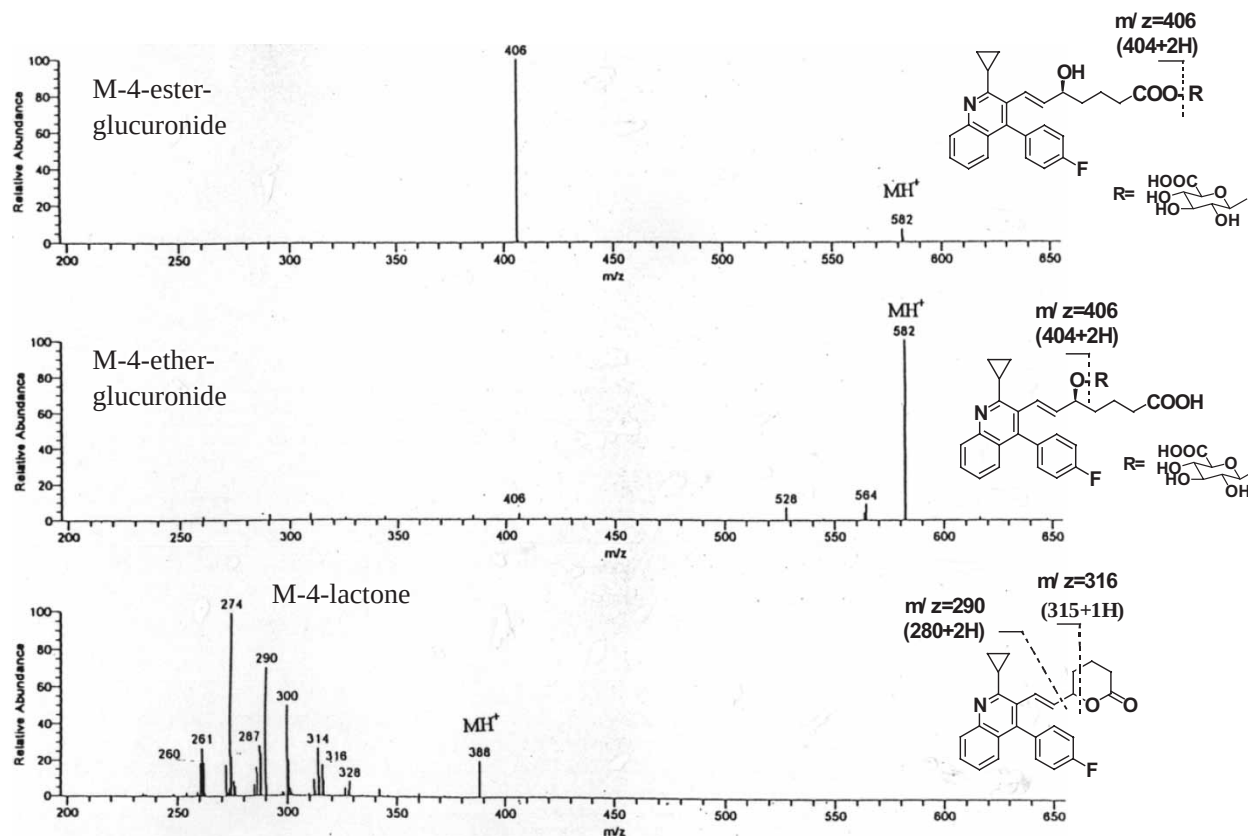


Figure 5. LC/MS/MS spectra of three metabolites derived from M4 after incubation with monkey microsomes in the presence of UDPGA.

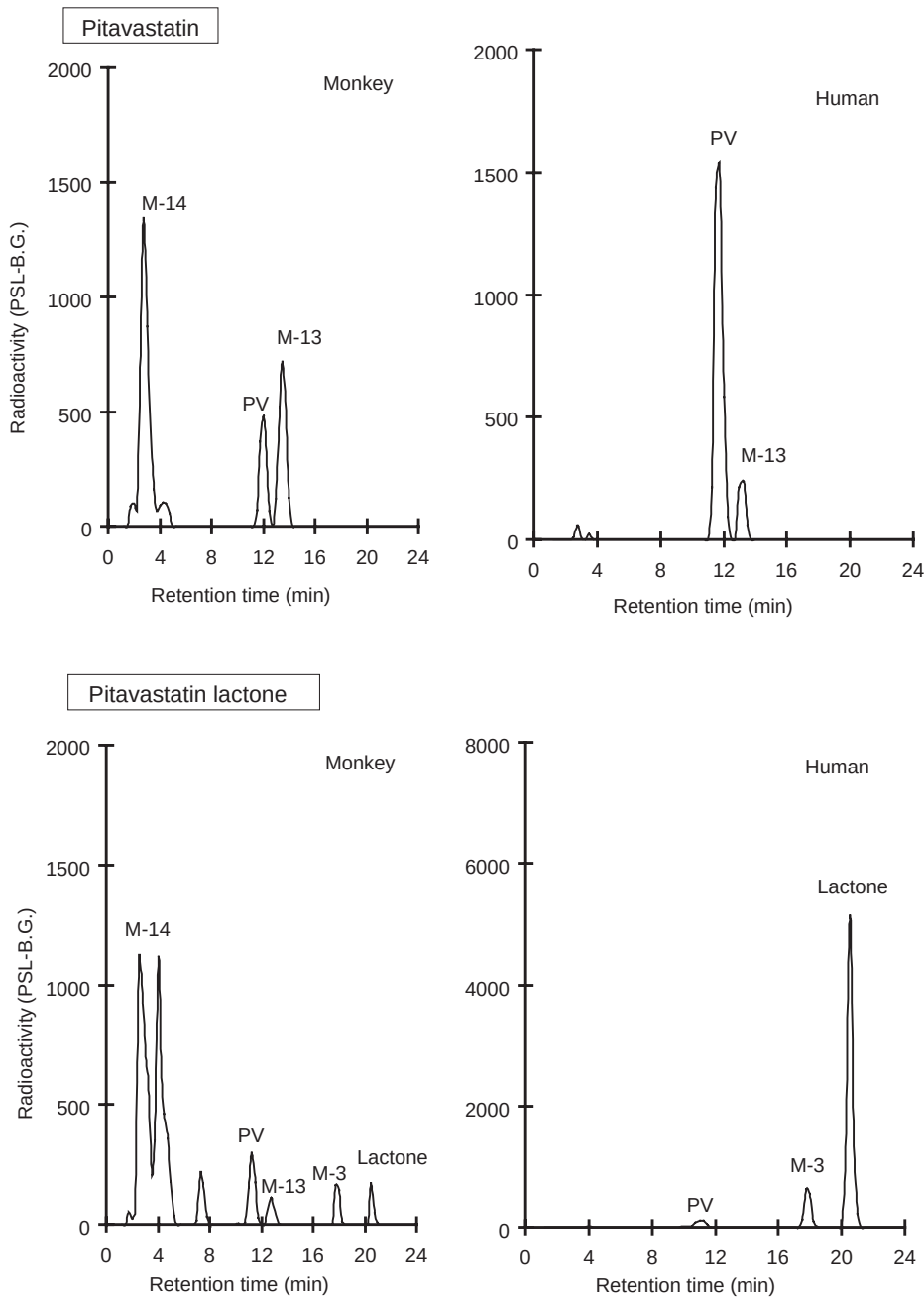


Figure 6. HPLC radiochromatograms of reaction samples after a 2-h incubation of pitavastatin and pitavastatin lactone with monkey and human hepatic microsomes, in the presence of an NADPH-regenerating system (phase I reaction). PV, pitavastatin; lactone, pitavastatin lactone.

hepatic microsomes than in other animal microsomes, suggesting this is the reason for the low bioavailability in monkeys *in vivo*.

Two metabolic pathways of pitavastatin lactone, M13 and M14 formation by CYP2D6-mediated oxidation, and M3 formation by CYP3A4-mediated oxidation

Table 4. Metabolic clearance of pitavastatin and its lactone after incubation with human and monkey hepatic microsomes, in the presence of an NADPH regenerating system.

Substrate	Cl ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{protein}$)	
	Human*	Monkey
Pitavastatin	3.4	26.8
Pitavastatin lactone	4.6	76.1

*Quoted from Fujino *et al.* 2003.

were reported by Fujino *et al.* (2003). In the current study, M14 was mainly observed after the incubation of pitavastatin lactone in the presence of an NADPH-regenerating system by monkey microsomes. Furthermore, a substantial difference in debrisoquine 4-hydroxylation activity exists between humans and monkeys (Weaver *et al.* 1999). Indinavir, an HIV protease inhibitor, has been investigated, and the rate of metabolism mediated by the CYP2D subfamily in monkeys was fourfold greater than in humans (Chiba *et al.* 2000). These results indicate that the substantial species difference in metabolism between monkey and human microsomes may relate to CYP2D activity.

In previous reports, the mechanism of the formation of pitavastatin lactone was suggested to be initial glucuronidation via UGT, and subsequent lactonization by the spontaneous elimination of the glucuronide acid moiety (Fujino *et al.* 2003). In the current study, to re-evaluate the mechanism of lactonization, pitavastatin and its metabolites M3 and M4 (with different side chains) were investigated. Pitavastatin and M4 were good substrate for lactonization, but M3 was metabolized to both the 3-*O*-ether glucuronide and the 1-*O*-acyl glucuronide, but not to the lactone. These findings indicate that the hydroxyl moiety at the 5-position in the side chain, which pitavastatin and M4 both have, is the appropriate structure to produce the lactone form. Prueksaritanont *et al.* (2002) reported that acyl glucuronide formation from the hydroxy acid forms of the statins, simvastatin, atorvastatin and cerivastatin in rat, dog and human liver microsomes *in vitro* undergo cyclization to their lactone forms. Furthermore, the interconversion between the lactone and the carboxylate forms of irinotecan occurs in human plasma (Xie *et al.* 2002), suggesting that lactonization of irinotecan has the same mechanism as statin, related to its 5-hydroxy pentanoic acid-like structure. The present results suggest that drugs containing the 5-hydroxy pentanoic acid-like moiety, such as statins and irinotecan, may be converted to their lactone forms via their acyl glucuronides catalysed by UGT.

The postulated metabolic pathway of pitavastatin is shown in figure 7. It appears that the enzymes responsible for pitavastatin metabolism are the phase I reactions catalysed by cytochrome P450 and lactonization by UGT. *In vivo*, pitavastatin is mainly converted to its lactone by UGT in humans, monkeys and dogs. On the other hand, cytochrome P450 participated in pitavastatin metabolism in all animals, although a species difference in the metabolic rate occurred. For instance, M13 and M14, produced by cytochrome P450, are abundant metabolites in monkeys compared with humans and another animals (Fujino *et al.* 1999a). Interestingly, in rats, β -oxidative metabolites (M6, M8) are major products instead of pitavastatin lactone (Kojima *et al.* 1999a). Thus, the role of the metabolism of pitavastatin by enzymes seems to differ between rodents and non-rodents,

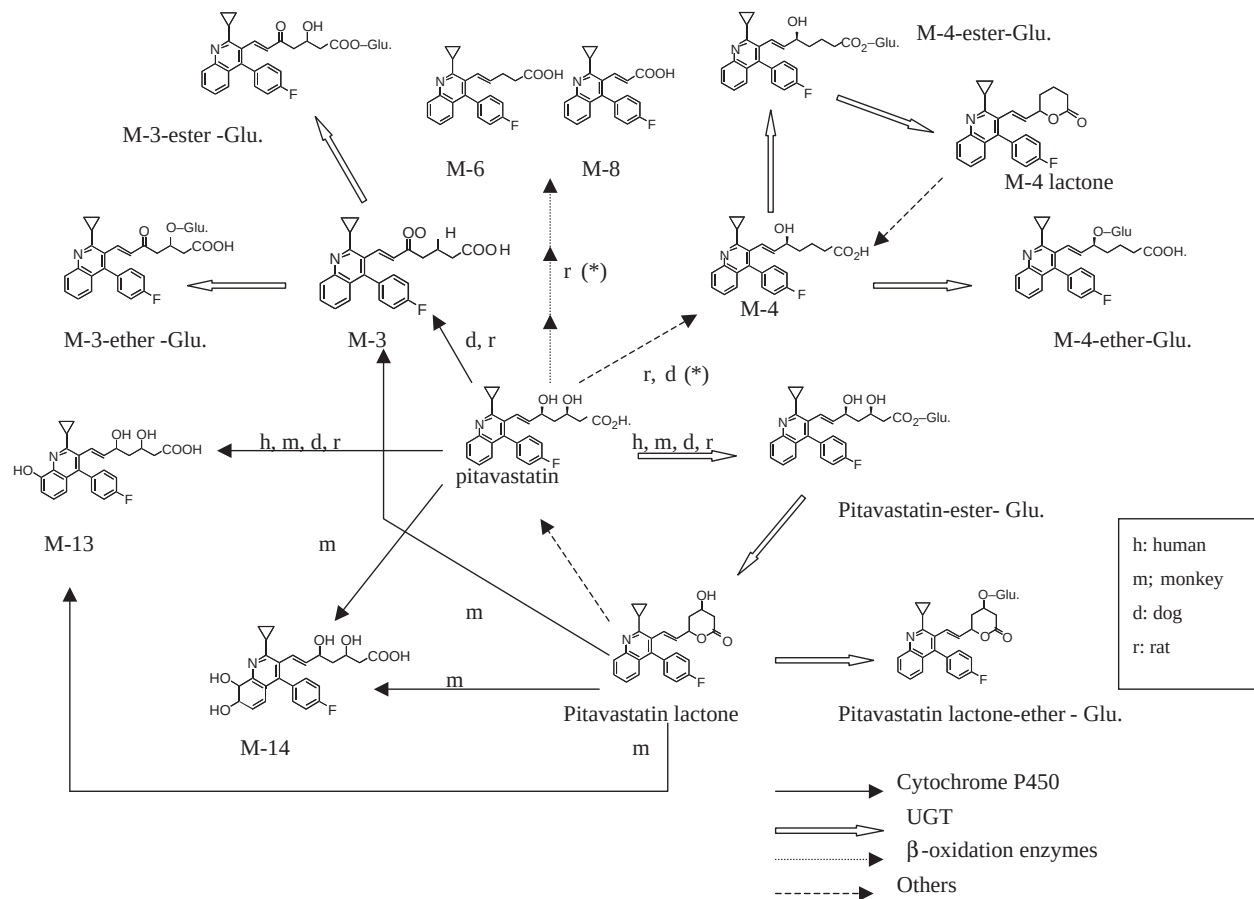


Figure 7. Postulated metabolic pathway of pitavastatin in liver catalysed by cytochrome P450, UGT and β -oxidation enzymes. *Quoted from Kojima *et al.* (1999a).

assuming that the metabolism difference is related not only to cytochrome P450 and UGT, but also to β -oxidation of the side chain.

In conclusion, UGT is the enzyme responsible for pitavastatin lactonization in all animal species, including humans, and the metabolism of pitavastatin by cytochrome P450 and its lactone in monkeys was greater than in humans.

Acknowledgements

The authors are grateful to Ms Keiko Maekawa for technical assistance.

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Comparative metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and dog

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Received 7 February 2003

1. There is a significant species difference in the toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D). The oral no overall adverse effect level (NOAEL) for chronic toxicity of 2,4-D in rat is $5 \text{ mg kg}^{-1} \text{ day}^{-1}$ and in dog is $1 \text{ mg kg}^{-1} \text{ day}^{-1}$. The maximum tolerated dose (MTD) in rat is 150 and $75 \text{ kg}^{-1} \text{ day}^{-1}$ for male and females, respectively. The MTD in dog is $7.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ for males and females.

2. In an attempt to explain the increased sensitivity to 2,4-D in dog, male and female rats and dogs were orally dosed with either 5 or $50 \text{ mg kg}^{-1} \text{ }^{14}\text{C}$ -2,4-D. The rates and routes of excretion were investigated along with plasma toxicokinetics and biotransformation of the compound.

3. Elimination of the radioactive dose of 2,4-D from rat plasma was significantly faster than in dog. The approximate $t_{1/2}$ were 1.3–3.4 h for rat and 99–134 h for dog following a 5 or 50 mg kg^{-1} dose, respectively. This led to large differences in the calculated $\text{AUC}_{(0-\infty)}$ $21\text{--}57 \mu\text{eq. h g}^{-1}$ for rat and $4889\text{--}5298 \mu\text{eq. h g}^{-1}$ for dog at 5 mg kg^{-1} , and $122\text{--}2358 \mu\text{eq. h g}^{-1}$ for rat and $34\,110\text{--}44\,296 \mu\text{eq. h g}^{-1}$ for dog at 50 mg kg^{-1} .

4. In rat, the major route of excretion was in the urine. Excretion was essentially complete after 24 h for the low dose and after 48 h for the high dose. For dog, elimination was incomplete over the sampling period with only about 50% of the dose recovered. Urine was the principal route of excretion at the low dose, but about equal amounts were excreted in urine and faeces at the high dose over 120 h.

5. In rat, 2,4-D was unmetabolized and excreted in urine as the parent compound. In dog, the dose was excreted mainly following metabolism. 2,4-D in dog was conjugated forming the taurine, serine, glycine, glutamic acid, cysteine, sulphate and glucuronide conjugates, plus an unidentified metabolite, which were excreted in urine. Plasma, however, only contained unmetabolized 2,4-D.

6. The results show that the body burden of 2,4-D in dog is significantly higher than in rat for an equivalent dose, which is consistent with the increased sensitivity of dog to 2,4-D toxicity.

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D, CAS No. 94-75-7) is a plant growth regulator type herbicide that was introduced in 1946 and rapidly became the most widely used herbicide in the world. The compound shows a marked species difference in toxicity. The overall chronic (24-month) no overall adverse effect level (NOAEL) in rat was $5 \text{ mg kg}^{-1} \text{ day}^{-1}$, and the maximum tolerated dose (MTD) was $75 \text{ mg kg}^{-1} \text{ day}^{-1}$ for female and $150 \text{ mg kg}^{-1} \text{ day}^{-1}$ for male rats

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(Charles *et al.* 1996a). In dog, the overall NOAEL was $1 \text{ mg kg}^{-1} \text{ day}^{-1}$ and the MTD was $7.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (Charles *et al.* 1996b, c). The toxicological symptoms between rat and dog were markedly different. The MTD in rats and dogs was based primarily on body weight loss, but with little or no organ pathology. In the case of the dog, however, at dose levels above $10 \text{ mg kg}^{-1} \text{ day}^{-1}$, there was an occurrence of severe systemic toxicity as follows: increased mortality, body weight loss, reduced food consumption, anaemia, increased relative kidney weights and cellular alterations in the proximal convoluted tubules of the kidneys.

The toxicological profile of 2,4-D is much like that of 4-chloro-2-methylphenoxyacetic acid (MCPA), where the chronic NOAEL of MCPA in rat is $1.3 \text{ mg kg}^{-1} \text{ day}^{-1}$ and for dog is $0.2 \text{ mg kg}^{-1} \text{ day}^{-1}$. A previous study was performed to investigate whether there was any relationship between the comparative metabolism and toxicokinetics of MCPA in rat and dog, which might explain the differences in toxicity between the species (Lappin *et al.* 2002). The latter study showed that the dog was deficient in its ability to eliminate MCPA and/or its metabolites from plasma with concomitant slower excretion in urine and faeces. The present study was designed to investigate whether similar conclusions could be drawn for 2,4-D.

2,4-D was orally dosed to male and female Fischer rats and dogs. The Fischer strain of rat was chosen as it was used in the above toxicology studies. Animals were dosed at either 5 or 50 mg kg^{-1} . The lower dose was chosen as it was equal to the NOAEL in rat and was within the chronically tolerable range for the dog. The upper dose was chosen as it was approximately the mean chronic MTD for rats and tolerated as a single dose by the dog, although it was well above the tolerated dose for repeat administration in dog.

Materials and methods

Chemicals

2,4-Dichlorophenoxy acetic acid (2,4-D, purity 99%) and ^{14}C -2,4-D (universally labelled in the aromatic ring, specific activity $9.206 \text{ MBq mg}^{-1}$, radiopurity 99%) were obtained from BASF (Ludwigshafen, Germany). General-purpose reagents and solvents of Analar grade were obtained principally from Merck Ltd (Poole, UK). Liquid scintillants (Emulsifier-Safe[®], Hionic-Fluor[®], Ultima flo[®]) were obtained from Canberra Packard (Pangbourne, UK). Permafluor[®] E+, Carbo-Sorb[®] and (^{14}C)-Spec Chec[®] for use in the sample oxidizer were obtained from Canberra Packard. (^{14}C)-toluene was obtained from Amersham Pharmacia Biotech (Aylesbury, UK).

Animals

Twenty-two male and 22 female Fischer (CDF Cr1:BR(F-344)) strain rats (158–253g, 6–12 weeks old) were obtained from Charles River (UK) Ltd (Margate, UK). Rats were fed water and rat diet (SQC rat and mouse maintenance diet No. 1, Special Diet Services, Stepfiled, UK) *ad libitum*. Four male and four female Beagle dogs (about 6–12 months of age and 9–15 kg) were obtained from Bantin and Kingman Universal Ltd (Hull, UK). Dogs were fed 400 g dog maintenance diet daily (SQC Laboratory Diet A Special Diets Services) and water *ad libitum*.

Dosing

Rats were given an oral dose, by gavage, of either 5 or 50 mg kg^{-1} body weight ($4\text{--}5 \text{ MBq kg}^{-1}$) ^{14}C -2,4-D, suspended in 1% aqueous carboxymethylcellulose (CMC). Dogs were given an oral dose, by capsule, of 5 mg kg^{-1} (4 MBq kg^{-1}) ^{14}C -2,4-D dried onto CMC. After a 70-day washout period, the same dogs were given an oral dose of 50 mg kg^{-1} (4 MBq kg^{-1}) ^{14}C -2,4-D. ^{14}C -2,4-D was stable in the dosing preparation over the dosing period.

Sample collection

Eight male and eight female rats (four per sex per dose level) were group housed, by sex, in wire-floored cages. Blood (about 200 μ l) was sampled from the lateral caudal vein at the following times after dosing: 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 h.

Another eight male and eight female rats (four per sex per dose level) were housed in separate glass metabolism cages. Urine and faeces were collected at the following times after dosing: 12, 24, 48, 72, 96 and 120 h.

Six male and six female rats were group housed, by sex, in wire-floored cages. Three male and three female rats received the 5 mg kg⁻¹ dose and were killed and exsanguinated 4 h after dosing. The remaining rats, which received the 50 mg kg⁻¹ dose, were killed and exsanguinated 8 h after dosing. Blood was centrifuged to produce plasma, which was used in the determination of protein binding (below).

Four dogs were housed in metabolism cages. Blood (about 10 ml) urine and faeces were collected at the same time points as those for rats.

For both species, collection vessels for excreta were cooled by solid carbon dioxide. Where animals were housed in metabolism cages, the cages were rinsed with water after each collection of excreta. At the end of experiment, after the animals had been removed, the cages were rinsed with methanol.

Radioanalysis

Blood samples were centrifuged to produce plasma. Portions of urine, plasma and cage washings were added directly to Emulsifier-Safe[®] scintillant and assayed by liquid scintillation counting. Faeces were homogenized in an appropriate volume of deionized water and added to ashless floc in Combusto Cones[®] and combusted in oxygen using a Packard Sample Oxidizer. The combusted products were absorbed in Carbo-Sorb[®], mixed with Permafluor[®] E⁻, and the radioactivity determined by liquid scintillation counting. Radioassays were performed at least in duplicate. Radioactivity was measured for 5 min or 1% 2 σ (95.5% confidence level) using Packard Tri-Carb liquid scintillation counters (Canberra Packard) with the facilities for computing quench-corrected disintegrations per minute (dpm). Efficiency correlation curves were prepared for organic, aqueous and combusted sample types and was routinely checked by the use of (¹⁴C)-toluene standards.

Calculation of toxicokinetic data

Non-compartmental toxicokinetic evaluations of plasma data were performed with the aid of WinNonlin V3.0 (Pharsight Corporation, Camino Real, Mountain View, CA, USA). The input consisted of: sample time (h); sample concentration; units of concentration; dose administered; and units of dose administered. Blood and plasma half-lives of total radioactivity following administration of test article were calculated by regression analysis of the linear portion of the individual concentration-time curves when plotted semilogarithmically. The area under the plasma concentration-time curves (AUC) were calculated using the trapezoidal rule.

Identification of metabolites

Samples of urine were centrifuged to remove particulate matter and subjected to HPLC analysis. For the rat and dog, individual urine samples were analysed for males and females at both dose levels. Owing to the amounts of rat blood sampled and levels of 2,4-D-related compounds contained therein, it was not possible to analyse rat plasma by HPLC or LC/MS. Dog plasma was analysed, however, from the low-dose group at the 12-h time point. Before HPLC, plasma protein was precipitated with acetonitrile and centrifugation. Radiolabelled and non-radiolabelled 2,4-D were used as authentic standards. The HPLC column was a Zorbax SB-C8 (250 $\times$ 4.6 mm i.d., particle size 5 μ m). The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Solvent A was held at 70% for 10 min, then a linear gradient to 40% A, 60% B over 10 min then to 100% B over 1 min, which was held isocratically for 5 min. The flow rate was 1 ml min⁻¹ and detection was by UV at 230 nm and by radiodetector using a 500- μ l liquid cell.

Mass spectra were acquired using a Finnigan LCQ Deca quadrupole ion-trap mass spectrometer with electrospray LC-MS interface coupled to a HP 1100 binary gradient HPLC system and a radiodetector. The flow was split 15:1 into the mass spectrometer. Negative-ion electrospray (ESP)-mass spectrometric analysis was carried out with a capillary temperature of 250°C, a capillary voltage of -7 V and a spray voltage of 4.8 kV. The scan range was from m/z 125-600.

Plasma protein binding

Plasma protein binding was determined using equilibrium dialysis (Spectrum® Equilibrium Dialyser using 12–14 K MWCO Spectra/Por® precut membranes). Non-specific binding and equilibration time were determined using 0.1 and 100 µM 2,4-D in 0.02 M sodium phosphate-buffered saline (0.9% w/v, pH 7.4) incubated at about 37°C for up to 6 h. Non-specific binding was <10% and equilibrium time was 4 h. Plasma taken 4 or 8 h after dosing from rats and dogs was pooled by species and dose group and analysed for total protein, albumin and α1-acid glycoprotein. Portions of each pooled plasma sample were used to determine the plasma protein binding using equilibrium dialysis over 4 h.

Results

For rat, the mean total recovery of radioactivity (urine, faeces and cage washings) was between 95.49 and 102.21% for all dose groups. Mean total recoveries for the dog ranged from 41.89 to 50.60% over the time course of the sample collection (table 1).

For rat, the vast majority of the radioactivity, irrespective of dose level or sex, was excreted in urine (86.77–99.36%) with only 2.32–8.26% in faeces. It was likely that the radioactivity detected in rat faeces arose from contamination with urine in the metabolism cages during sample collection. Excretion was essentially complete 24 h after dosing (figure 1).

For dogs administered 5 mg kg⁻¹ ¹⁴C-2,4-D, the majority of the excreted dose was found in urine (37.57–38.21%) with 10.08–13.03% in faeces (table 1). For dogs administered 50 mg kg⁻¹ ¹⁴C-2,4-D, about equal amounts of radioactivity were excreted in urine (20.62–23.02%) and faeces (21.27–25.00%). Excretion was incomplete after the final, 120-h sampling time, which almost certainly explains the lower recoveries in dog. For the dog, there were no apparent sex differences in rates or routes of excretion (figures 2 and 3).

Radioactivity was rapidly eliminated from rat plasma. The *t*_½ was similar for males and females at the 5 mg kg⁻¹ dose (1.7 and 1.29 h, respectively), but was slightly longer for females at the 50 mg kg⁻¹ dose (1.5 and 3.35 h for males and females, respectively) (table 2).

These results were in sharp contrast to those in dog, where the very much increased *t*_½ were 99.3 and 107 h for males and 104 and 134.4 h for females at

Table 1. Recovery of radioactivity, as the per cent of administered dose from rats and dogs orally dosed at either 5 or 50 mg ¹⁴C-2,4-D kg⁻¹ body weight.

Sample	5 mg kg ⁻¹		50 mg kg ⁻¹	
	Male	Female	Male	Female
Rat				
Urine*	99.36 ± 17.31	96.83 ± 16.15	98.68 ± 13.06	86.77 ± 15.84
Faeces	2.36 ± 2.90	3.36 ± 2.23	2.32 ± 0.57	8.26 ± 4.61
Carcass	0.49 ± 0.049	0.40 ± 0.12	0.75 ± 0.52	0.46 ± 0.16
Total recovery	102.21	100.59	101.75	95.49
Dog				
Urine*	37.57 ± 11.41	38.21 ± 9.55	23.02 ± 6.29	20.62 ± 4.60
Faeces	13.03 ± 1.95	10.08 ± 1.20	25.00 ± 1.91	21.27 ± 2.96
Total recovery	50.60	48.29	48.02	41.89

*Urine includes cage washings.

Data are the mean ± standard deviation from four animals per group.

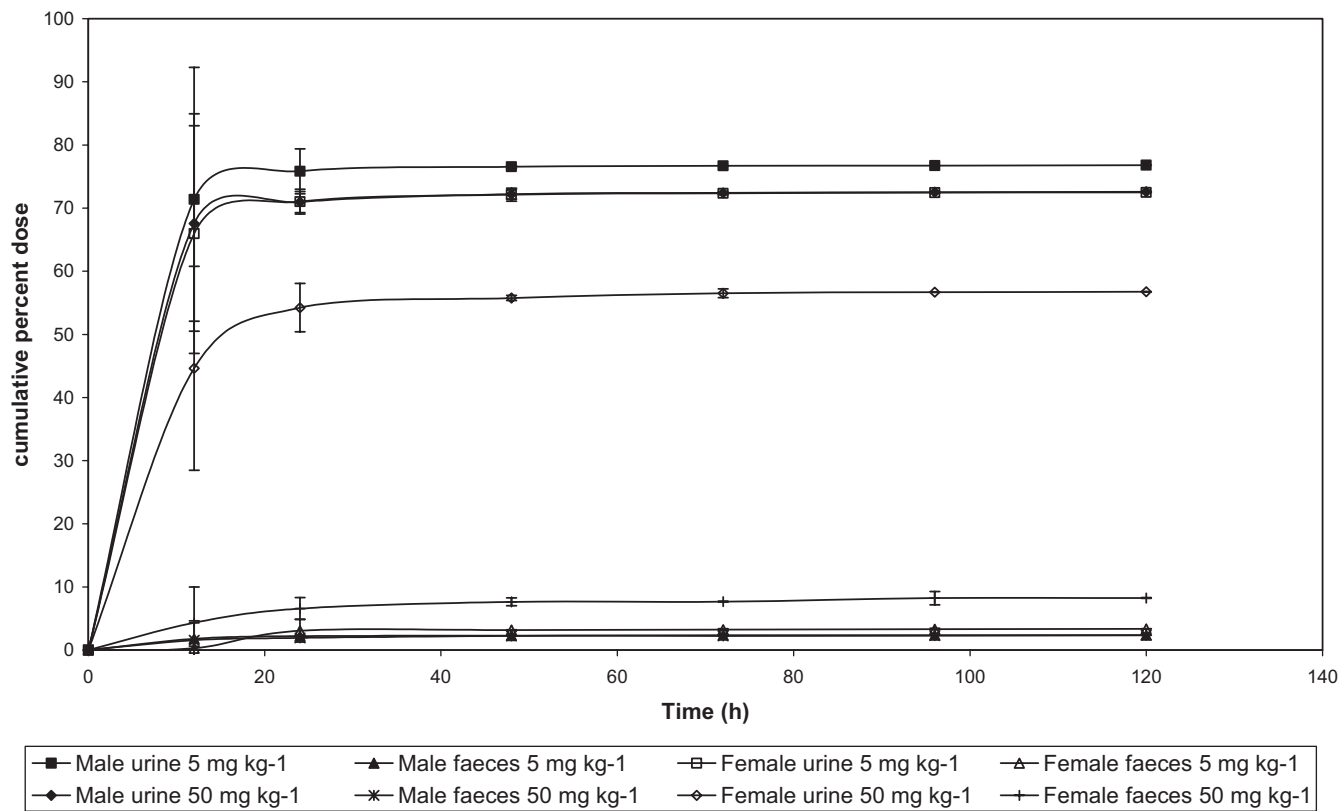


Figure 1. Excretion of radioactivity in urine and faeces from rats orally dosed at either 5 or 50 mg ^{14}C -2,4-D kg $^{-1}$ body weight. Error bars are $\pm$ standard deviation ($n - 1$) from four animals.

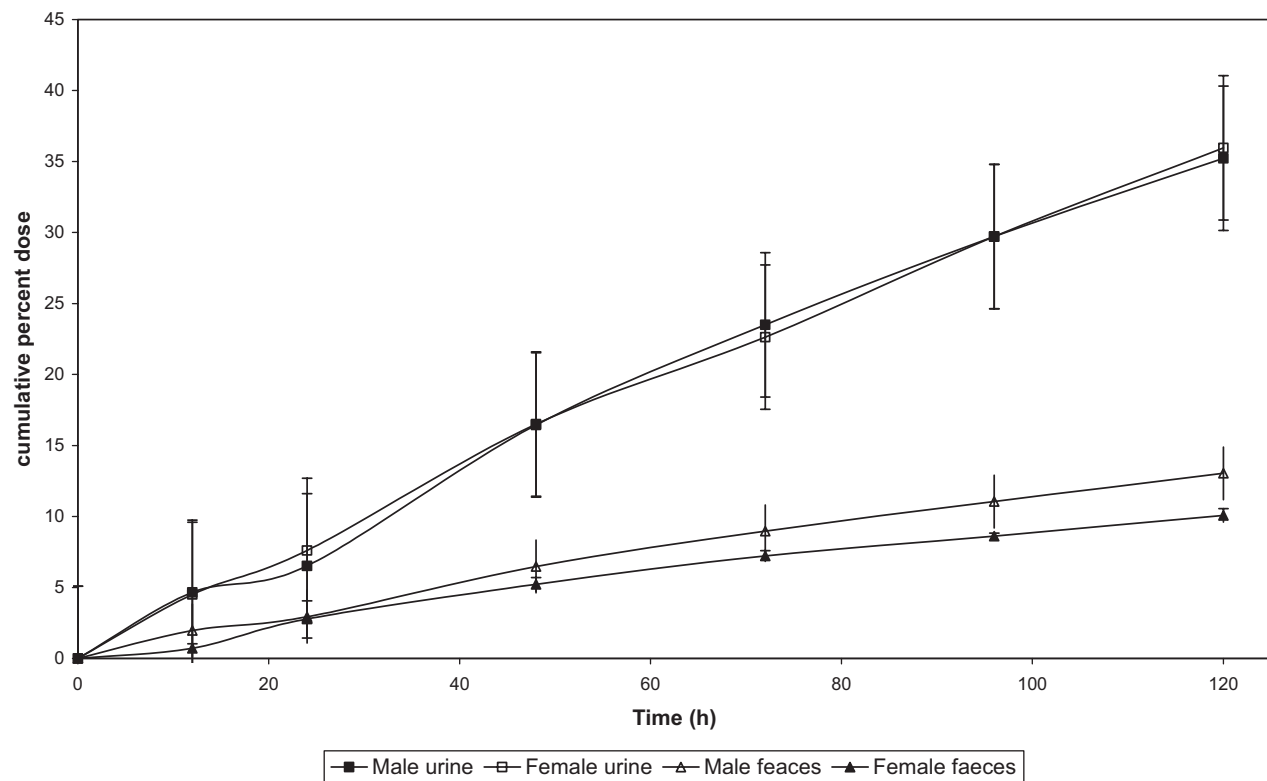


Figure 2. Excretion of radioactivity in urine and faeces from dogs dosed at $5 \text{ mg } ^{14}\text{C-2,4-D kg}^{-1}$ body weight. Error bars are $\pm$ standard deviation ($n - 1$) from four animals.

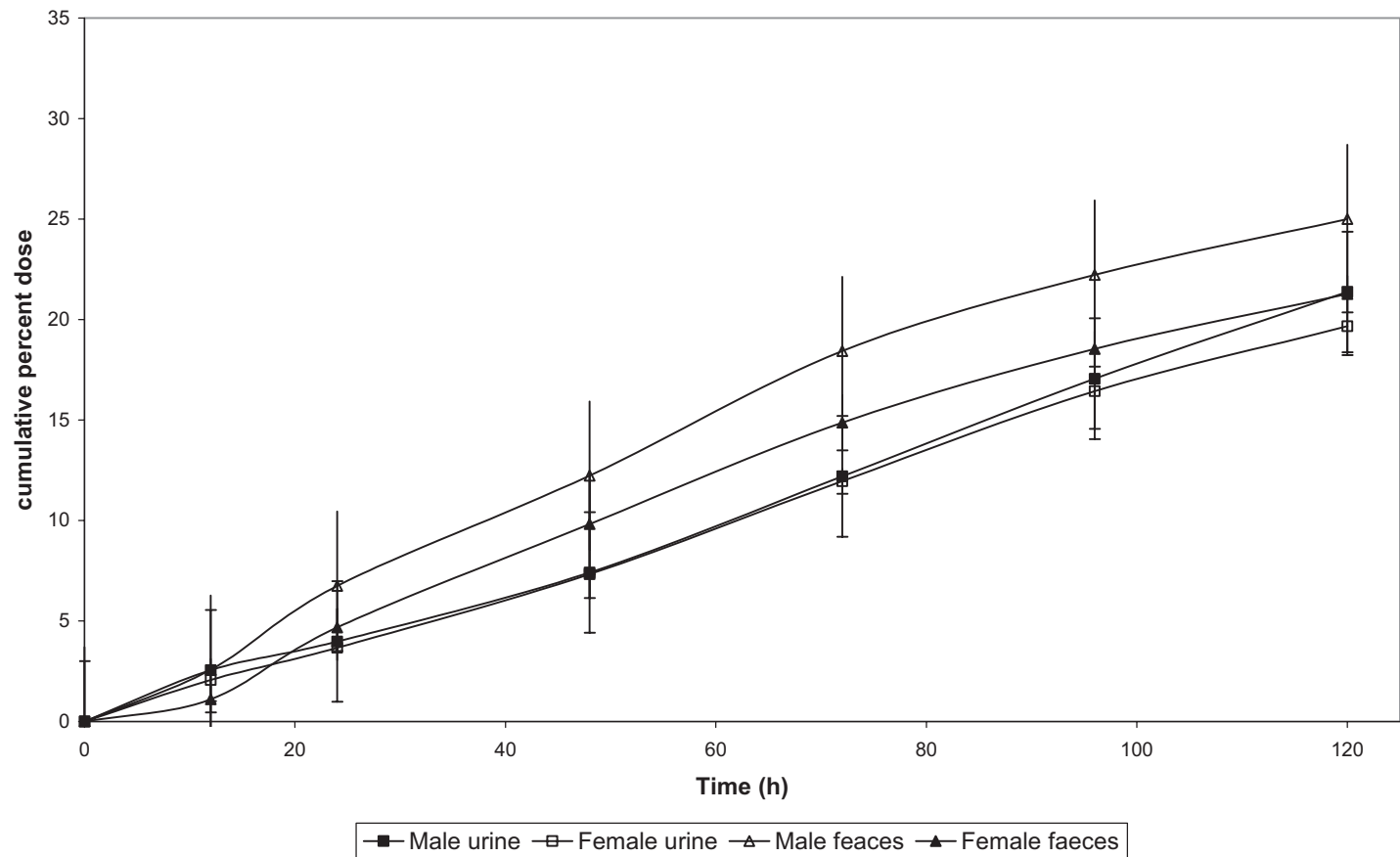


Figure 3. Excretion of radioactivity in urine and faeces from dogs dosed at $50 \text{ mg } ^{14}\text{C-2,4-D kg}^{-1}$ body weight. Error bars are $\pm$ standard deviation ($n - 1$) from four animals.

Table 2. Plasma toxicokinetic parameters calculated for rats and dogs orally dosed at either 5 or 50 mg ¹⁴C-2,4-D kg⁻¹ body weight.

Pharmacokinetic parameter (units)	5 mg kg ⁻¹		50 mg kg ⁻¹	
	Male	Female	Male	Female
Rat				
C _{max} (µg eq. g ⁻¹)	9.84 ± 3.38	14.26 ± 3.28	189.8 ± 21.71	266.6 ± 60.41
T _{max} (h)	1	1	2	2 ± 1.4
t _{1/2} (h)	1.7 ± 1.14	1.29 ± 0.80	1.50 ± 0.68	3.35 ± 1.32
AUC _(0-t) (µg eq.h g ⁻¹)	20.5 ± 8.06	56.86 ± 8.34	1222 ± 133.9	2358 ± 2359
AUC _(0-∞) (µg eq.h g ⁻¹)	21.04 ± 7.5	56.9 ± 8.36	1228 ± 137.4	2359 ± 797.2
Dog				
C _{max} (µg eq. g ⁻¹)	32.41 ± 4.62	34.24 ± 2.41	233.1 ± 22.45	224.2 ± 12.92
T _{max} (h)	5.5 ± 1.9	6 ± 2	8	10 ± 2.3
t _{1/2} (h)	99.3 ± 42.34	104 ± 19.55	106.8 ± 19.65	134.4 ± 10.24
AUC _(0-t) (µg eq.h g ⁻¹)	2579 ± 660.1	2853 ± 344.9	18 310 ± 1226	19 956 ± 459.6
AUC _(0-∞) (µg eq.h g ⁻¹)	4889 ± 2519	5298 ± 1196	34 110 ± 6217	44 296 ± 3097

Data are the mean ± standard deviation from 4 animals per group.

the 5 and 50 mg kg⁻¹ dose levels, respectively (table 2 and figures 4 and 5). Other toxicokinetic parameters also reflected the marked species differences in elimination of radioactivity from the plasma. AUC_(0-t) values in the male dog were 125- and 15-fold higher for the 5 and 50 mg kg⁻¹ doses than the corresponding values in rat. Similarly, AUC_(0-t) values in the female dog were 50- and 8-fold higher for the 5 and 50 mg kg⁻¹ doses than the corresponding values in rat. In addition, for the rat, AUC_(0-t) and AUC_(0-∞) were almost identical for males and females for both the 5 and 50 mg kg⁻¹ doses confirming that elimination was virtually complete. For dog, however, AUC_(0-∞) were extrapolated as being 180 and 220% of the corresponding AUC_(0-t), confirming that elimination was not complete over the sampling period.

Plasma protein binding values determined using equilibrium dialysis were 95.5 and 92.9% for rats dosed at 5 and 50 mg kg⁻¹, respectively. For dogs, the values were 95.7 and 86.7% for the 5 and 50 mg kg⁻¹ doses, respectively. Total protein for all plasma samples ranged from 55 to 58 g l⁻¹. Albumin ranged from 33 to 36 g l⁻¹ and α1-acid glycoprotein ranged from 21 to 22 g l⁻¹.

Plasma was de-proteinated with acetonitrile before HPLC analysis. Recovery of radioactivity from plasma for both rat and dog following de-proteination was approximately 95%. Analysis of both dog plasma and rat urine by HPLC and LC/MS showed that the only radiolabelled peak of any significance was 2,4-D.

In rat urine, some very minor additional peaks were seen but none of these exceeded 2% of the administered dose and were not identified. For dog plasma, no peaks other than that of 2,4-D could be distinguished. Dog urine, however, contained at least nine radiochromatographic peaks and unchanged 2,4-D accounted for about 1% of the total metabolites present. The metabolite profiles varied considerably between animals but a typical chromatograph for dog urine is shown in figure 6. Structures for seven metabolites and the parent compound were assigned based on their mass spectra, namely: the taurine, serine, glycine, glucuronide, glutamic acid, sulphate and cysteine conjugates (figure 7). One metabolite with a retention time slightly longer (thus suggesting the metabolite was more lipophilic than the parent) was also present but remained unidentified (figure 8).

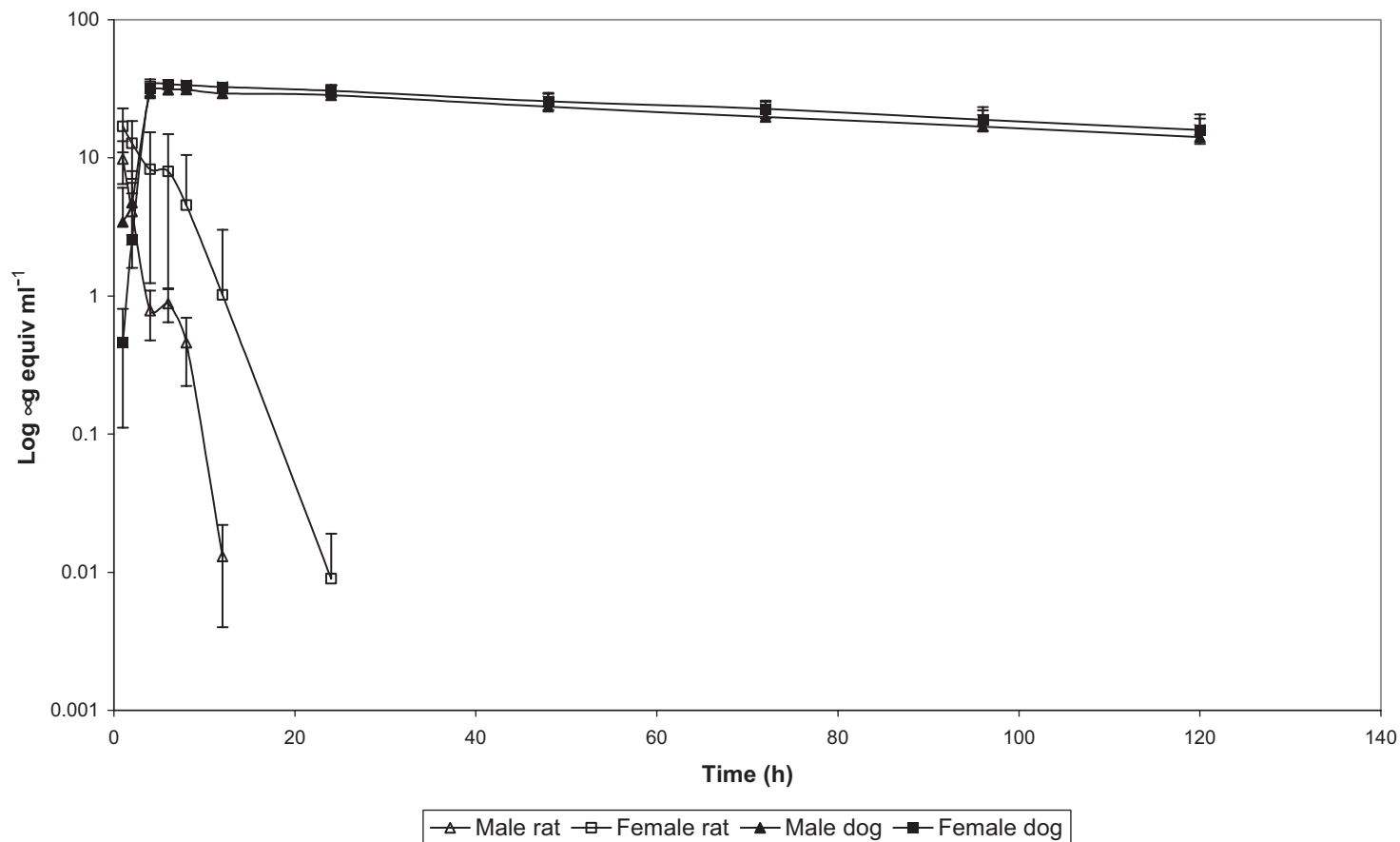


Figure 4. Semilog plot of concentration ($\mu\text{g equivalents } 2,4\text{-D g}^{-1}$) in plasma over time for rats and dogs dosed at $5 \text{ mg } ^{14}\text{C}\text{-}2,4\text{-D kg}^{-1}$ body weight. Error bars are $\pm$ standard deviation ($n = 4$) from four animals.

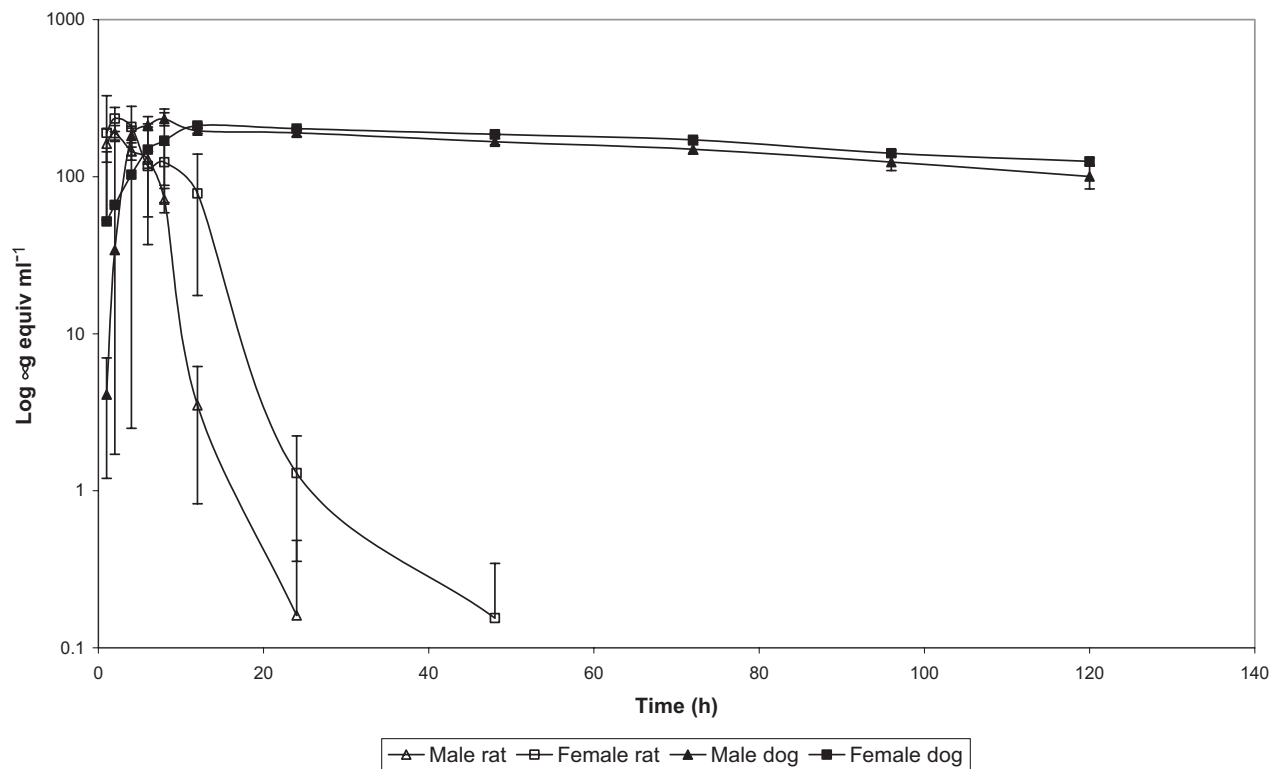


Figure 5. Semilog plot of concentration ($\mu\text{g equivalents } 2,4\text{-D g}^{-1}$) in plasma over time for rats and dogs dosed at $50 \text{ mg } ^{14}\text{C-}2,4\text{-D kg}^{-1}$ body weight. Error bars are $\pm$ standard deviation ($n - 1$) from four animals.

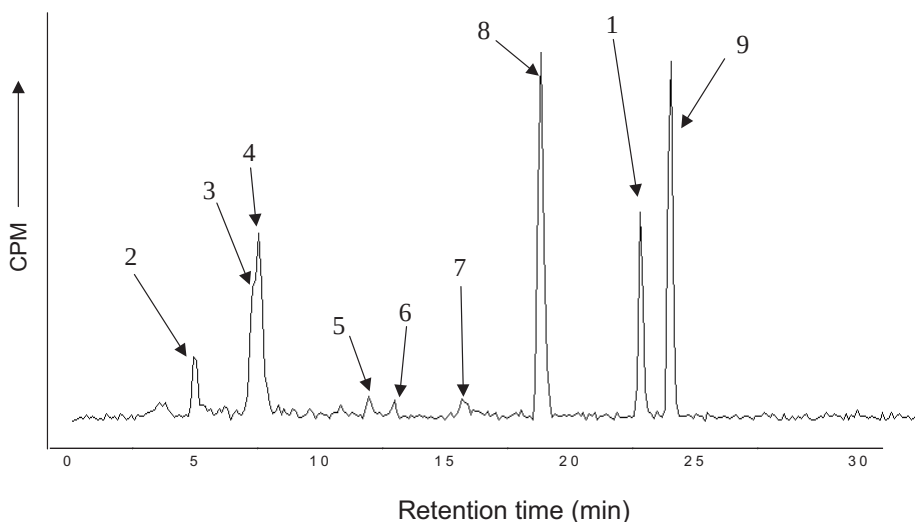


Figure 6. Representative HPLC radiochromatogram of dog urine showing at least seven metabolites, six of which were identified. Metabolite numbers and structures are shown in figure 7.

Discussion

There were marked differences in rates and routes of excretion and the rate of elimination of radioactivity from plasma, between rat and dog. These differences could be explained either by differences in the rate of absorption, or in rate of elimination, although the first explanation is very unlikely as phenoxyacetic acids are known to be readily absorbed upon oral dosing. Fischer rats have been previously given a range of doses of ^{14}C -2,4-D and absorption was only saturated at doses above 100 mg kg^{-1} (Gorzinski *et al.* 1987). Likewise, the structurally related compound triclopyr has been shown to be well absorbed in dog (Timchalk and Nolan 1997). ^{14}C -2,4-D (1 mg kg^{-1}) has been previously administered to Fischer rats by the intravenous route. Although the data reported were limited with respect to pharmacokinetics, the $t_{1/2(\text{elim})}$ could be estimated to be about 2 h and elimination of radioactivity was essentially complete in under 20 h (Kim *et al.* 1994). The similarity of results between the intravenous and oral routes supports the fact that 2,4-D and other similar phenoxyacetic acids are rapidly absorbed through the gastrointestinal tract.

Once absorbed in rat, radioactivity was rapidly eliminated almost entirely as unchanged 2,4-D in rat urine. Owing to the small amount of blood that could be sampled at each time point, it was not possible to analyse rat plasma for the presence of 2,4-D or its metabolites. There have been a number of studies, however, which have shown that 2,4-D is not metabolized in rat (Schulze *et al.* 1985, Gorzinski *et al.* 1987, Pelletier *et al.* 1989). These previous reports are entirely consistent with the results of the present study as the only radiolabelled compound of any note in rat urine, for both the 5 and 50 mg kg^{-1} dose levels, was 2,4-D. The circulating chemical species in rat, therefore, was almost certainly unchanged 2,4-D. There was sufficient dog plasma for HPLC analysis and this showed that the only radiolabelled compound present was 2,4-D. Hence for both rat and dog, measurement of total radioactivity could be taken as a surrogate for body burden of circulating 2,4-D.

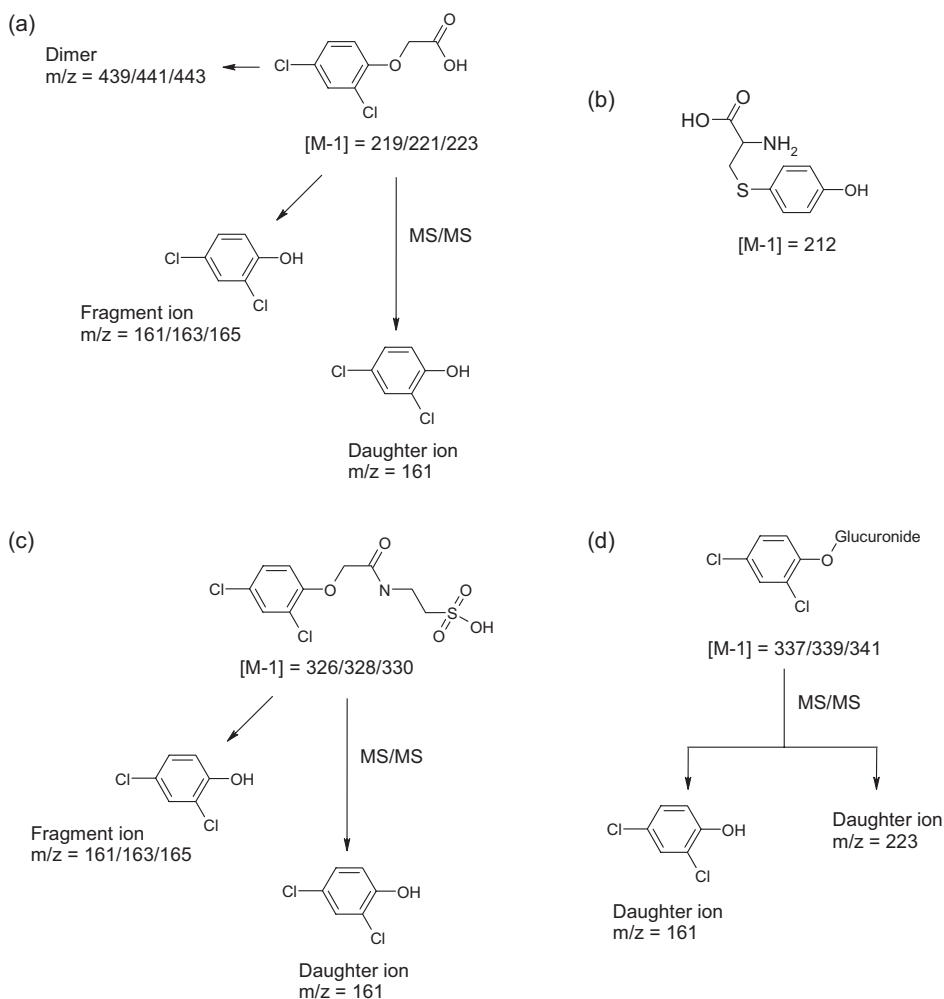
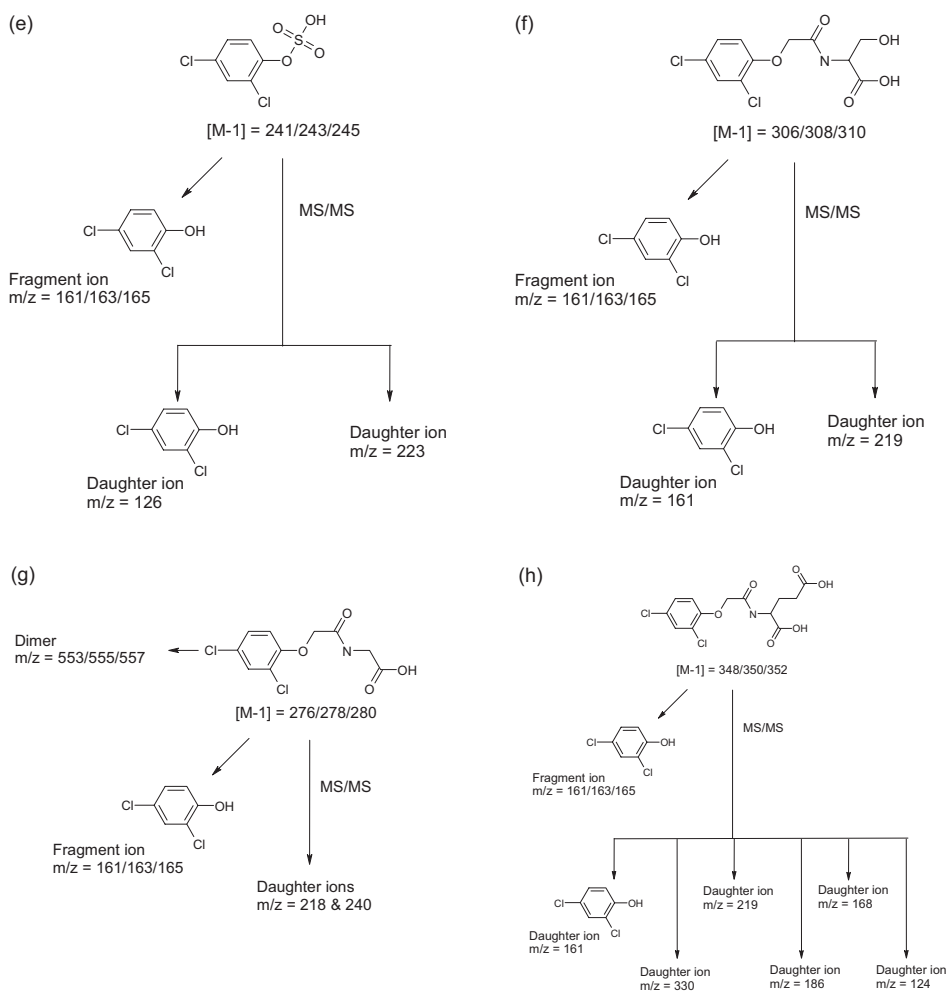


Figure 7. Metabolites identified in dog urine and their identification using LC-MS/MS. Quantities of metabolites are given as the per cent of administered dose recovered over the experimental period (120 h). (a) Identification of metabolite 1 (parent 2,4-D, ca 1%); (b) Tentative identification of metabolite 2 (cysteine conjugate, ca 1%); (c) Identification of metabolite 3 (taurine conjugate, ca 1%); (d) Identification of metabolite 4 (glucuronide conjugate, ca 7%); (e) Identification of metabolite 5 (sulphate conjugate, ca 1%); (f) Identification of metabolite 6 (serine conjugate, ca 1%); (g) Identification of metabolite 8 (glycine conjugate, ca 34%); (h) Identification of metabolite 7 (glutamic acid conjugate, ca 1%).

Comparison of the $AUC_{(0-\infty)}$ showed that the values for dog were about 27 (high dose) to 232 (low dose) times higher than the corresponding values in rat (table 2). The fact that in dog $AUC_{(0-\infty)}$ was extrapolated by such a large margin from the $AUC_{(0-t)}$, together with the low recovery of radioactivity in this species, showed that excretion in dog was far from complete after 120 h. This was in sharp contrast to the rat where excretion was completed after 24 h for the 5 mg kg^{-1} dose and 48 h for the 50 mg kg^{-1} dose.

The higher systemic exposure in dog, therefore, is consistent with the increased sensitivity of this species to 2,4-D toxicity. It is also worth noting that female rats

Figure 7. *Continued.*

excreted less in urine compared with males and had a longer elimination half-life at the 50 mg kg^{-1} dose level, which is consistent with the MTDs for this species (150 and $75 \text{ mg kg}^{-1} \text{ day}^{-1}$ for males and females, respectively). Also, comparing the $\text{AUC}_{(0-\infty)}$ for rats, it can be seen that a 10-fold increase in dose resulted in a more than proportional increase of the AUC, indicating that a dose of 50 mg kg^{-1} in rat cannot be eliminated as efficiently as at a dose of 5 mg kg^{-1} .

Although the present study was based on a single dose design, given the long $t_{1/2}$ in dog, it can be envisaged that repeat daily dosing in a toxicity study, would lead to a steady increase in body burden until a point where renal clearance mechanisms would be overwhelmed. The difference in proportions of radioactivity in urine and faeces for dogs between the 5 and 50 mg kg^{-1} doses suggest that the capacity of the kidney to eliminate 2,4-D and/or its metabolites is saturated at, or below, a single oral dose of 50 mg kg^{-1} .

The structures of seven metabolites and parent found in dog urine were tentatively assigned from their mass spectra. Three principal routes of metabolism

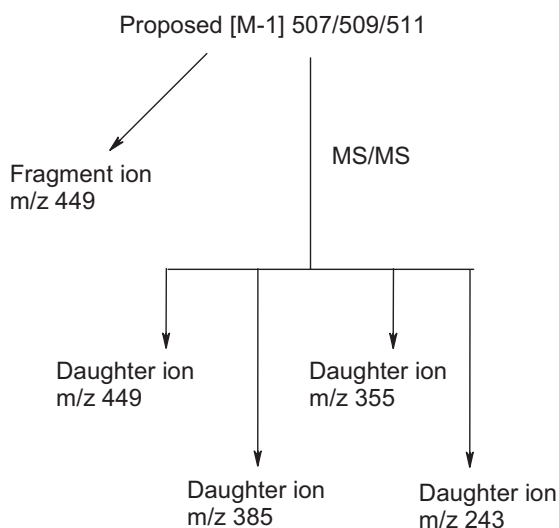


Figure 8. LC-MS/MS of unidentified metabolite 9. Over the duration of the experiment (120 h), about 1% of the administered dose was recovered as metabolite 9.

were evident: (1) conjugation of the acidic moiety of 2,4-D to form the taurine, serine, glutamic acid or glycine conjugates; (2) cleavage of the ether moiety to form the dichlorophenol which was then conjugated to the glucuronide or sulphate conjugates, and (3) there was evidence of glutathione conjugation, ultimately forming a cysteine conjugate of phenol, accompanied by dechlorination. A small amount of unchanged 2,4-D was also present in dog urine. A proposed metabolic pathway for 2,4-D in dog is shown in figure 9. The formation of taurine and glycine conjugates as major metabolites of MCPA in dog has been previously reported (Lappin *et al.* 2002). Following an oral dose of 200 mg kg⁻¹ ¹⁴C-2,4-D, the glycine conjugate was found in the bile of the rat, mouse and hamster the taurine conjugate in the bile of the mouse and the glucuronide conjugate was tentatively identified in the bile of rat and mouse (Griffin *et al.* 1997). In all cases, the conjugates were present in only very small amounts. Serine conjugation of phenyl and *o*-cresyl glycidyl ether in rats has been previously reported (de Rooij *et al.* 1998) and serine and glutamic acid have been shown to be substrates for glycine *N*-acyltransferase *in vitro* (van der Westhuizen *et al.* 2000). The presence of the serine and glutamic acid conjugates in dog urine is nevertheless an unusual occurrence.

The presence of the cysteine conjugate in dog urine in the present study implies glutathione metabolism. Glutathione *S*-transferase mediated metabolism may occur with 2,4-D, presumably via a 2,4-D aryl oxide (Dierickx 1988) but in the absence of any other conjugates along the glutathione pathway, metabolism of 2,4-D by this route remains tentative.

It has been previously postulated that the dog is deficient in the ability to excrete organic acids, although the reasons for this remains obscure (Timchalk *et al.* 1997 and Lappin *et al.* 2002). One postulated reason, in the case of 2,4,5-trichlorophenoxyacetic acid, was that excretion was inhibited by plasma protein binding (Hook *et al.* 1976). This can be ruled out in the present study as there was a higher rate of plasma 2,4-D binding in the case of the rat compared with dog. Given that 2,4-D is acidic, then the urinary pH could have an effect on the rates of

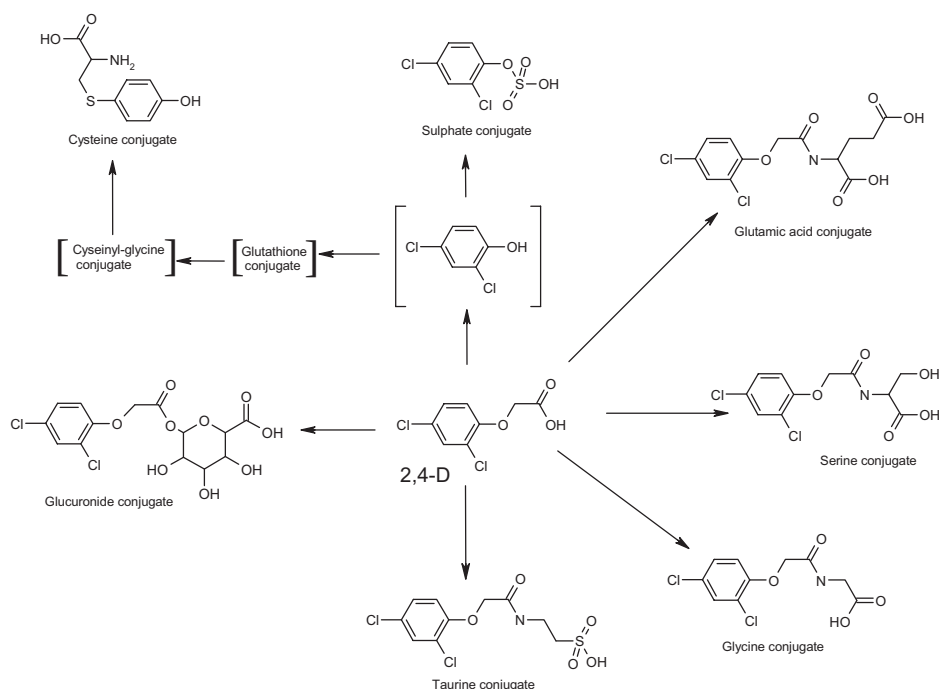


Figure 9. Proposed metabolic pathway of 2,4-D in dog. In the rat, only 2,4-D was identified. Postulated intermediates are shown in brackets.

elimination. In the toxicology studies with 2,4-D, however (Charles 1996a, b), the urinary pH was 7.5–8.0 and remained unchanged throughout. The urinary pH in the present study was not measured during the experimental stage but retrospective measurement showed the pH of rat urine to be 7 and for dog it was 8. Urinary pH therefore seems an unlikely explanation for the very significant differences renal elimination between the species.

Enterohepatic recirculation in dog but not the rat is another possible explanation for the observed species differences. In the present study, it is reasonable to assume that the entire dose was absorbed by the dog and therefore the presence of radioactivity in dog faeces would imply that the biliary route of excretion was significant. Bile has been shown to be only a minor route of excretion of ^{14}C -2,4-D in rat, to the extent of only 3% of a 200 mg kg^{-1} dose (Griffin *et al.* 1997). It is possible, therefore, that extended enterohepatic recirculation in dog, but not the rat led to proportionally prolonged systemic levels in the former species. Enterohepatic recirculation, however, seems an unlikely explanation as the characteristic fluctuation in plasma radioactivity commonly associated with enterohepatic recirculation was completely absent. It was not possible to conduct a bile duct cannulation experiment for the dog in the present study due to licensing constraints for animal experimentation at the performing laboratory.

Although enterohepatic recirculation could not be ruled out, based upon the current evidence, the most likely reason for the wide differences in rate of elimination of organic acids between rat and dog are related to differences in the active renal transport mechanisms between species. Dichlorprop and 2,4,5-T are known to be excreted by active renal mechanisms (Beitz *et al.* 1985). Basolateral

accumulation of 2,4-D has been shown to be mediated by 2,4-D/ α -ketoglutarate (α -KG) exchange, coupled indirectly to Na^+ / α -KG co-transport in the same way as *p*-aminohippurate and fluorescein are (Villalobos *et al.* 1996). Moreover, it has been known for some time that 2,4-D concentrates in kidney slices by an active transport mechanism (Berndt and Koschier 1973). Species differences in such renal mechanisms are known although they have been difficult to characterize (Pritchard 1987) and so have not been extensively studied in dog.

The results presented here and elsewhere (Lappin *et al.* 2002, *passim*) show clearly that dogs are deficient in their ability to excrete a range of organic acids. In the present study, 2,4-D in dog appears to require metabolism before it can be excreted. This does not seem to be required in rats, monkeys or in man (Timchalk and Nolan 1997). A precise mechanism explaining the species differences is not known but there is evidence to support the fact that active transport renal mechanisms are involved and the difference are not due to simple explanations such as plasma protein binding or differences in urinary pH. In the case of certain organic acids therefore, the rat is a better model for assessing the relevance to man of the toxicity of these compounds than the dog.

Acknowledgements

The study was sponsored by The Industry Task Force II on 2,4-D Research Data. For full information and publications on 2,4-D, see the Task Force website: <http://www.24d.org/>

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High-throughput screening to estimate single or multiple enzymes involved in drug metabolism: microtitre plate assay using a combination of recombinant CYP2D6 and human liver microsomes

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Received 20 February 2003

1. The purpose of this study was to estimate readily involvement of single or multiple enzymes in the metabolism of a drug through inhibitory assessment. Inhibitory effects of various compounds on CYP2D6 activity assayed by formation of fluorescent metabolite from 3-[2-(*N,N*-diethyl-*N*-methyl-ammonium)ethyl]-7-methoxy-4-methyl-coumarin (AMMC) were assessed using microtitre plate (MTP) assays with a combination of recombinant CYP2D6 and human liver microsomes (HLM).

2. Among various compounds studied, antipsychotic drugs extensively inhibited recombinant CYP2D6 activity and the IC_{50} values were generally lower than those of antidepressants and antiarrhythmic drugs.

3. After pre-incubation, the IC_{50} values of mianserin, chlorpromazine, risperidone, thioridazine, alprenolol, propafenone and dextromethorphan increased but the values of timolol, *S*-metoprolol and propranolol substantially decreased compared with those in case of co-incubation

4. The IC_{50} values of typical substrates of CYP2D6 (bufuralol and dextromethorphan at lower substrate concentration) in inhibition studies using HLM, were similar to those in the case of recombinant CYP2D6, but the values of the compounds that are metabolized by multiple CYP forms (perphenazine and chlorpromazine) in HLM were much larger.

5. If the ratio (HLM/rCYP ratio) of IC_{50} values between HLM and recombinant CYP2D6 exceeds approximately 2, it suggests that other CYP forms in addition to CYP2D6 might be involved in the metabolism of the test compounds. From the advantage such as speed, high throughput and ease of the technique, the MTP assay using a combination of the recombinant CYP2D6 and HLM is useful to estimate the involvement of single or multiple enzymes in the metabolism of drugs at the stage of drug discovery.

Introduction

Orally administrated xenobiotics and drugs if significantly absorbed are generally metabolized by oxidative enzymes such as cytochrome P450s (CYPs) present in the human liver (Gonzalez 1988). The marker CYPs involved in drug metabolism are CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Guengerich 1995, Rendic and Di Carlo 1997). Among these, CYP3A4 and CYP2D6 are responsible for the metabolism of approximately 80% of all marketed drugs (Shimada *et al.* 1994, Guengerich 1996, Lewis *et al.* 1997). It has been

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reported that CYP2D6 catalyses the metabolism of many drugs that act on circulatory and central nerve systems (Fromm *et al.* 1997, Michalets 1998). Since there are some patients with no CYP2D6 activity due to a genetic polymorphism, it is most important to identify the contribution of CYP2D6 if the metabolism of the drug is mainly catalysed by CYP2D6.

The frequency of poor metabolizers with deficient CYP2D6 activity is reported to be 5–10% in Caucasians and to be less than 1% in the Japanese (Horai *et al.* 1989, Broly *et al.* 1991). Therefore, when the drugs that are predominantly metabolized by CYP2D6 are administered to the poor metabolizers, serious side effects might occur due to large increases in plasma concentrations. Drug–drug interactions also present clinical problems. The inhibition of CYPs related to drug metabolism is the biggest cause of many drug–drug interactions (Guengerich 1997). The withdrawal of terfenadine and mibefradil from the market due to inhibition-based interactions involving CYP3A4 and CYP2D6 show the medical relevance of CYP inhibition (von Moltke *et al.* 1994, SoRelle 1998). The withdrawal of the compounds was also highly dependent on the therapeutic margin and severity of the adverse events. To avoid such situations in advance, many pharmaceutical companies have been selecting drug candidates that do not have such properties at the stage of drug discovery.

In general, identification of the CYP forms involved in the metabolism of a drug is carried out using combinations of several approaches. One is identification by measurement of the disappearance rate of a parent drug in recombinant CYP microsomes. However, since various recombinant CYPs and analysis using high-performance liquid chromatography (HPLC) or liquid chromatography/tandem mass spectrometry (LC/MS/MS) is used to identify the involvement of a single CYP or that of multiple CYP forms, such methods are time and labour consuming for the estimation of CYP forms involved in the metabolism of a drug.

In the stage of drug discovery, various high-throughput screenings (HTS) are used to optimize drug properties. The microtitre plate (MTP) assay, which consists of recombinant CYP microsomes and fluorescent probes (Crespi *et al.* 1997), is often used as an HTS method for the evaluation of CYP inhibition. It was reported that the MTP assay is useful when evaluating the inhibitory effect of drug candidates on CYP activities and readily to estimate inhibition patterns (Yamamoto *et al.* 2002). Although selection of drug candidates based on inhibition studies using recombinant CYPs is important, this is not true for all cases. The actual inhibitory effect of a drug candidate in HLM might be smaller than that on recombinant CYPs, because multiple enzymes in HLM could be involved in the metabolism of a drug.

Nomeir *et al.* (2001) compared the IC_{50} values from recombinant CYPs microsomes using the MTP assay with those obtained from HLM using a conventional method (HPLC analysis), from an inhibition study of CYP3A4 and 52 new drug candidates. For 26 of the compounds studied, the latter authors reported that the IC_{50} values from recombinant enzyme were lower than those for HLM, and that the difference could be attributed to metabolism by other CYPs present in HLM. Chauret *et al.* (2001) reported that 3-[2-*N,N*-diethyl-*N*-methyl-ammonium]ethyl]-7-methyl-4-methyl-coumarin (AMMC), which is used as substrate of CYP2D6 in the MTP assay, is selectively metabolized by CYP2D6 and that the substrate is useful in inhibition studies as a typical probe in HLM as well as in recombinant CYP2D6 microsomes.

For various drugs metabolized by CYP2D6, the inhibitory effects of these compounds on CYP2D6 activity were evaluated by using the MTP assay with combinations of recombinant CYP2D6 and HLM. It has been confirmed that this combination assay is as useful as an HTS method to estimate the involvement of single or multiple CYP forms in the metabolism of drug candidates.

Materials and methods

Chemicals

3-Cyano-7-ethoxycoumarin (CEC) and 3-cyano-7-hydroxycoumarin (CHC) were purchased from Molecular Probes (Eugene, OR, USA). 7-Hydroxy-4-trifluoroethylcoumarin (HFC), 7-methoxy-4-(trifluoromethyl)-coumarin (MFC), 3-[2-(*N,N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methyl-coumarin (AMMC), 3-[2-(*N,N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC), 7-benzyl-oxyquinoline (7-BQ) and 7-hydroxyquinoline were purchased from BD Gentest Co. (Woburn, MA, USA). Quinidine and bufuralol were purchased from Ultrafine Chemicals Co. (Manchester, UK). Amitriptyline, clomipramine, clozapine, imipramine, haloperidol, desipramine, nortriptyline, trimipramine, maprotiline, mianserin, amoxisapine, perphenazine, risperidone, thioridazine, chlorpromazine, alprenolol, taxol, propafenone, dextromethorphan, *S*-metoprolol and propranolol were purchased from Sigma-Aldrich (St Louis, MO, USA). Compounds A and B (developmental drugs as new drug candidates) were synthesized in Taisho Pharmaceutical Co. Ltd (Saitama, Japan). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺ were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and solvents used were of the highest commercially available quality.

Enzymes

Microsomes from baculovirus insect cells expressing human CYP forms, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Supersomes), and pooled human liver microsomes (H161 lot. 12) were purchased from BD Gentest Co.

Inhibitory effects of various test compounds on recombinant CYP2D6 activity

The inhibitory effects of various compounds (nine antidepressants, six antipsychotics, seven antiarrhythmics, dextromethorphan, compounds A and B) on recombinant human CYP2D6 activity were evaluated using the MTP assay. The MTP assay was conducted in a 96-well (final volume 200 µl) microtitre plate (Corning Coster, Cambridge, MA, USA), based on a method given on the BD Gentest Co. website (<http://www.gentest.com>) (Yamamoto *et al.* 2002). According to the data sheet provided by the manufacturers, P450 contents in the incubation medium were 7.5 pmol P450 ml⁻¹, which corresponded to a protein concentration of 0.073 mg protein ml⁻¹. Concentration of AMMC used as a fluorescent probe was 1.5 µM, which is similar to the *K_m* of AMMC for recombinant CYP2D6 (Miller *et al.* 2000). The various test compounds examined included: amitriptyline, clomipramine, imipramine, desipramine, nortriptyline, trimipramine, maprotiline, mianserin, amoxapine, clozapine, haloperidol, perphenazine, risperidone, thioridazine, chlorpromazine, propafenone dextromethorphan, alprenolol, timolol, *S*-metoprolol, propranolol, bufuralol, compounds A and B, and quinidine (a potent inhibitor of CYP2D6, but which is predominantly metabolized by CYP3A4). It was reported that CYP2D6 was predominantly involved in part or all of the metabolism of these compounds (Fromm *et al.* 1997, Michaelis 1998). All test compounds except for maprotiline, propafenone and *S*-metoprolol were dissolved in acetonitrile. Maprotiline, propafenone and *S*-metoprolol were dissolved in de-ionized water, and these compounds were diluted 50:50 with acetonitrile. In this study, most of the test compounds, except for quinidine, compound A and haloperidol, were examined in a range of eight concentrations from 0.05 to 100 µM or from 0.09 to 200 µM as the final concentration. Haloperidol, compound A and quinidine were tested in the range 0.06–133, 0.002–5 and 0.0002–0.5 µM, respectively.

The final acetonitrile concentration was 2%. Serial dilution of test compound and addition of all reagents to the 96-well plates were done by manual pipetting. Incubation mixtures containing the serially diluted inhibitors and NADPH-generating solution was prewarmed at 37°C for 10 min. The reactions were initiated by adding 100 µl enzyme/substrate mixtures and terminated by adding stop solution (4:1 v/v of acetonitrile/0.5 M Tris base) after incubation for 30 min. The AMMC used for the MTP assay was reported to be a selective substrate of CYP2D6 in HLM (Chauret *et al.* 2001, Stresser *et al.* 2002). This report showed that the actual metabolite of AMMC formed in HLM is AMHC (3-[2-(*N,N*-diethyl-*N*-methylammonium)ethyl]-7-hydroxy-4-methyl-coumarin). In the present study,

since AMHC as a standard for quantification could not be commercially obtain, AHMC was used based on the assumption that it had the same fluorescent properties as AMHC. The fluorescence in wells was measured, using a multilabel counter, 1420 ARVOsx (Wallac, Finland), with excitation wavelengths of 390 nm and emission wavelengths of 460 nm. Control reactions were assessed in the wells containing no inhibitor. All reactions were carried out in duplicate.

Effect of pre-incubation on inhibition of CYP2D6

The effects of pre-incubation on the inhibitory potential of 24 test compounds (except compound B) on recombinant CYP2D6 activity assayed by the formation of fluorescent metabolite from AMMC were studied using the MTP assay with the pre-incubation method described by Yamamoto *et al.* (2002). The range of concentration of test compounds used was the same as described above. After NADPH generating solution and serially diluted test compounds had been added to each well, pre-incubation was carried out by adding CYP2D6 enzyme to each well at 37°C for 30 min. After pre-incubation, the reaction was initiated by adding 100 µl substrate. Subsequent procedures were as described above.

Inhibitory effects of tricyclic antidepressants, β -blockers and antipsychotic drugs on five recombinant CYP forms

The inhibitory effects of amitriptyline, imipramine, nortriptyline, desipramine, clozapin, perphenazine, chlorpromazine, timolol, *S*-metoprolol and bufuralol on five recombinant CYP forms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4) were studied as described by Yamamoto *et al.* (2002). A total of 5 µM CEC (for CYP1A2), 75 µM MFC (CYP2C9), 25 µM CEC (CYP2C19), 1.5 µM AMMC (CYP2D6) and 40 µM 7-BQ (CYP3A4) were used as substrates for the MTP assay, respectively. The maximum concentrations of the inhibitors used were 100 µM (nortriptyline, desipramine, propranolol, *S*-metoprolol, propafenone) and 200 µM (amitriptyline, imipramine, clozapin, perphenazine, chlorpromazine, bufuralol, timolol).

Inhibitory effects on CYP2D6 activity in HLM

The incubation mixtures contained 0.5 mg protein ml⁻¹ (corresponding to 5 pmol P450 ml⁻¹) HLM, an NADPH-generating system (8.2 µM NADP⁺, 0.41 mM glucose 6-phosphate, 0.41 mM MgCl₂, 0.4 unit ml⁻¹ glucose 6-phosphate dehydrogenase), 100 mM potassium phosphate buffer (pH 7.4), 1.5 µM AMMC and each test compound (as inhibitor) in a final volume of 0.2 ml per well of the microtitre plate. Concentration of AMMC as fluorescent probe was 1.5 µM, which was similar to the K_m of AMMC for CYP2D6 in HLM (Chauret *et al.* 2001). Experimental conditions such as reaction time, substrate and inhibitor concentrations, and procedures in this study were the same as described in the inhibition study on recombinant CYP, except for the use of 0.5 mg protein ml⁻¹ HLM instead of recombinant CYP2D6 as the microsomal enzyme. The control reaction was assessed in the wells containing no inhibitor. All reactions were carried out in the linear ranges of protein concentration and incubation time.

Data analysis

Formation activity of the individual fluorescence metabolite was proportional to the incubation time. Data were analysed using an Excel spreadsheet (Microsoft, USA). The inhibition effect at each concentration of test compounds on the specific activity was expressed as a percentage of control value. The IC₅₀ values were calculated by linear interpolation. For amitriptyline, clomipramine, clozapine, imipramine, desipramine and nortriptyline, the inhibition constants (K_{i-cal}) were calculated from IC₅₀ values, assuming competitive inhibition, according to the following relationship described by Bapiri *et al.* (2001) and Yamamoto *et al.* (2002):

$$K_i = (K_m \times IC_{50}) / (K_m + S),$$

where K_m and S are the affinity constants for each fluorescence metabolic activity and substrate concentration used. The K_{i-cal} values from recombinant microsomes were compared with the K_m values (K_{m-rep}) from recombinant CYPs reported in the literature, using conventional methods. IC₅₀ ratios (HLM/rCYP IC₅₀ ratio) of various test compounds were calculated as the ratio of the IC₅₀ in HLM with that of recombinant CYP.

Results

Inhibitory effects of various test compounds on recombinant CYP 2D6 activity

Inhibitory effects of various test compounds on recombinant CYP2D6 activity are shown in figure 1. Here, control activity of AHMC formation was $0.83 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ P450}$ and was similar to the activity reported by Stresser *et al.* (2002). The IC_{50} ($0.005 \mu\text{M}$) of quinidine used as positive control was in agreement with previous data (Chauret *et al.* 2001, Yamamoto *et al.* 2002). Among the antidepressants assessed, the inhibitory effects were extensive in the order: mianserin > clomipramine > trimipramine > maprotiline > imipramine = nortriptyline = amitriptyline > desipramine > amoxapine. Although mianserin showed the greatest inhibitory effect on CYP2D6, the IC_{50} was 100 times larger than that of quinidine. The IC_{50} values of these antidepressants were in the range of 0.55 (mianserin) to $3.64 \mu\text{M}$ (amoxapine).

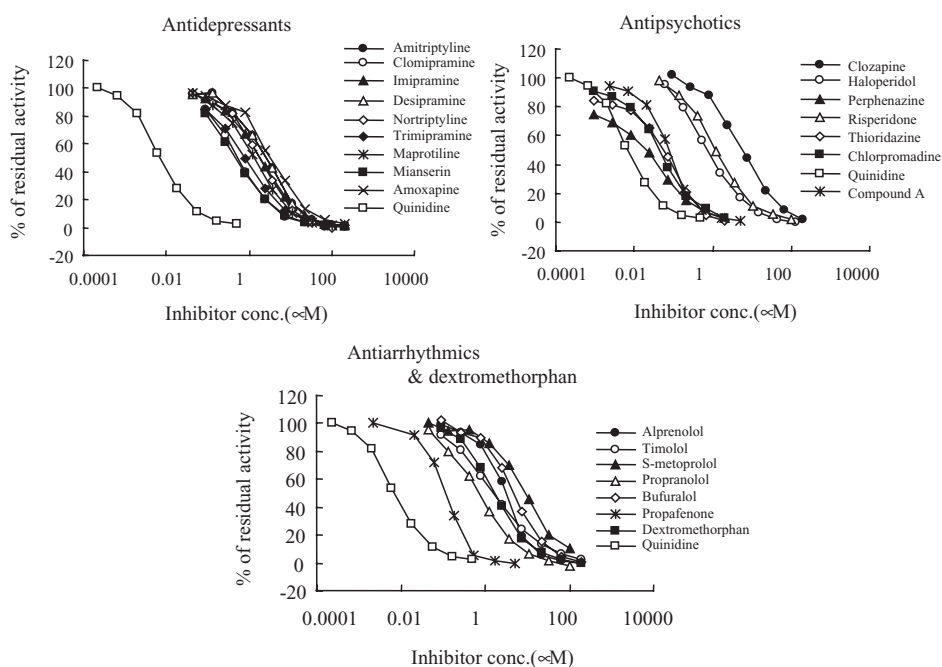


Figure 1. Inhibitory effects of antidepressants, antipsychotics and antiarrhythmics on recombinant CYP2D6 activity assayed by formation of fluorescent metabolite from 3-[2-(*N,N*-diethyl-*N*-methyl-ammonium)ethyl]-7-methoxy-4-methyl-coumarin (AMMC). The inhibition study using the MTP assay was carried out as described in the Materials and methods. Test compounds used as inhibitors were mainly substrates of CYP2D6. Although quinidine is classified as an antiarrhythmic drug, it is a positive control inhibitor due to comparison with the inhibitory effect of other compounds. Although the β -blockers used have many clinical actions, they are classed as antiarrhythmic drugs. Since compound B did not inhibit CYP2D6 activity at the maximum concentration used, the corresponding data are not shown. The concentration of AMMC as the fluorescent probe was $1.5 \mu\text{M}$, which was similar to the K_m of AMMC for recombinant CYP2D6 (Miller *et al.* 2000). Control activity of fluorescent metabolite formation in this assay was $0.83 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ P450}$. P450 contents in the incubation medium were 7.5 pmol ml^{-1} and correspond to a protein concentration of $0.073 \text{ mg protein ml}^{-1}$ (the data sheet was provided by the manufacturers). Each point is the average of duplicate determinations.

Among the antiarrhythmic drugs tested (except for quinidine), propafenone was the most potent inhibitor of CYP2D6 activity ($IC_{50} = 0.13 \mu M$), followed by propranolol ($IC_{50} = 0.79 \mu M$). The inhibitory effects of β -blockers such as timolol, alprenolol, bufuralol and *S*-metoprolol on CYP2D6 activity were slightly weaker than those of antidepressants, and IC_{50} values were 1.81, 3.49, 5.49 and $9.79 \mu M$, respectively. The value for dextromethorphan, a typical CYP2D6 substrate, was similar to β -blockers such as timolol. On the other hand, the inhibitory effects of antipsychotic drugs on recombinant CYP2D6 activity were generally substantial compared with those of antidepressants and antiarrhythmic drugs. Perphenazine, chlorpromazine, thioridazine and compound A inhibited CYP2D6 activity with IC_{50} values of 0.02, 0.05, 0.07 and $0.09 \mu M$, respectively. Haloperidol inhibited CYP2D6 activity to approximately the same extent as the antidepressants, but the inhibitory effect of clozapine was weak compared with effects of the antidepressants.

Effect of pre-incubation on inhibition of CYP2D6

Figure 2 shows IC_{50} values of 24 test compounds on recombinant CYP2D6 activity after co-incubation (0 min) and pre-incubation (30 min). Control activity of AHMC formation after pre-incubation was $0.42 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ P450. Among the antidepressants examined, mianserin extensively inhibited CYP2D6 after co-incubation and the IC_{50} after pre-incubation ($1.98 \mu M$) was 3.5 times larger than that before co-incubation. The IC_{50} values of other antidepressants except for imipramine, maprotiline and amoxapine showed a tendency to increase after pre-incubation. For the antipsychotic drugs, the IC_{50} values of other compounds after pre-incubation (except for clozapine, haloperidol and perphenazine) were 2.3 times greater than the co-incubation value. For compound A (under development as a new drug candidate), the IC_{50} after pre-incubation was 3.76 times greater than that for co-incubation.

In contrast, the IC_{50} values of haloperidol and perphenazine did not change after pre-incubation, but the value for clozapine was two times lower than that after co-incubation. As for the antiarrhythmic drugs assessed, the IC_{50} values of propafenone, alprenolol, bufuralol and dextromethorphan were 3.71-, 3.06-, 1.41- and 2.60-fold greater than those with co-incubation. On the other hand, β -blockers such as timolol, propranolol and *S*-metoprolol extensively inhibited CYP2D6 activity approximately 9.0-, 2.5- and 7.0-fold after pre-incubation and the IC_{50} values were 0.20, 0.11 and $3.08 \mu M$, respectively. The IC_{50} of quinidine, a selective CYP2D6 inhibitor, was least influenced by pre-incubation.

Comparison between K_{i-cal} and K_{m-rep}

Figure 3 shows the inhibitory effects of amitriptyline, imipramine, nortriptyline, desipramine and clozapine on five recombinant CYPs activities. Amitriptyline extensively inhibited CYP2D6 and CYP2C19 activities and slightly inhibited CYP2C9 and CYP3A4 activities. Clozapine inhibited all the activities of CYP forms over the concentration range used and extensively inhibited CYP2D6 activity at the low concentration. Imipramine and nortriptyline inhibited CYP2D6, CYP2C19 and CYP3A4 activities. Desipramine inhibited only CYP2D6 activity among the CYP forms studied. The IC_{50} values obtained from

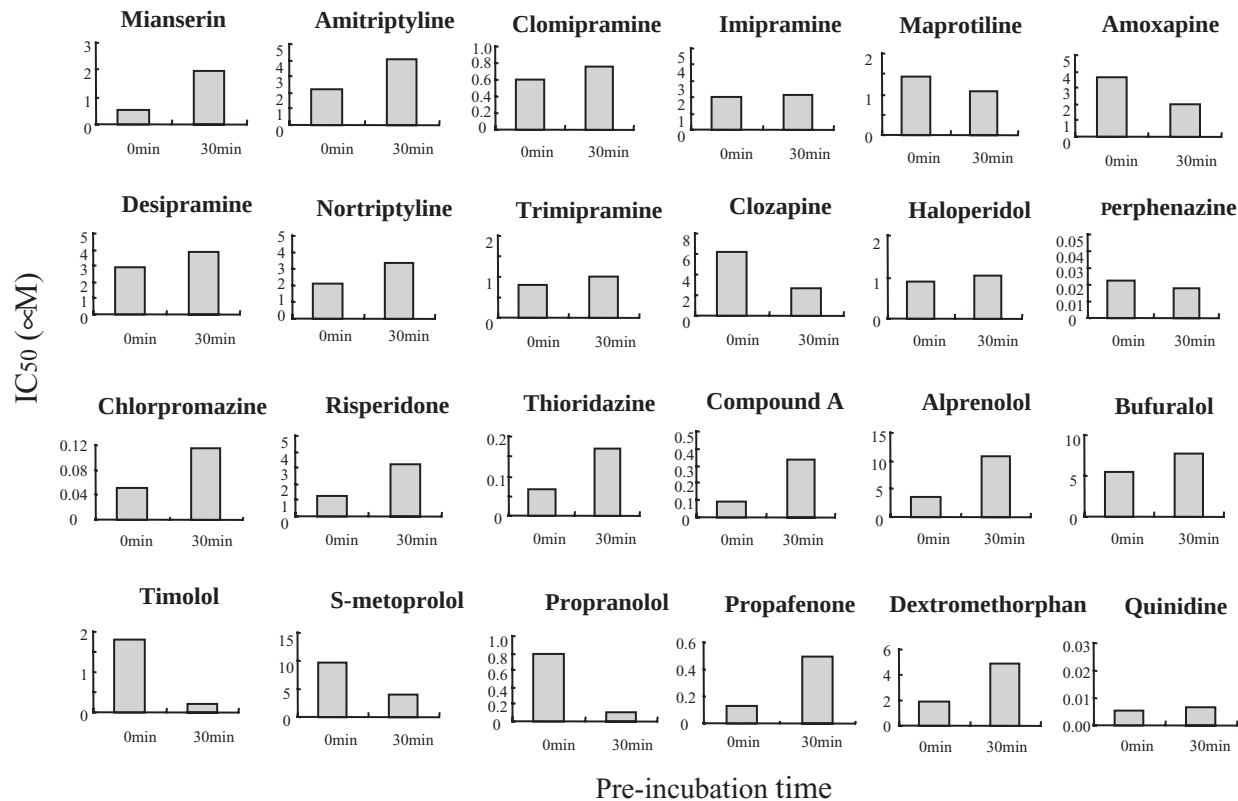


Figure 2. Effect of pre-incubation on the inhibitory potential against recombinant CYP2D6 activity. Zero minutes represents the IC₅₀ values in the case of co-incubation with test compound and enzyme and 30 min represents the IC₅₀ values after pre-incubation for 30 min. The concentration of AMMC as fluorescent probe was 1.5 μ M. Each datum point is the average of duplicate determinations. The control activity of fluorescent metabolites formation in pre-incubation method was 0.42 pmol min⁻¹ pmol⁻¹ P450.

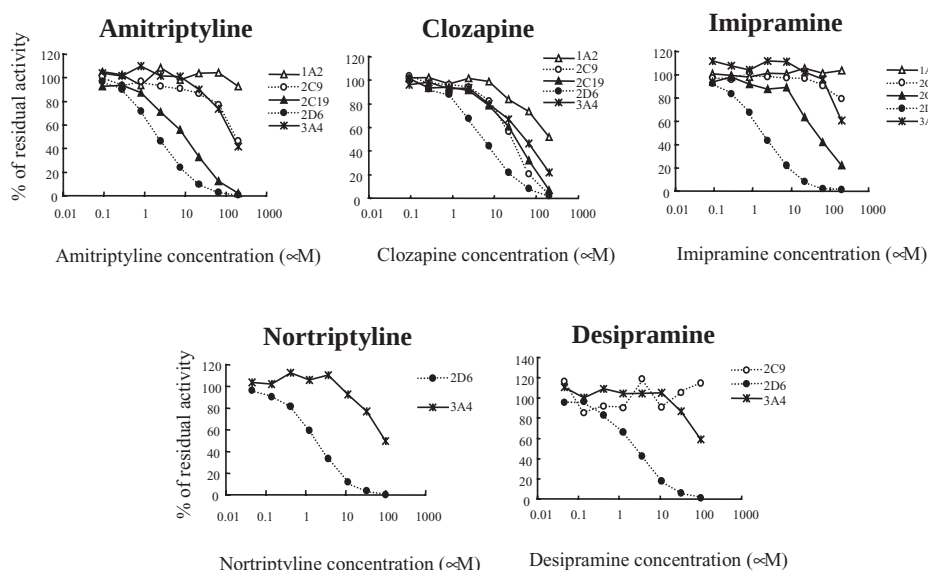


Figure 3. Inhibitory effect of antidepressants and clozapine on five recombinant CYPs' (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4) activities. Inhibition studies using the MTP assay were done as described by Yamamoto *et al.* (2002). A total of 5 μM CEC (for CYP1A2), 75 μM MFC (for CYP2C9), 25 μM CEC (for CYP2C19), 1.5 μM AMMC (for CYP2D6) and 40 μM 7-BQ (for CYP3A4) were used as substrates for the MTP assay, respectively. Control activity of the fluorescent metabolite formation in this assay was 10.39 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 (for CYP1A2), 1.35 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 (CYP2C9), 1.83 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 (CYP2C19), 0.85 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 (CYP2D6) and 52.87 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 (CYP3A4), respectively. The test compounds used were mainly metabolized by multiple CYP forms in HLM. Each point is the average of duplicate determinations.

this assay were converted to K_i values ($K_{i-\text{cal}}$), as described in the Materials and methods, and the $K_{i-\text{cal}}$ values were compared with the $K_{m-\text{rep}}$ in each recombinant CYP that has been reported elsewhere (Koyama *et al.* 1997, Linnet and Olesen 1997, von Moltke *et al.* 1998, Venkatakrishnan *et al.* 1999, 2001).

Table 1 shows a comparison between these $K_{i-\text{cal}}$ and $K_{m-\text{rep}}$ values. The $K_{i-\text{cal}}$ (0.89 μM) of amitriptyline for CYP2D6 was the lowest among CYP forms inhibited by this compound, followed by CYP2C19 ($K_{i-\text{cal}}$ = 6.23 μM). The values for CYP2C9 and CYP3A4 were estimated as 94.54 and 81.38 μM , respectively. Ranking of inhibitory effects of amitriptyline for each CYP form well matched those for $K_{m-\text{rep}}$ values for each CYP. The $K_{i-\text{cal}}$ values of clozapine, which inhibited all CYP forms, were 2.5, 15.37, 20.65, 28.55 and 82.35 μM for CYP2D6, CYP2C9, CYP2C19, CYP3A4 and CYP1A2, respectively. Imipramine, nortriptyline and desipramine also inhibited CYP forms involved in the metabolism of these compounds, and the $K_{i-\text{cal}}$ values closely corresponded to the $K_{m-\text{rep}}$ values.

Inhibitory effects of various test compounds on CYP2D6 activity in human liver microsomes: comparison between IC_{50} values derived from HLM and recombinant CYP2D6

The production of fluorescent metabolites in HLM was 3.56 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein (0.36 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450) and matched well the activity (3.12 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein) reported by Stresser *et al.* (2002). Table 2 shows

Table 1. Comparison between the literature $K_{m\text{-rep}}$ and $K_{i\text{-cal}}$ values from IC_{50} values using the MTP assay.

			CYP forms				
Drugs			CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Amitriptyline	$K_{m\text{-rep}}(\mu\text{M})$	(a)	63.5	50.5	8.52	7.12	69.3
	$K_{i\text{-cal}}(\mu\text{M})$		> 200*	94.5	6.23	0.89	81.4
Clozapine	$K_{m\text{-rep}}(\mu\text{M})$	(b)	25	15	13	25	230
	$K_{i\text{-cal}}(\mu\text{M})$		82.4	15.4	20.7	2.50	28.6
Imipramine	$K_{m\text{-rep}}(\mu\text{M})$	(c)	301	—	24.7	2.15	869
	$K_{i\text{-cal}}(\mu\text{M})$		> 200*	> 200*	26.7	0.83	> 200*
Nortriptyline	$K_{m\text{-rep}}(\mu\text{M})$	(d)	—	—	—	2.08	37.4
	$K_{i\text{-cal}}(\mu\text{M})$		—	—	—	0.85	46.7
Disipramine	$K_{m\text{-rep}}(\mu\text{M})$	(e)	—	—	—	11.7	—
	$K_{i\text{-cal}}(\mu\text{M})$		—	> 100*	—	1.15	> 100*

The $K_{i\text{-cal}}$ of these compounds were calculated from the IC_{50} in the MTP assay, based on the following equation: $K_{i\text{-cal}} = (K_m \times IC_{50} / K_m + S)$, where $K_{i\text{-cal}}$, IC_{50} , K_m and S are inhibition constants calculated from IC_{50} , 50 % in inhibition percentage, affinity of each typical substrate on recombinant CYPs and concentrations of each typical substrate used in this study, respectively. Each point is the average of duplicate determinations. The $K_{i\text{-cal}}$ values for each CYP were compared with $K_{m\text{-obs}}$ values with recombinant CYPs from the following reports previously described: (a) Venkatakrishnan *et al.* (2001); (b) Olesen Linnert (1997); (c) Koyama *et al.* (1997); (d) Venkatakrishnan *et al.*, (1999); (e) von Moltke *et al.* (1998).

* Since the IC_{50} values could not be obtained in this study due to weak inhibition, we did not calculate the $K_{i\text{-cal}}$. Therefore, > 100 or > 200 means that the IC_{50} values exceed 100 or 200 μM .

—, Data are not shown in the reports (for $K_{m\text{-rep}}$), and the experiment not done (for $K_{i\text{-cal}}$).

Table 2. Comparison between IC_{50} values derived from recombinant CYP2D6 (rCYP) and human liver microsomes (HLM).

CYP2D6 Inhibitors	$IC_{50}(\mu\text{M})$		HLM/rCYP Ratio	CYP2D6 inhibitors	$IC_{50}(\mu\text{M})$		HLM/cDNS Ratio
	rCYP	HLM			rCYP	HLM	
Perphenazine	0.02	0.33	14.77	amitriptyline	2.23	3.37	1.51
Chlorpromazine	0.05	1.71	33.61	clozapine	6.24	8.19	1.31
Timolol	1.81	35.58	19.71	imipramine	2.04	5.58	2.73
Quinidine	0.01	0.04	5.39	haloperidol	0.89	1.14	1.28
Mianserin	0.55	3.56	6.48	desipramine	2.89	2.21	0.77
Risperidone	1.26	5.74	4.57	nortriptyline	2.12	2.50	1.18
Thioridazine	0.07	0.36	5.60	trimipramine	0.81	2.47	3.06
Propranolol	0.79	3.96	5.00	maprotiline	1.44	3.54	2.46
Clomipramine	0.61	2.75	4.54	amoxapine	3.64	8.20	2.26
Bufuralol	5.49	8.97	1.64	alprenolol	3.49	1.92	0.55
S-metoprolol	9.79	14.63	1.49	compound A	0.09	0.15	1.63
Dextromethorphan	1.89	4.02	2.13	compound B	> 200	> 200	1.00
Propafenone	0.13	0.22	1.63				

HLM/rCYP ratio represents the ratio of IC_{50} values using HLM or rCYP. Compound B did not inhibit CYP2D6 activities with recombinant CYP2D6 or HLM at the maximum concentration used (200 μM). Therefore, the IC_{50} values are represented as > 200. Concentration of AMMC as fluorescent probe was 1.5 μM .

the IC_{50} values of various test compounds obtained from recombinant CYP2D6 and HLM. Quinidine, a selective CYP2D6 inhibitor, extensively inhibited CYP2D6 activity in HLM as well as recombinant CYP2D6 and the IC_{50} (0.04 μM) was 5.4 times lower than for recombinant CYP2D6. Following quinidine, compound A extensively inhibited the CYP2D6 activity in HLM but the IC_{50} (0.15 μM) was only 1.6 times larger than that in recombinant CYP2D6.

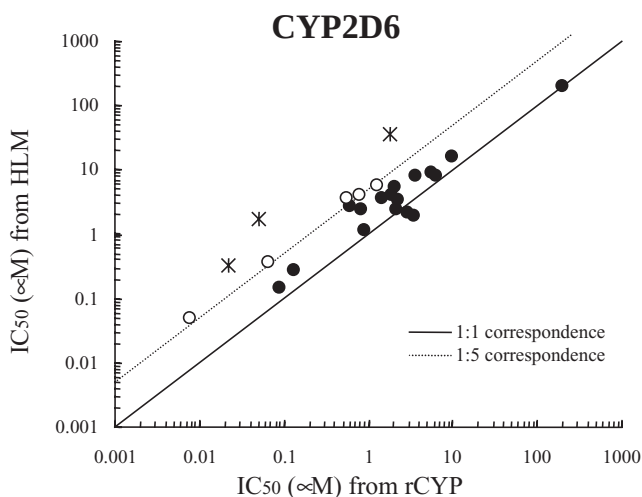


Figure 4. Correlation of IC_{50} values on recombinant CYP2D6 and IC_{50} in human liver microsomes. The solid line represents a 1:1 correspondence of the IC_{50} values between rCYP and HLM. The dotted line represents values of five. ●, Compounds showing an IC_{50} ratio (HLM/rCYP) = 2; ○, compounds showing an IC_{50} ratio (HLM/rCYP) > 5; *, compounds showing an IC_{50} ratio (HLM/rCYP) > 10.

Figure 4 shows the correlation between the IC_{50} values of various test compounds derived from HLM and those for recombinant CYP2D6. The IC_{50} values of perphenazine, chlorpromazine and timolol on HLM were 14.8-, 33.6- and 19.7-fold higher than these on recombinant CYP2D6, respectively. The IC_{50} values of miaserin, thioridazine, propranolol, risperidone, clomipramine and quinidine were approximately five times larger than those for recombinant CYP2D6. However, the IC_{50} values for other compounds exhibited slight differences between HLM and recombinant CYP2D6 and showed a good correlation ($r^2 = 0.993$) between both IC_{50} values for these compounds.

Inhibitory effects of perphenazine, chlorpromazine, propranolol, bufuralol, S-metoprolol, timolol and propafenone on five recombinant CYP activities

The inhibitory effects of seven compounds on five recombinant CYPs activities were evaluated using the MTP assay (figure 5). Four of these compounds (perphenazine, chlorpromazine, timolol, propranolol) exhibited a large difference in the IC_{50} ratio (HLM/rCYP ratio) between HLM and recombinant CYPs. Three compounds (bufuralol, S-metoprolol, propafenone) exhibited similar HLM/rCYP ratios. Perphenazine inhibited all CYP forms examined and the IC_{50} values were very low for CYP2D6 (0.04 μ M), followed by CYP1A2 (0.52 μ M). The IC_{50} values for perphenazine were greater for CYP2C9, CYP2C19 and CYP3A4. Chlorpromazine also inhibited all CYP forms as well as perphenazine. The IC_{50} values for each recombinant CYP activity were; 0.06 μ M (CYP2D6), 2.31 μ M (CYP1A2), 13.65 μ M (CYP2C19), 58.96 μ M (CYP3A4) and 62.17 μ M (CYP2C9). Propranolol inhibited CYP2D6 and CYP1A2 activities over the range of the concentrations examined. In contrast, timolol and S-metoprolol only inhibited CYP2D6 with IC_{50} values of 1.7 and

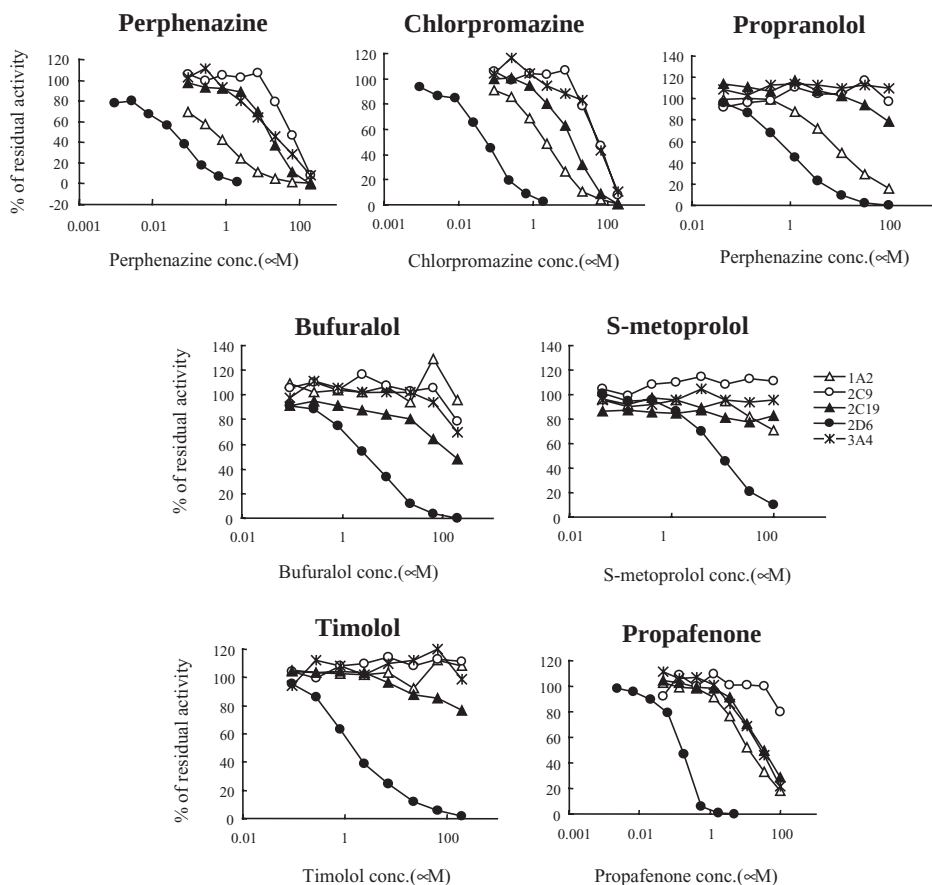


Figure 5. Inhibitory effects of perphenazine, chlorpromazine, propranolol, bufuralol, *S*-metoprolol, timolol and propafenone on five recombinant CYPs' (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4) activities. An inhibition study using an MTP assay was done as described by Yamamoto *et al.* (2002). Perphenazine, chlorpromazine, timolol and propranolol showed large differences in the IC_{50} ratio between HLM and recombinant CYPs (HLM/rCYP ratio) and bufuralol, *S*-metoprolol and propafenone showed small differences of the HLM/rCYP ratio (table 2). Each point is the average of duplicate determinations.

9.79 μ M, respectively. Bufuralol extensively inhibited CYP2D6 and had a marginal effect on CYP2C19 with IC_{50} values of 3.44 and 178.15 μ M, respectively. Propafenone inhibited four CYP activities but only inhibited CYP2D6 at a low concentration.

Discussion

The main purpose of this study was to assess the involvement of single or multiple enzymes in the metabolism of a drug by using an inhibitory assessment approach. The IC_{50} values of antidepressants for recombinant CYP2D6 activity were similar to the IC_{50} values of antiarrhythmic drugs (except for propafenone), but the values of antipsychotic drugs such as perphenazine, chlorpromazine, thioridazine and compound A were smaller than 0.1 μ M. These findings imply that the antipsychotic drugs readily interact with drugs metabolized by CYP2D6.

After pre-incubation, the IC_{50} values of many antidepressants were greater than values for co-incubation, but the effect on imipramine did not change. Imipramine was metabolized to 2-hydroxyimipramine and desipramine by CYP2D6. Although desipramine is a minor metabolite, it inhibits CYP2D6 activity as well as imipramine (Ball *et al.* 1997, Koyama *et al.* 1997, Yang *et al.* 1999). The IC_{50} values for chlorpromazine, risperidone, thioridazine, compound A and typical substrates of CYP2D6 such as bufuralol and dextromethorphan were several fold greater after pre-incubation. This result agrees with a previous report that the IC_{50} values of terfenadine and phenacetin (typical substrates of CYP3A4 and CYP1A2, respectively) increase after pre-incubation (Yamamoto *et al.* 2002). Therefore, the increase in the IC_{50} after pre-incubation suggests that the tested compounds were substrates of CYP2D6 due to decreases in concentrations of the compound in incubation medium during the pre-incubation period (figure 6B).

After pre-incubation, the IC_{50} values of alprenolol and propafenone for CYP2D6 activity increased but those of timolol, *S*-metoprolol and propranolol decreased. According to a previous report that the IC_{50} values of troleandomycin and furafylline (mechanism-based inhibitors) decreased after pre-incubation (Yamamoto *et al.* 2002), thereby indicating that the metabolite formed during pre-incubation might inhibit CYP2D6 activity more extensively than the parent compound or that a mechanism-based inhibition occurs (figure 6C).

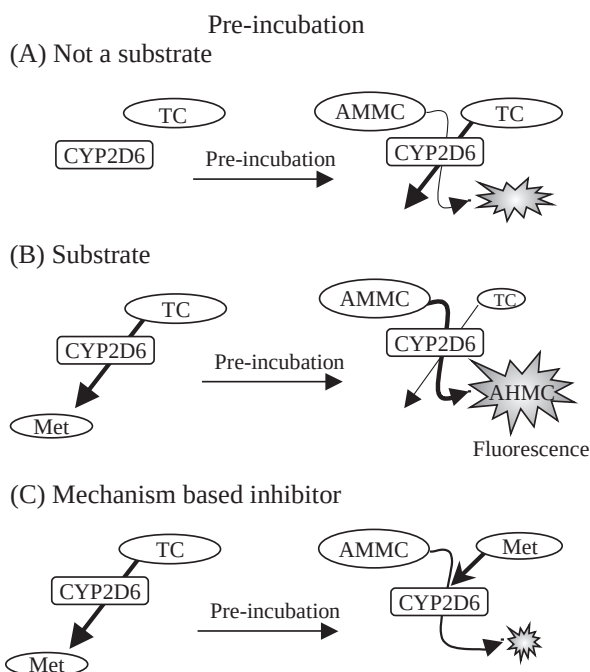


Figure 6. Scheme of inhibition patterns in the incubation medium including recombinant CYP2D6 microsomes after pre-incubation. TC, test compound; AMMC, 3-[2-(*N,N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (substrate of CYP2D6); AHMC, 3-[2-(*N,N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (fluorescent metabolite from AMMC); Met, metabolite of the test compound metabolized by CYP2D6. (A) This case shows that the test compound is not a substrate of CYP2D6. (B) This case shows that the test compound is a substrate of CYP2D6. (C) This case shows that the test compound is a substrate of CYP2D6 and that the formed metabolite is a mechanism-based inhibitor.

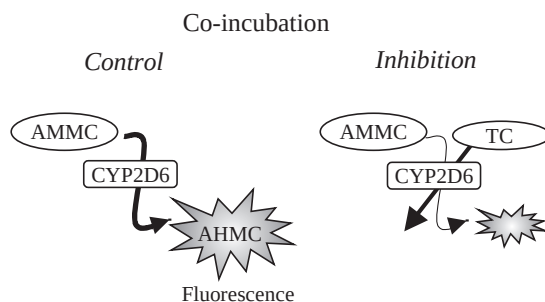
When the inhibitory effects of amitriptyline, clozapine, imipramine, nortriptyline and desipramine, which are mainly metabolized by multiple CYP forms, on five recombinant CYP activities were assessed, all five compounds inhibited all activities of CYP forms involved in the metabolism of these compounds (figure 3). As shown table 1, the $K_{i\text{-cal}}$ values for these compounds for each recombinant CYP corresponded closely with the $K_{m\text{-rep}}$ values reported in the literature (Koyama *et al.* 1997, Linnet and Olesen, 1997, von Moltke *et al.* 1998, Venkatakrishnan *et al.* 1999, 2001). Since these compounds are substrates of each CYP form, correspondence between the $K_{i\text{-cal}}$ and $K_{m\text{-rep}}$ suggests that affinity for each CYP form of the compounds was estimated by determination of the inhibitory potential. Although there are some exceptions such as quinidine, fluconazole and fluoxetine, if the CYP inhibitory patterns are competitive inhibition based on metabolism, the MTP assay for assessment of the inhibitory effect of a test compound on recombinant CYP activity and the increase in the IC_{50} values after pre-incubation leads us to conclude that the test compound was a substrate for the CYP forms.

The inhibitory effects of various substrates of CYP2D6 on CYP2D6 activity in HLM were also compared with the results for recombinant CYP2D6. Among the compounds studied, the HLM/rCYP ratios (IC_{50} ratio of HLM to rCYP) of perfenazine, chlorpromazine and timolol were substantially larger than for other compounds. For quinidine, mianserin, risperidone, thioridazine, propranolol and clomipramine, the HLM/rCYP ratios were about 5. The IC_{50} values of other compounds including bufuralol and dextromethorphan, typical substrates of CYP2D6, in HLM closely corresponded to those in recombinant CYP2D6 and the HLM/rCYP ratio was approximately 2 or less. Therefore, when the HLM/rCYP ratio is greater than approximately 2 in our evaluation system, it was concluded that test compounds in HLM are likely metabolized by other CYP forms with HLM in addition to CYP2D6 (figure 7, multiple metabolism). This consideration is supported by the reports describing perphenazine, chlorpromazine, mianserin, risperidone, propranolol and clomipramine are substrates of multiple CYP forms in HLM (Yoshimoto *et al.* 1995, Koyama *et al.* 1996, Nielsen *et al.* 1996, Fang *et al.* 1999, Olesen and Linnet 2000, Yoshii *et al.* 2000).

Quinidine is a selective inhibitor of CYP2D6, but the HLM/rCYP ratio exceeded 5. This result is based on the finding that quinidine is mainly metabolized by CYP3A4 in HLM (Nielson *et al.* 1995) contrary to the case of the recombinant CYP2D6 microsomes that does not express other P450s. Although thioridazine is mainly metabolized by CYP2D6, the HLM/rCYP ratio exceeded 5. One reason for this may be that metabolism is catalysed by other enzymes as well as by CYP2D6, because it has been reported that flavin-containing monooxygenase (FMO) is involved in the metabolism in mouse liver (Blake *et al.* 1995). Although amitriptyline is metabolized by many CYP forms (Venkatakrishnan *et al.* 2001), the inhibitory effects on CYP2D6 activity in HLM did not change compared with findings with recombinant CYP2D6 microsomes. For this reason, it is suggested that the metabolites formed might inhibit CYP2D6 activity in HLM.

On the other hand, the inhibitory effects of many compounds including bufuralol, dextromethorphan and *S*-metoprolol in HLM were similar to that in the case of recombinant CYP2D6. As shown in figure 7 (single metabolism), this is based on findings that these compounds (except for metabolism of dextromethorphan by CYP3A4 at high concentration) are not metabolized by other CYPs. These considerations are further indirectly supported from the observation that

(A) Recombinant CYP2D6 microsomes



(B) Human liver metabolism

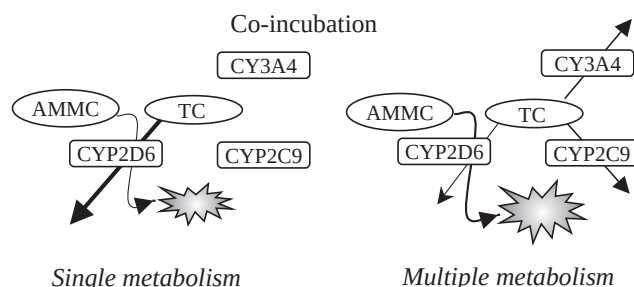


Figure 7. Comparison of possible inhibition patterns between the incubation medium including recombinant CYP2D6 microsomes (A) and HLM (B) using the MTP assay. TC, test compound; AMMC, 3-[2-(*N,N*-diethyl-*N*-methyl-ammonium)ethyl]-7-methoxy-4-methyl-coumarin (substrate of CYP2D6); AHMC, 3-[2-(*N,N*-diethylamino)ethyl-7-hydroxy-4-methyl-coumarin (fluorescent metabolite from AMMC); Met, metabolite of a test compound metabolized by CYP2D6.

perphenazine, chlorpromazine and propranolol inhibit multiple CYP activities forms but bufuralol, *S*-metoprolol and propafenone (at a low concentration) inhibited only CYP2D6 activity among the five CYP forms examined. There are reports that each compound was metabolized by CYP forms in which these compounds showed inhibitory activity (Otton *et al.* 1988, Olesen and Linnet 2000, Yoshii *et al.* 2000, Yoshimoto *et al.* 1995, Mankowski 1999, Anzenbacherova *et al.* 2000, McGinnity *et al.* 2000). Therefore, the IC_{50} values of perphenazine and chlorpromazine for CYP2D6 activity in the HLM seem to be larger than those on recombinant CYP2D6.

In the current study, timolol inhibited CYP2D6 activity in recombinant CYP and the IC_{50} decreased after pre-incubation. This result suggests that the formed metabolites in recombinant CYP2D6 extensively inhibit CYP2D6 activity as compared with timolol. However, the IC_{50} of timolol in HLM was about 20 times greater than that for recombinant CYP2D6. The increase in the IC_{50} suggests that timolol in HLM is likely metabolized by other enzyme as well as by CYP2D6 and that the metabolites formed do not inhibit CYP2D6 activity. Ishii *et al.* (2000) reported that timolol was partially metabolized by CYP2D6 in HLM, but there is no information to support this result. If there is large difference in protein binding of timolol in the incubation medium between HLM and recombinant CYP2D6, the increased protein binding might contribute to the increased IC_{50} values in HLMs.

In conclusion, in recombinant CYP2D6, the increase in IC₅₀ values observed after pre-incubation strongly implies that the test compound is a substrate of CYP2D6 (figure 6B). When the HLM/rCYP ratio of IC₅₀ values obtained from the MTP assay using the combination of rCYP and HLM exceeds approximately 2, the compound is likely metabolized by other CYP forms, in addition to CYP2D6 (figure 7). In drug development, if an orally administered drug is predominantly eliminated by metabolism, it is very important to determine whether the metabolism is mediated by single or multiple CYPs in the liver. The considerable involvement of CYP2D6 in the metabolism of drug often is considered to be a negative property in the development of a drug. However, if new drug candidates are metabolized by multiple enzymes, this negative property observed from recombinant CYPs might not be a large problem *in vivo*. To estimate CYP forms involved in the metabolism of drug using HPLC or LC/MS/MS at an early stage of drug discovery is time and labour consuming. Therefore, the MTP assay using a combination of HLM and recombinant CYP2D6 is useful readily to estimate the involvement of single or multiple CYPs in the metabolism of new drug candidates at an early stage of drug discovery because the MTP assay has the advantage such as speed, high throughput and ease of the technique. However, more definitive studies are needed in HLM to confirm the initial findings of the screening process and to identify the contribution of CYP forms involved in the metabolism of drugs or the inhibition mechanism to predict the drug–drug interactions at an early stage of drug development.

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Identification of CYP3A4 and CYP2C8 as the major cytochrome P450s responsible for morphine *N*-demethylation in human liver microsomes

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Received 8 January 2003

1. The aim was to identify the cytochrome P450 (CYP) enzymes responsible for the *N*-demethylation of morphine *in vitro*.

2. In human liver microsomes, normorphine formation followed Michaelis–Menten kinetics with mean K_m and V_{max} of 12.4 ± 2.2 mM and 1546 ± 121 pmol min⁻¹ mg⁻¹, respectively. In microsomes from a panel of 14 human livers phenotyped for 10 CYP enzymes, morphine *N*-demethylation correlated with testosterone 6 β -hydroxylation ($r = 0.91$, $p < 0.001$) and paclitaxel 6- α hydroxylation ($r = 0.72$, $p < 0.001$), two specific markers of CYP3A4 and CYP2C8, respectively. Normorphine formation decreased when incubated in the presence of troleandomycin or quercetin (by 46 and 33–36%, respectively), which further corroborates the contribution of CYP3A4 and CYP2C8.

3. Among eight recombinant human CYP enzymes tested, CYP3A4 and CYP2C8 exhibited the highest intrinsic clearance. More than 90% of morphine *N*-demethylation could be accounted for via the action of both CYP3A4 and CYP2C8.

4. The *in vitro* findings suggest that hepatic CYP3A4, and to a lesser extent CYP2C8, play an important role in the metabolism of morphine into normorphine.

Introduction

Morphine is a potent opioid analgesic traditionally used for the treatment of moderate to severe pain (Levy 1996). In humans, it is metabolized by three distinct metabolic pathways: glucuronidation, sulfation and *N*-demethylation (figure 1) (Milne *et al.* 1996). Irrespective of the route of administration, the majority of morphine clearance can be attributed to its biotransformation into morphine 3-glucuronide (M-3-G) and to a lesser extent, into morphine 6-glucuronide (M-6-G), morphine 3-sulfate and normorphine (Yeh *et al.* 1977, Hasselström and Säwe 1993).

Normorphine possesses analgesic properties (Lasagna and Kornfeld 1958) and may contribute to morphine-induced toxicity (Glare *et al.* 1990). This metabolite is found in urine from normal and addicted subjects (Yeh *et al.* 1977, Hasselström and Säwe 1993, Zhou *et al.* 1994, Fromm *et al.* 1997) and is formed in greater amounts when morphine is administered orally (Brunk and Delle 1974). This may account for the difference in free morphine concentrations found in urine after oral and

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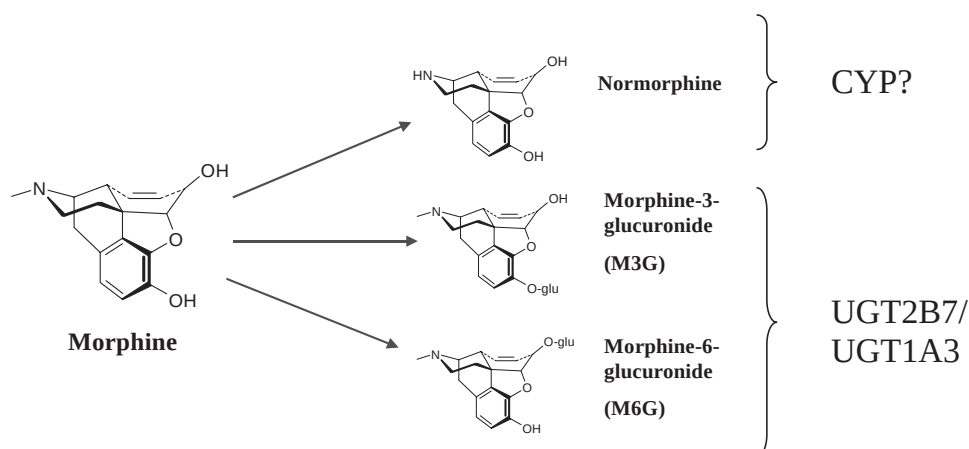


Figure 1. Morphine and its main metabolic pathways in humans.

intravenous administration (Glare and Walsh 1991). However, because the enzyme(s) mediating the formation of normorphine in humans is unknown, the influence of pathophysiological conditions or drug interactions on the metabolism of morphine cannot be entirely predicted.

Although the metabolic profile of morphine is relatively well described *in vivo*, only the enzymes involved in its glucuronidation (e.g. UGT2B7 for the conversion of morphine into M-3-G and M-6-G and less importantly UGT1A3 for the formation of M-3-G) have been identified (Coffman *et al.* 1998, Green *et al.* 1998). In contrast, the enzymes responsible for the sulfation and *N*-demethylation reactions remain to be identified. Although this has never been proven experimentally in human liver microsomes, morphine is thought to be *N*-demethylated via CYP3A4 (Milne *et al.* 1996, Fromm *et al.* 1997). The purpose of the present study was to identify, *in vitro*, the CYP(s) responsible for normorphine formation in humans.

Material and methods

Chemicals

Morphine sulfate was obtained from BDH (Toronto, Ontario, Canada) and naloxone bis-hydrochloride was from RBI (Natick, MA, USA). Normorphine hydrochloride was obtained from Sigma-Aldrich (St Louis, MO, USA). Other compounds were from the following sources: magnesium dichloride hexahydrate (MgCl_2), glucose 6-phosphate, glucose 6-dehydrogenase, nicotinamide adenosine di-phosphate (NADP^+), troleandomycin (TAO), sulphaphenazole (SPZ), quinidine hydrochloride and quercetin were from Sigma-Aldrich (St Louis, MO, USA); *S*(+)-mephenytoin (*S*-meph) was from Ultrafine (Manchester, UK). Stock solutions of morphine, normorphine and naloxone were prepared in distilled water and stored in amber glass tubes at -20°C . Working solutions were prepared in appropriate solvents immediately before the experiments. All other chemicals and reagents were purchased from Sigma-Aldrich.

Human liver microsomes

General incubation conditions. Human liver microsomes were purchased from Xenotech LLC (Kansas City, KS, USA). Human livers were obtained from approved sources within the USA, in compliance with United Network for Organ Sharing policies. All incubations (human liver microsomes (0.4 mg ml^{-1} proteins), morphine ($7\text{--}13\,000\,\mu\text{M}$), 0.1 M potassium phosphate buffer at pH 7.4) were performed in duplicate at 37°C . After a 5-min pre-incubation, the reaction was started by the addition

of an NADPH-regenerating system (1 mM NADP⁺, 5 mM glucose 6-phosphate, 1 unit ml⁻¹ glucose 6-phosphate dehydrogenase, 3.3 mM MgCl₂). The final incubation volume was 500 µl. Reactions were stopped after 30 min by the addition of an equal volume of ice-cold acetonitrile acidified with 0.1% formic acid containing 750 nM naloxone (internal standard). Samples were then vortex-mixed for 1 min, put on ice for 10 min and centrifuged (10 000 *g* for 20 min at 4°C). Supernatants (350 µl) were frozen at -80°C until analysis. Control incubations were carried out without the NADPH-regenerating system or without microsomes.

Enzyme kinetics. The kinetics of normorphine formation were determined using three sets of pooled human liver microsomes obtained from 10 to 15 donors (Xenotech LLC, Kansas City, KS, USA). Morphine was incubated at concentrations ranging from 7 to 13 000 µM. Morphine could not be incubated at higher concentrations than 13 mM because of solubility limitations (stock solutions precipitated at concentrations above 130 mM). Enzyme kinetic parameters ($V_{\max}$, K_m) were assessed using computerized iterative non-linear regression (Prism, GraphPad Software, Inc., San Diego, CA, USA). The *in vitro* intrinsic clearance (CL_{int}) was calculated as the ratio of $V_{\max}$ over K_m (Houston and Kenworthy 2000). Morphine *N*-demethylation CL'_{int} was scaled up to an *in vivo* intrinsic clearance (C'_{int}) as described in equation (1) and using previously published values of scaling factors (45 mg microsomal protein g⁻¹ liver and 20 g liver kg⁻¹ body weight) (Carlile *et al.* 1997, Obach 1999):

$$CL'_{\text{int}} = CL_{\text{int}} \cdot (45 \text{ mg microsomes g}^{-1} \text{ liver}) \cdot (20 \text{ g liver kg}^{-1} \text{ bodyweight}) \quad (1)$$

The CL'_{int} of morphine *N*-demethylase was entered in equation (2), along with estimates of human hepatic blood flow ($Q = 20 \text{ ml min}^{-1} \text{ kg}^{-1}$; Obach 1999) and morphine-free fraction (f_u) in human plasma (0.65; Glare and Walsh 1991) to predict the *in vivo* partial clearance ($CL_{(N-DM)}$) of morphine *N*-demethylase using the well-stirred model (Obach 1999):

$$CL_{(N-DM)} = Q \cdot f_u CL'_{\text{int}} / (Q + f_u CL'_{\text{int}}) \quad (2)$$

Chemical inhibition studies. Morphine (50 µM) was incubated with human liver microsomes (three different pools) in the presence or absence of two different concentrations of specific inhibitors or substrates of CYPs (see figure 3 for concentrations), namely SPZ (CYP2C9, Newton *et al.* 1995), quercetin (CYP2C8, Rahman *et al.* 1994, Masimirembwa *et al.* 1999), *S*-meph (CYP2C19, Loft *et al.* 1991), quinidine (CYP2D6) and TAO (CYP3A, both Newton *et al.* 1995). Stock solutions of CYP inhibitors/substrates were prepared in methanol and diluted in methanol immediately before the incubations. The final methanol concentrations never exceeded 1% v/v and appropriate incubation controls (with morphine only and appropriate amounts of methanol) were used. The mechanism-based inhibitor TAO was pre-incubated with microsomes (for 15 min in the presence of the NADPH-regenerating system) before the addition of morphine. All other inhibitors/substrates were pre-incubated with morphine before the addition of an NADPH-regenerating system, as described in the section on general incubation conditions.

Regression studies. The *N*-demethylation of morphine (50 µM) was investigated in a panel of 15 human liver microsomes (Reaction Phenotyping[®] kit v.5, Xenotech LLC). The microsomal preparations were characterized for the following activities: NADPH-P450 reductase, 7-ethoxyresorufin *O*-dealkylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), *S*-mephenytoin *N*-demethylation (CYP2B6), *S*-mephenytoin 4-hydroxylation (CYP 2C19), paclitaxel (taxol) 6 α -hydroxylation (CYP2C8), tolbutamide methyl-hydroxylation (CYP2C9), dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), testosterone 6 β -hydroxylation (CYP3A4/5) and lauric acid 12-hydroxylation (CYP4A11). Statistical analyses were performed with Sigma Stat 2.0 (SPSS, Inc., San Rafael, CA, USA). The statistical significance of any correlation between normorphine formation and specific CYP activities was evaluated by simple linear regression using a Student's *t*-test ($\alpha = 0.05$).

cDNA-expressed human CYPs

Morphine *N*-demethylation was evaluated in microsomes prepared from insect cells transfected with cDNA encoding for human CYP1A2, CYP2A6, CYP2C8, CYP2C9-Arg₁₄₄, CYP2C19, CYP2D6, CYP2E1 or CYP3A4. The recombinant enzymes and microsomes from untransfected insect cells were purchased from Gentest (Supersome[®]; Woburn, MA, USA). All incubations (microsomes (100 pmol CYP ml⁻¹), morphine (50 µM), 0.1 M potassium phosphate buffer at pH 7.4) were performed at 37°C. After a 5-min pre-incubation, the reaction was started by the addition of the NADPH-regenerating system (1 mM NADP⁺, 5 mM glucose 6-phosphate, 1 unit ml⁻¹ glucose 6-phosphate dehydrogenase, 3.3 mM MgCl₂). The final incubation volume was 500 µl. Reactions were stopped after 20 min by the

addition of an equal volume of ice-cold acetonitrile acidified with 0.1% formic acid and containing 750 nM naloxone (internal standard). Samples were then vortex-mixed for 1 min, put on ice for 10 min and centrifuged (10000g for 20 min at 4°C). Supernatants (350 µl) were frozen at -80°C until analysis. Control incubations were carried out without NADPH-regenerating system, without protein or in microsomes from untransfected insect cells. Normorphine formation rates, expressed as pmol min⁻¹ pmol⁻¹ CYP, were corrected for the basal rate of formation of normorphine (0.01 pmol min⁻¹ pmol⁻¹ CYP) when morphine was incubated in microsomes from untransfected insect cells.

Enzyme kinetics and relative contribution of CYPs. CYPs found to contribute to at least 1% of total relative activity were subjected to enzyme kinetics investigations, as described above. The Michaelis-Menten kinetics of morphine *N*-demethylation by CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 were investigated using substrate concentrations ranging from 7 to 13 000 µM. Morphine could not be incubated at higher concentrations than 13 mM because of solubility limitations (stock solutions precipitated at concentrations above 130 mM). The relative contribution of individual CYPs (RC_i of CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) to morphine *N*-demethylation was estimated using the relative activity factor (RAF) approach suggested by Crespi and Miller (1999), Strömer *et al.* (2000a, b) and Venkatakrishnan *et al.* (2001a). The individual CYP reaction velocity ($v_i(S)$) of the isoform *i* at the morphine concentration [S] was obtained using the Michaelis-Menten equation (3). RAF_{*i*} values for specific isoforms (8.3 for CYP2C8, 4.0 for CYP2C9, 230 for CYP2C19, 42 for CYP2D6, 3.5 for CYP3A4) were obtained from Gentest and entered with $v_i(S)$ in equation (4) and (5) to estimate RC_{*i*}:

$$v_i(S) = (V_{\max,i}[S]) / (K_{m,i} + [S]) \quad (3)$$

$$v = \sum_i v_i(S) / \text{RAF}_i \quad (4)$$

$$\text{RC}_i(\%) = \{v_i(S) / (\text{RAF}_i \times v) \times 100\}. \quad (5)$$

LC/MS analysis of normorphine. Normorphine was quantified using a modified version of an HPLC-MS method developed in our laboratory (Projean *et al.* 2003). Briefly, acetonitrile-diluted (200 µl) supernatants were collected and evaporated to dryness under a stream of nitrogen. Dry residues were dissolved in 200 µl 0.1% v/v formic acid and 30 µl were injected into the LC/MS system. Chromatographic separations were achieved on a reversed-phase HPLC column (3 µm particle size Synergi polar-RP 75 × 4.6 mM; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% v/v formic acid in acetonitrile (A) and 0.1% v/v formic acid in water (B). After 1 min at 100% of B, a linear gradient of A into B was run (0–75% v/v) from 1 to 10 min. The column was allowed to re-equilibrate for 2.5 min between injections. The flow rate was 1 ml min⁻¹. Retention times were 3.2 min for normorphine, 4.2 min for morphine and 5.1 min for the internal standard naloxone. The MS was operated in scan mode for the qualitative analysis of the incubates (m/z 200–500) and in selected ion monitoring (SIM) mode for the quantitative studies (m/z = MH⁺, M being the mass of the parent compound; m/z 272 for normorphine, m/z 286 for morphine, m/z 328 for the internal standard). Calibration curves (9.7–10 000 nM) were constructed by linear regression of the analyte/internal standard peak area ratio versus concentration curves (using a weighting factor of 1/concentration). Mean intraday precision was less than 10%, mean accuracy was approximately 95%. The limit of quantification was 9.7 nM.

Results

Kinetic studies in human liver microsomes

The NADPH-dependent metabolism of morphine resulted in the formation of normorphine in all incubations. No other oxidative metabolite could be detected by mass spectrometry in SCAN mode. Normorphine formation required microsomal proteins and the NADPH-regenerating system. The reaction was linear with protein concentration and time. The kinetic parameters describing normorphine formation in pooled human liver microsomes are presented in table 1.

In all cases, normorphine formation followed apparent first-order Michaelis-Menten kinetics (figure 2). Mean apparent K_m and $V_{\max}$ were 12.4 ± 2.2 mM and

Table 1. Apparent Michaelis–Menten kinetic parameters for morphine *N*-demethylation in pooled human liver microsomes. All incubations were performed in duplicate.

	Morphine <i>N</i> -demethylation		
	K_m mM	V_{max} pmol min ⁻¹ mg ⁻¹ protein	CL_{int} μl min ⁻¹ mg ⁻¹ protein
Pool 1	13.9	1606	0.116
Pool 2	13.7	1625	0.118
Pool 3	9.6	1407	0.147
Mean ± SD	12.4 ± 2.2	1546 ± 121	0.127 ± 0.02

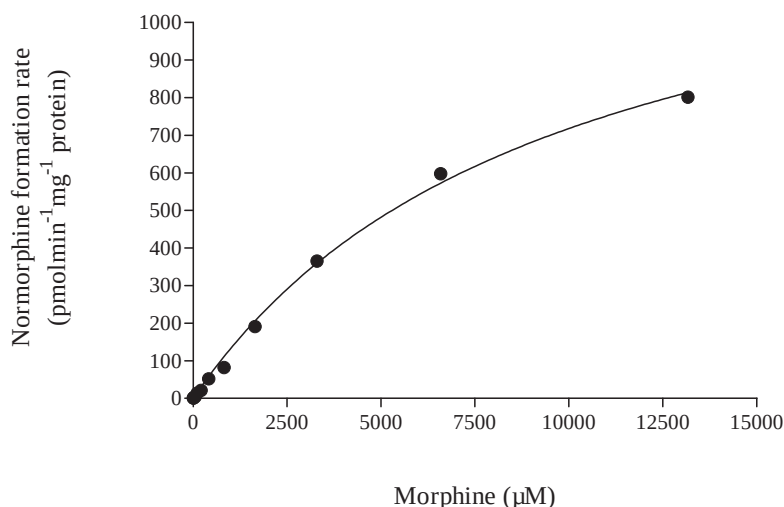


Figure 2. Kinetics of normorphine formation from morphine in pooled human liver microsomes (HLM3). Symbols represent observed data (average of duplicate samples), and the curve was fitted by non-linear regression. Kinetic parameters are shown in table 1.

1546 ± 121 pmol min⁻¹ mg⁻¹, respectively, yielding a mean apparent CL_{int} = 0.127 ± 0.017 μl min⁻¹ mg⁻¹ protein. Normorphine formation kinetics in HLM predicted a $CL_{(N-DM)}$ of 0.08 ± 0.01 ml min⁻¹ kg⁻¹ in man.

Effect of chemical CYP inhibitors

Results from the co-incubation of morphine with various CYP inhibitors/substrates in human liver microsomes are shown in figure 3. Inhibitors/substrates selective for CYP2C19 (*S*-meph), CYP2D6 (quinidine) and a low dose of SPZ (25 μM) had little (<10%) or no effect on the biotransformation of morphine into normorphine. A 20% inhibition was observed with high (50 μM) but less selective concentrations of SPZ. Low concentrations of quercetin, a CYP2C8 inhibitor, decreased normorphine formation by approximately 31–36%. TAO, a selective mechanism-based inhibitor of CYP3As, inhibited normorphine formation by 46%.

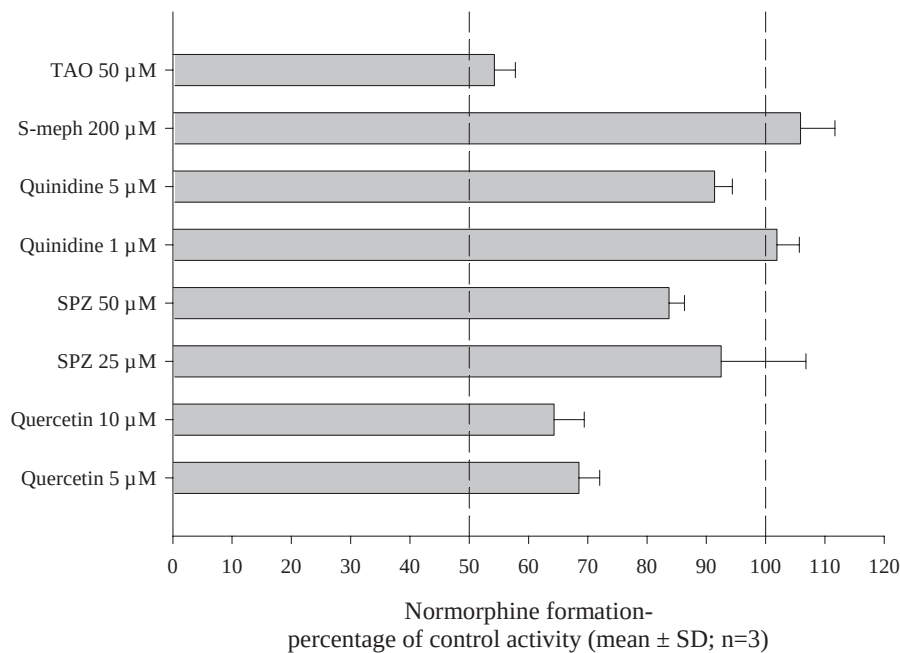


Figure 3. Effect of chemical substrates/inhibitors on morphine *N*-demethylation in pooled human liver microsomes. Morphine was incubated at 50 µM. SPZ, sulphaphenazole; *S*-meph, *S*-mephenytoin; TAO, troleandomycin. Data were derived from three different determinations; the 100% value is 7.5 pmol min⁻¹ mg⁻¹.

Table 2. Correlation between morphine *N*-demethylase activity and various CYP-specific activities following the incubation of morphine (50 µM) in a panel of 15 different human liver microsomal preparations.

CYP-specific catalytic activities	Morphine <i>N</i> -demethylase activity	
	CYP	Correlation coefficient (<i>r</i>)
Ethoxyresorufin <i>O</i> -deethylase	1A2	0.09
Coumarin 7-hydroxylase	2A6	0.57
<i>S</i> -mephenytoin <i>N</i> -demethylase	2B6	0.33
Paclitaxel 6α-hydroxylase	2C8	0.72 ***
Tolbutamide methylhydroxylase	2C9	0.45
<i>S</i> -mephenytoin 4'-hydroxylase	2C19	0.49
Dextrometorphan <i>O</i> -demethylase	2D6	0.18
Chlorzoxazone 6-hydroxylase	2E1	0.03
Testosterone 6β-hydroxylase	3A	0.91 ***
Lauric acid 12-hydroxylase	4A11	0.10

p* < 0.05, *p* < 0.01, ****p* < 0.001, Student's *t*-test.

Regression studies

In characterized microsomal preparations from 15 human livers, normorphine formation rates showed a 10-fold interindividual variability. Data from simple regression analyses (table 2) suggest that CYP3A (by the formation of 6β-hydroxy-testosterone, figure 4A) and CYP2C8 (by the formation of 6-α-hydroxy-paclitaxel, figure 4B) are likely to be involved in normorphine formation.

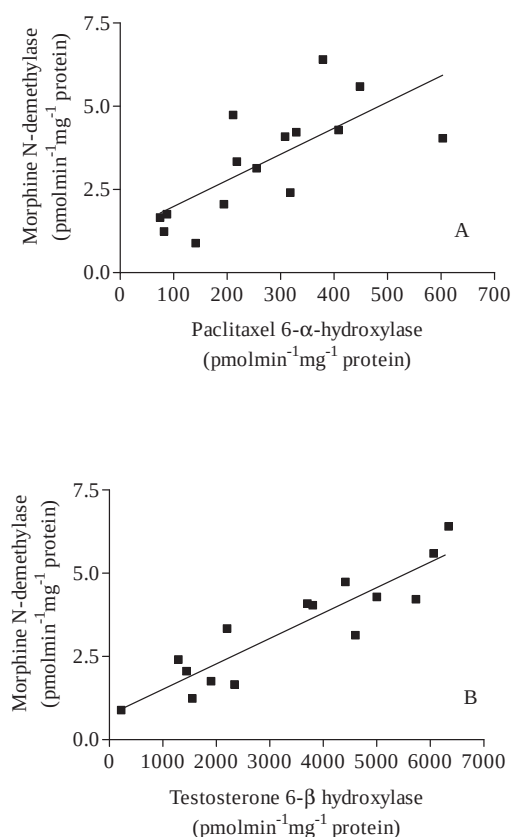


Figure 4. Relationship between paclitaxel 6- α -hydroxylase (A), testosterone-6 β -hydroxylase (B) and morphine *N*-demethylase activity. Morphine (50 μ M) was incubated in 15 different human liver microsomal preparations. Regression coefficients (*r*) are shown in table 3. Symbols represent observed data (average of duplicate samples).

Human CYPs expressed in insect cells

Recombinant human CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 had the capacity to catalyse normorphine formation (figure 5). Under these conditions, CYP3A4 *N*-demethylated morphine more efficiently than any other CYPs. Recombinant CYP2D6, CYP2C19 and CYP2C8 followed CYP3A4 in decreasing order of importance. The least effective enzyme was CYP2C9. The other CYP enzymes exhibited no significant metabolic activity.

The kinetics of morphine *N*-demethylation were investigated further with recombinant CYP2D6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4. Normorphine formation followed apparent first-order Michaelis–Menten kinetics in all recombinants tested (table 3 and figure 6). In agreement with the K_m obtained in human liver microsomes, the K_m obtained in recombinant CYPs ranged from 4.8 to 14 mM. CYP2C8 and CYP2C19 exhibited the highest affinity for morphine, whereas CYP3A4 showed the highest intrinsic clearance. Consistent with the results obtained using a single 50 μ M concentration of morphine, CYP2C9 was the least efficient enzyme to catalyse normorphine formation. CYP2D6 efficiency was lower than that obtained with CYP2C8 and CYP2C19.

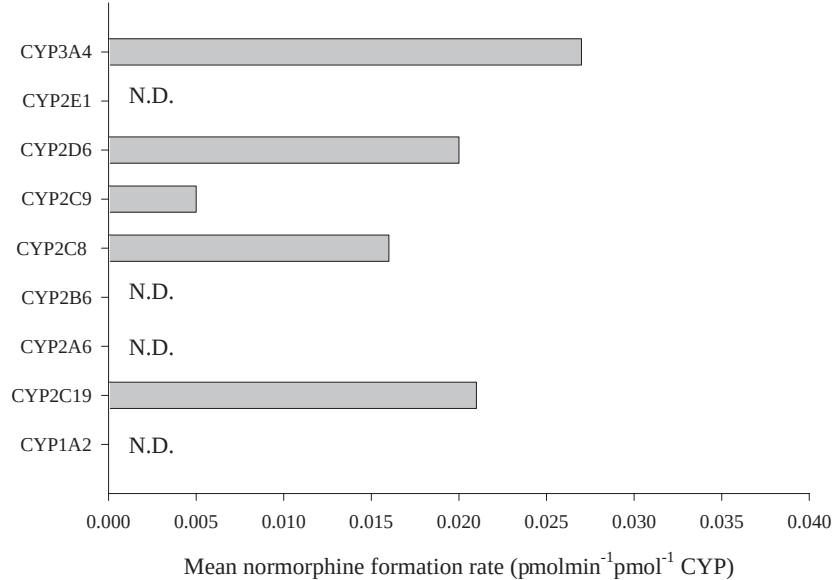


Figure 5. Normorphine formation rate following the incubation of 10 μ M morphine in microsomes prepared from insect cells expressing individual human CYPs. Samples were assayed in triplicate. N.D., no detectable formation of normorphine by the enzyme.

Table 3. Apparent Michaelis–Menten kinetic parameters for morphine *N*-demethylation in microsomes from cDNA-expressed CYPs in insect cells. Incubations were performed in duplicate.

	Morphine <i>N</i> -demethylation		
	K_m mM	V_{max} pmol min ⁻¹ pmol ⁻¹ CYP	CL_{int} μ l min ⁻¹ pmol ⁻¹ CYP
CYP2C8	4.8	5.41	0.0011
CYP2C9	25.4	5.32	0.0002
CYP2C19	6.4	7.67	0.0012
CYP2D6	10.1	6.57	0.0007
CYP3A4	14.15	28.13	0.0020

Predicted contribution of CYP enzymes to morphine N-demethylation

The relative contributions (RCi) of CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 to morphine *N*-demethylation in HLM were estimated using enzyme kinetic parameters obtained in recombinant human CYPs (table 3) over a concentration range determined to be representative of *in vivo* liver concentrations (0–100 μ M). For the most part, morphine *N*-demethylation was accounted for by CYP3A4 (about 60%) and CYP2C8 (about 30%) (figure 7). CYP2C19, CYP2C9 and CYP2D6 appeared to be of minor importance, accounting globally for less than 10% of the total reaction.

Discussion

This is the first investigation of morphine oxidative metabolism in human liver microsomes. Mass spectrometry analysis of the microsomal incubates revealed that normorphine was the only metabolite formed in our incubation conditions.

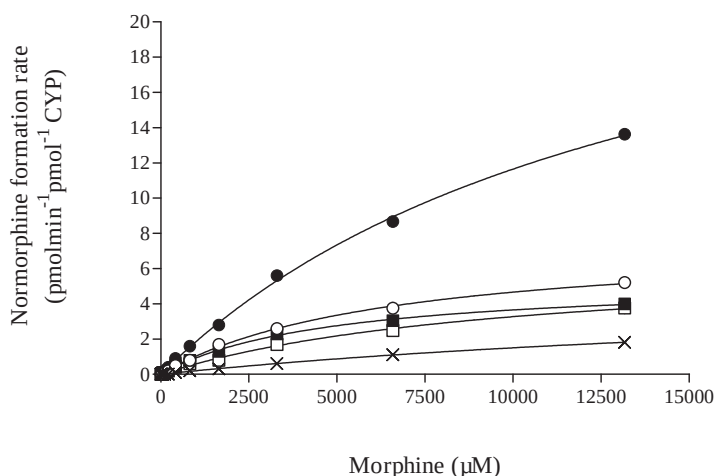


Figure 6. Kinetics of normorphine formation from morphine in cDNA-expressed human CYP enzymes. ●, CYP3A4; ■, CYP2C8; ○, CYP2C19; □, CYP2D6; ×, CYP2C9. Symbols represent observed data (average of duplicate samples); lines represent formation rates predicted with non-linear regression. Kinetic parameters are shown in table 3.

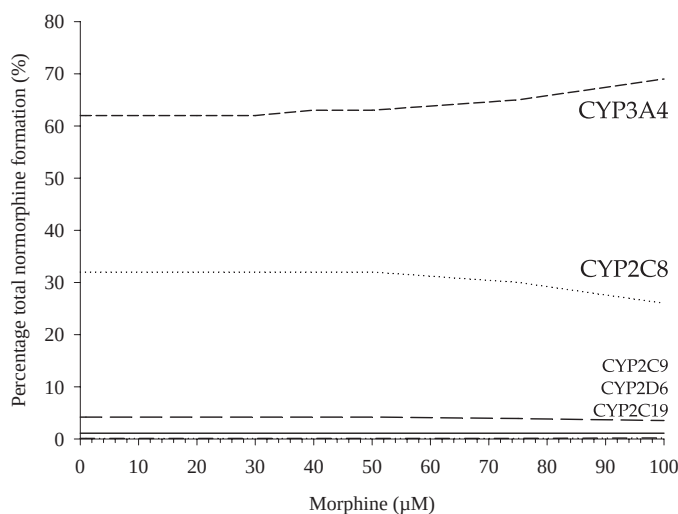


Figure 7. Percentage contribution to morphine *N*-demethylation for CYP3A4, CYP2C8, CYP2C9, CYP2D6 and CYP2C19 predicted from recombinant CYP using RAF normalization. Calculations were based on kinetic parameters obtained from recombinant CYP shown in table 3.

Clinically, in chronically treated subjects, morphine peak-to-trough plasma concentrations rarely exceed the micromolar range (Faura *et al.* 1996). However, distribution studies in animals that received single or multiple doses of morphine have shown that morphine tends to concentrate in tissues such as the kidney, liver, lung, spleen and muscle (Woods 1954). Accordingly, in patients, morphine concentrations in liver are likely to be higher than plasma. Intrahepatic concentrations can be approximated by multiplying plasma concentrations by a partition factor obtained from animal distribution studies or clinical autopsies (Schmider *et al.* 1999). Post-mortem analyses reported a morphine liver to blood partition

ratio of approximately 4 (Felby *et al.* 1974). In addition, local liver concentrations may be even higher shortly after oral administration (Shen *et al.* 1997). Morphine is rapidly and completely absorbed from the gastrointestinal tract and is subjected to extensive first-pass metabolism (Milne *et al.* 1996). Liver concentrations of 10^{-6} – 10^{-5} M are therefore relevant to study the *in vitro* metabolism of morphine.

The mean apparent K_m of 12.4 ± 2.2 mM evidences that liver microsomal enzymes metabolizing morphine to normorphine have a poor affinity for the substrate. The mean apparent K_m is consistent with values previously obtained for the *N*-demethylation of structurally related opioids. Thus, the *N*-demethylation of dihydrocodeine is characterized by apparent K_m ranging from 4 to 14 mM (Kirkwood *et al.* 1997).

Using *in vitro* CL_{int} data in human liver microsomes, the predicted $CL_{(N-DM)}$ in man is 0.08 ± 0.01 ml min⁻¹ kg⁻¹, which would only contribute to 0.4% of total morphine clearance. The low predicted clearance obtained in the present experiments suggests that the CYP(s) implicated in normorphine formation would have little contribution to morphine total clearance. These observations are in good agreement with that obtained from *in vivo* data since the major metabolites recovered from biological fluids are morphine glucuronides and not oxidation products (Hasselström and Sawe 1993). In addition, according to urinary data in normal subjects, $CL_{(N-DM)}$ is also expected to contribute to less than 1% of total morphine clearance (Zhou *et al.* 1994).

Although a one-enzyme model best fitted normorphine formation, one cannot exclude the involvement of more than one isoform. Studies using cDNA-expressed systems showed that CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 could all catalyse the formation of normorphine. However, unlike microsomal preparations that contain all CYPs in their native proportions, cDNA-expressed enzymes alone cannot determine whether an enzymatic pathway makes a quantitatively important contribution to the overall metabolism of a compound (Rodrigues 1999). The use of the RAFs has been successfully applied to bridge the gap between recombinant systems and liver microsomes. The RAF approach is based on the assumption that any effect on the rate of formation of a metabolite is independent of the substrate, and that any factor that may affect the rate of metabolism for one substrate will equally affect other substrates (Crespi and Miller 1999, Strömer *et al.* 2000a, b). Since this approach has the advantage of taking into account the inherent differences between the recombinant systems and the liver microsomes, it represents, when used in conjunction with other methods (inhibition/correlation studies), a powerful tool to estimate CYP contribution to drug metabolism (Venkatakrishnan *et al.* 2001b, Nakajima *et al.* 2002).

Using the RAF approach, CYP3A4 was identified as the most active CYP enzyme contributing to more than 60% of total morphine *N*-demethylation over a wide range of concentrations. These observations are consistent with those obtained in human liver microsomes where the formation of normorphine correlated strongly with the 6 β -hydroxylation of testosterone, a CYP3A-mediated reaction (figure 6A) (Waxman *et al.* 1991). Further corroboration of the involvement of CYP3A4 in morphine *N*-demethylation was obtained when the microsomal reaction was extensively inhibited by TAO, a potent inhibitor of CYP3As (Newton *et al.* 1995).

CYP3A4 is recognized as the principal enzyme involved in the *N*-demethylation of many drugs (Smith and Jones 1992). The present results are consistent with

previous findings on the *N*-demethylation of other opioids structurally similar to morphine, including codeine (Caraco *et al.* 1996) and dihydrocodeine (Kirkwood *et al.* 1997), which are also *N*-demethylated by CYP3A4. The present results are also in agreement with clinical findings evidencing a significant increase in total normorphine urine concentrations when morphine was administered orally to healthy volunteers pretreated with rifampin, a CYP3A inducer (Fromm *et al.* 1997).

Although normorphine formation in human liver microsomes was best fitted by a one-enzyme kinetic model, a combination of enzymes having similar kinetic behaviours could lead to the determination of one apparent K_m . In addition, if one CYP is clearly predominant for a particular reaction, this could also result in apparent simple kinetics (Clarke 1998). Therefore, in addition to CYP3A4, other CYPs might contribute to normorphine formation.

Direct evidence that CYP2C8 could also *N*-demethylate morphine was first provided by cDNA-expressed enzymes. Recombinant CYP2C8 had a higher affinity for morphine (table 2 and figure 4) but a lower capacity than CYP3A4. The RAF approach predicted CYP2C8 contribution to account for approximately 30% of total normorphine formation. The results were confirmed by incubations in a panel of human liver microsomes, where there was a significant correlation between normorphine formation and taxol 6 α -hydroxylation, a CYP2C8-mediated reaction (Rahman *et al.* 1994). In addition, low concentrations of quercetin (CYP2C8-inhibitor), inhibited morphine *N*-demethylation by 30–35%, which is also consistent with a contribution of CYP2C8 to normorphine formation.

A modest inhibition of normorphine formation (20%) was observed when morphine was incubated with high concentrations of SPZ. However, recombinant CYPs and correlation studies failed to evidence any significant contribution of CYP2C9. The RAF approach predicted the sum of CYP2D6, CYP2C19 and CYP2C9 to account for less than 7% of normorphine formation. Therefore, it is likely that the 20% inhibition observed with high SPZ concentrations in human liver microsomes may be due to the inhibition of a CYP other than CYP2C9. SPZ has been shown to inhibit CYP2C8 (Mancy *et al.* 1996) and may have partially decreased CYP2C8-mediated normorphine formation.

The contribution of CYP2C8 to the metabolism of drugs and endobiotics has recently gained interest (Ong *et al.* 2000). Thus far, morphine has not been involved in drug interactions with CYP2C8 substrates. This is not surprising since CYP2C8 would be expected to play a minor role, if any, in drug–drug interactions, given the major contribution from CYP3A4 and relatively small fraction of the total CYP contents in the liver expressed as CYP2C8 (Shimada *et al.* 1994, Inoue *et al.* 1997). However, our *in vitro* observations substantiate the hypothesis that a certain degree of overlap exists between CYP2C8 and CYP3A4 substrate specificity (Ong *et al.* 2000). Partial contribution from CYP2C8 to the metabolism of predominantly CYP3A4 substrates has been reported with drugs such as carbamazepine, zopiclone and verapamil (Kerr *et al.* 1994, Becquemont *et al.* 1998, Tracy *et al.* 1999). In light of these data, CYP2C8 may even contribute, albeit to a small extent, to the *N*-dealkylation of opioids identified as CYP3A4-specific substrates such as dihydrocodeine and codeine.

In summary, the present investigations evidence the role of CYP3A4 and CYP2C8 in the *N*-demethylation of morphine in human liver microsomes. However, since *N*-demethylation is a minor pathway of morphine metabolism,

CYP-mediated drug–drug interactions resulting from CYP3A4 or CYP2C8 should not be problematic in the clinic unless major pathways such as glucuronidation are also affected.

Acknowledgements

Denis Projean was supported by Research Studentship from AstraZeneca R&D Montréal.

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Detection of mono- and di-hexoses as metabolites of 4-bromoaniline using HPLC-TOF-MS/MS

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Received 13 February 2003

1. The metabolic fate of 4-bromoaniline (4-BrA) was investigated in rat following intraperitoneal administration at 50 mg kg⁻¹ using HPLC-TOF-MS/MS.
2. The sensitivity provided by the use of TOF-MS/MS, aided by the distinctive isotope pattern resulting from the presence of the bromine substituent in the molecule, enabled the detection of many previously uncharacterized metabolites in the samples.
3. Several groups of minor metabolites were detected in the urine that corresponded to a number of isomeric hexose and di-hexose-containing conjugates (possibly glucosides and diglucosides) of 4-BrA.
4. As well as hexose and di-hexose conjugates of 4-BrA, several further groups of metabolites that also contained either a sulphamate or sulphate group in addition to the sugar moieties were also detected.

Introduction

Anilines and their derivatives are widely used as intermediates in the production of a diverse range of important products including drugs and pesticides. Indeed, the pesticide Metobromuron is a substituted phenylurea based on 4-bromoaniline (4-BrA) and the free aniline has been shown to be produced during human exposure to the compound (Turcant *et al.* 2000). Exposure to anilines has been associated with a range of adverse effects that include haemolysis, methaemoglobin formation, specific organ toxicity (e.g. nephro- and hepatotoxicity) and carcinogenesis (Radomski 1979, Gorrod and Manson 1986). These adverse effects are often mediated through the formation of toxic/reactive metabolites (Gorrod and Manson 1986). An improved understanding of the metabolic fate of this class of compound may reveal the structural features of anilines which promote the formation of toxic metabolites. Such an understanding might then allow the prediction of the metabolic fate of individual anilines via the development of quantitative *in vivo* structure metabolism relationship (QSMR) models (e.g. Ghauri *et al.* 1992, Cupid *et al.* 1996, 1999, Scarfe *et al.* 2002a). As part of a

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series of studies, the metabolic fate of 4-bromoaniline (4-BrA) was investigated in rat (Nicholson *et al.* 2000, Abou-Shakra 2002, Scarfe *et al.* 2002b). In HPLC-NMR-MS studies, the major compound-related structure identified in urine following intraperitoneal (i.p.) administration was the *ortho*-ring-hydroxylated sulphate (Scarfe *et al.* 2002). Minor components also identified included *N*-acetyl-, *N*-oxanilic acid-, *N*-acetyl-cysteinyl- and glycolanilide-*O*-glucuronide metabolites. However, studies on the same samples, using HPLC-inductively coupled plasma mass spectrometry (ICPMS), exploiting the bromine-selective detection afforded by this technique, revealed a much more complex pattern of minor metabolites of 4-BrA than HPLC-NMR-MS (Nicholson *et al.* 2000). This finding prompted a further investigation using a combination of HPLC-ICPMS and orthogonal acceleration (oa)TOF-MS. This 'hyphenated' system provided Br- and S-selective detection using ICPMS and accurate mass data determination (and thus atomic composition determination) via the use of oaTOF-MS (Abou-Shakra *et al.* 2002). This study confirmed the complex pattern of 4-BrA-related components in urine, with a large number of minor metabolites. Subsequent studies, undertaken with the aim of characterizing these minor components have been performed using HPLC-TOF-MS and MS/MS, have identified a number of unusual pentose-containing conjugates (Major *et al.* 2003), probably ribosides. In the course of these investigations, a number of groups of isobaric conjugates characterized by the loss of 162 Da (characteristic of hexoses) from the molecular ion of a substantial number of Br-containing compounds were also detected. We present the results of work showing that these are novel metabolites of 4-BrA, comprised of a number of isomeric mono and di-hexoses (possibly glucosides) as well as group of mono and di-hexose-monosulphate conjugates.

Materials and methods

Chemicals and reagents

4-Bromoaniline (97% chemical purity) was obtained from Aldrich (Gillingham, UK) and used as received. Solvents used for chromatography were from Fisher Scientific (Loughborough, UK) and were of HPLC grade. D₂O (99.9% pure) was from Fluorochem Ltd (Old Glossop, UK). Ammonium formate was obtained from Sigma-Aldrich Co. Ltd (Gillingham, UK).

Samples

The urine samples analysed were obtained from the study described in detail by Scarfe *et al.* (2002). Briefly, following acclimatization (24 h) in metabolism cages (designed for the separate collection of urine and faeces), three male Sprague-Dawley rats (200–300 g) were administered 4-BrA at 50 mg kg⁻¹ i.p. in ethanol-water 1:1 (v/v). Urine was collected over solid CO₂ for various periods up to 48 h post-dose and stored frozen at -20°C until analysed. For the current study, 0–8-h samples were analysed.

Sample analysis

Aliquots (20 µl) of the 0–8-h urine samples were analysed by gradient reversed-phase HPLC on a Waters Symmetry C18 HPLC column (5 µm, 250 × 4.6 mm i.d.). The gradient, made up of an initial isocratic period followed by a series of linear gradients, was produced using 0.1 M ammonium formate (pH 7.0) (solvent A) and methanol (solvent B) as follows. 0–10 min 100% A; 10–35 min, 0–60% B; 35–40 min, 60–80% B; 40–45 min, 80–100% B. After 3 min at 100% B, a rapid gradient (2 min) was used to return the column to its original mobile phase composition of 100% A. A flow rate of 1 ml min⁻¹ was used with 160 µl min⁻¹ split to the mass spectrometer. In addition to studies with H₂O-based solvent systems, a series of experiments were also performed using the same chromatographic conditions, but

with D₂O instead in order to determine the number of exchangeable protons on the metabolites. For these experiments, the urine sample was diluted 1:1 with D₂O and left to stand for 24 h at 4°C in order partially to exchange the protons before chromatography and 50- μ l aliquots of this sample were used.

Mass spectrometry was performed using a Micromass Q-TOF Ultima mass spectrometer (Micromass, Wythenshawe, UK) in positive electrospray with lockspray (the lock mass was 556.2771, $[M + H]^+$ from leucine enkephalin, 0.2 ng μ l⁻¹ at 5 μ l min⁻¹, lock frequency 10 s). The capillary voltage was set to 3.0 kV, with a cone voltage of 35 V. The source temperature was 120° and the desolvation temperature 250°C. The cone and desolvation gases (nitrogen) were delivered at 50 and 500 l h⁻¹, respectively. For MS/MS, argon was used as the collision gas (about 2.8×10^{-3} mbar) and the gas cell was maintained at 22 eV.

Negative-ion electrospray MS studies were also performed with leucine enkephalin again being employed for lock mass correction, $[M - H]^-$ 554.2615, using the same conditions as described above. The capillary voltage was set to 2.7 kV, with a cone voltage of 30 V. All other parameters remained the same.

The method for determining neutral losses has been termed 'precursor ion discovery' and is achieved by operating the Q-ToF using a survey function with the quadrupole operating in R_f only wide band-pass mode and the collision cell pressurized with argon. Alternate low- and high-energy (fragment ion) spectra of the column eluent are acquired, using collision energy switching, with real-time lock mass correction being used for exact mass measurement. After the acquisition of each low/high energy pair the data are interrogated automatically to search for predefined neutral losses that are present above a set threshold. Candidate precursor ions satisfying these criteria are then selected by the quadrupole for MS/MS and product ion spectra acquired. The system then returns to survey mode. This cycle is carried out within the time frame of a chromatographic peak.

The low- and high-energy scans were performed at 5 and 25 eV, respectively, over the range 100–850 amu (0.4 s integration, 0.1 s interscan delay), and the MS/MS was performed over the same mass range (0.9 s integration, 0.1 s interscan delay). Neutral losses for hexose (C₆H₁₀O₅) of 162.0528 Da ($\pm$ 20 mDa) were monitored.

For studies employing D₂O as the aqueous modifier, the leucine enkephalin used to provide the lock reference was prepared in D₂O-acetonitrile (1 : 1) containing 0.1% v/v of formic acid at a concentration of 1 ng μ l⁻¹. The largest peak in the resulting isotope cluster in positive-ion electrospray was 563.3 (C₂₈H₃₁D₇N₅O₇, 563.3210) and this was used as the lock mass. The quadrupole resolution was set to allow the transmission of only one peak in the isotope cluster for these MS/MS experiments.

Results

A total ion current chromatogram for a 0–8-h urine sample from a 4-BrA dosed rat is shown in figure 1a. Preliminary HPLC-MS studies indicated a loss of 162 amu from a number of Br-containing compounds, presumed to be metabolites of 4-BrA. These losses were consistent with the presence of a number of hexose conjugates, possibly glucosides, in the samples. Whilst uncommon in mammals, *N*- and *O*-glucosides have been observed in a number of studies, with *N*-glucosides generally more abundant (e.g. Duggan *et al.* 1974, Tang *et al.* 1978, 1979, Soine *et al.* 1986, Dahms *et al.* 1997, Boberg *et al.* 1998, Kirkman *et al.* 1998). On the assumption that these samples contained such glucosides, a series of experiments was therefore undertaken specifically to look for the loss of this moiety via neutral losses. This led to the detection of five clusters of compounds showing this loss, containing from two to four isobaric metabolites each (in varying proportions), with molecular masses of m/z 334/336, 414/416, 430/432 (447/449 as NH₃ adducts) 496/498 and 592/594 (609/611 as NH₃ adducts) (figure 1b–f). These substances were observed to elute between about 26–29 min for the ions at m/z 430/432 and 592/594 and about 31–35 min for the ions at m/z 334/336, 414/416 and 496/498, respectively.

Characterization of the metabolites of mass m/z 334/336

Analysis of the sample using HPLC-TOF-MS in positive-ion mode revealed a group of metabolites at m/z 334/336, exhibiting a loss of 162 Da, with retention

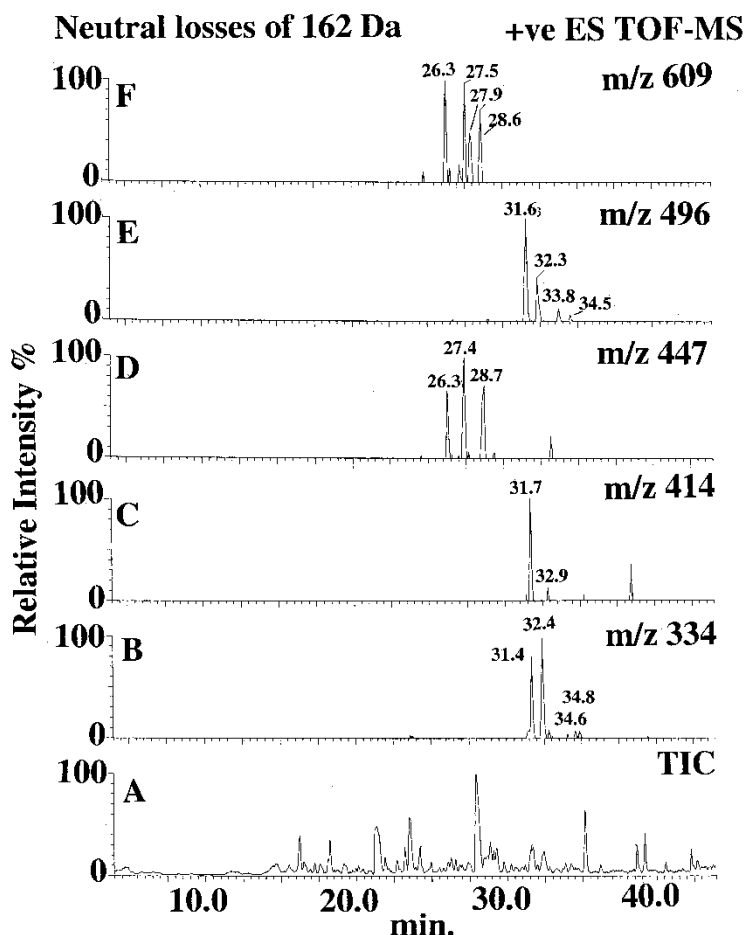


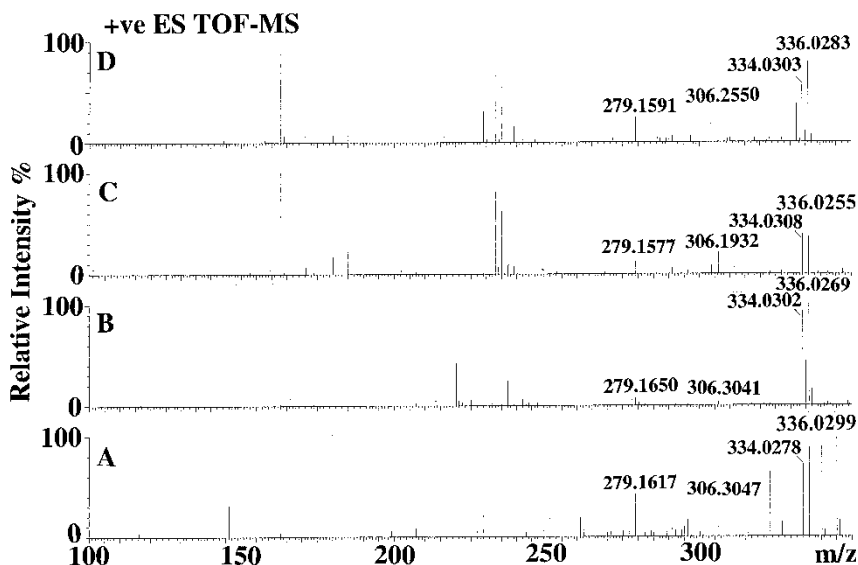
Figure 1. Positive-ion electrospray TOF-MS TIC (a) and mass chromatograms for compounds showing a neutral loss of 162 amu corresponding to the ions at (b) m/z 334, (c) m/z 414, (d) m/z 447 (NH_3 adduct of ion at m/z 430), (e) m/z 496 and (f) m/z 609 (NH_3 adduct of m/z 592). (The mass chromatograms were extracted from the low-energy survey scan.)

times of 31.4, 31.7, 32.4, 32.8, 34.6 and 34.8 min. Careful examination of the MS data showed that these ions resulted from two sources. Thus, the prominent peak at RT 32.4 min, and the minor peaks at 31.4, 34.6 and 34.8 min (figure 1b), were due to metabolites of 4-BrA with an atomic composition of $\text{C}_{12}\text{H}_{17}\text{NO}_5^{79}\text{Br}$. Weak $(\text{M} + \text{Na})^+$ ions were also observed at m/z 356/358. This atomic composition, combined with the observed neutral loss of 162 Da is best explained by the presence of a number of isobaric *N*-hexoses of 4-BrA. Further studies in negative-ion mode resulted in the detection of the $(\text{M} - \text{H})^-$ ions for these compounds at m/z 332/334. MS/MS data were obtained on the ^{81}Br isotope-containing form of the major 334/336 Da peak (eluting at 32.4 min) in order to confirm the presence of a hexose (probably glucose) moiety.

The ion current trace for m/z 334 is shown in figure 1b and the results for the MS experiments on the 334 Da metabolites are detailed in table 1. The mass spectra obtained for these components are given in figure 2a–d.

Table 1. MS and data for the putative monohexose conjugates of 4-bromoaniline (m/z 334).

RT (min)	m/z 334 observed	Error (mDa)	Error (ppm)	Comments
31.4	334.0278	− 1.2	− 3.6	also observed in negative, weak Na^+ in positive
32.4	334.0302	1.2	3.6	also observed in negative, weak Na^+ in positive
34.6	334.0308	1.8	5.4	also observed in negative, weak Na^+ in positive
34.8	334.0303	1.3	3.9	also observed in negative
		Calculated 334.0290	Formula $\text{C}_{12}\text{H}_{17}\text{NO}_5^{79}\text{Br}$	

Figure 2. Positive ES-TOF-MS data obtained for peaks giving ions at m/z 334 corresponding to monohexose conjugates of 4-BrA and eluting at 31.4, 32.4, 34.6 and 34.8 min (a–d, respectively).

In contrast to the hexoses eluting at 31.4, 32.4, 34.6 and 34.8 min observed in both positive- and negative-ion mode as discussed above, the major peak at 31.7 min and the minor peak at 32.8 min that were also detected as ions at m/z 334/336 were only seen in positive-ion mode. Further investigations revealed that these peaks resulted from in-source collision-induced dissociation (CID) of an ion of m/z 414/416 (see below).

This separation was repeated with D_2O in the mobile phase in an attempt to determine the number of exchangeable protons present in these metabolites. However, as a result of incomplete deuteration, which produced a number of partially deuterated species, and the small quantities of these metabolites present, it proved impossible to obtain meaningful results.

Characterization of the metabolites of mass m/z 414/416

As indicated above, as well as being the result of the presence of *N*-hexoses in the sample, the m/z 334/336 ion was also produced as an in-source CID fragment

Table 2. MS data for the putative hexose-sulphamate conjugates of 4-BrA (m/z 414 and 431).

RT (Min)	Observed	Error (mDa)	Error (ppm)	Calculated	Formula
	m/z 431				
31.7	431.0114	-0.9	-2.2	431.0124	$C_{12}H_{20}N_2O_8S^{79}Br$
32.9	431.0128	0.4	1.0		
	m/z 414				
31.7	413.9851	-0.7	-1.7	413.9858	$C_{12}H_{17}NO_8S^{79}Br$
32.9	413.9805*	-5.3	-12.9		
	m/z 334				
31.7	334.0298	0.8	2.4	334.0290	$C_{12}H_{17}NO_5^{79}Br$
32.9	334.0288	-0.2	-0.6		

*Very weak peak.

of an ion at m/z 414/416 (loss of 80 Da as SO_3). This metabolite was also observed as an ammonium adduct at m/z 431/433. Two peaks were observed that showed this behaviour, eluting at 31.7 and 32.9 min for the major and minor components, respectively (figure 1c). The exact mass measurements for the protonated molecular ion of the major peak gave 413.9851 Da and this, and the corresponding value for the ammonium adduct of 431.0114 Da are within 3 ppm of the calculated masses of 413.9858 ($C_{12}H_{17}NO_8S^{79}Br$) and 431.0124 ($C_{12}H_{20}N_2O_8S^{79}Br$), respectively, required for an *N*-sulphamate-*N*-hexose metabolite. These compounds were also observed when the studies were repeated in negative-ion mode as $[M-H]^-$ ions at m/z 412. Based on these data, the structure of these metabolites is perhaps best explained by postulating an *N*-hexose (probably glucose) *N*-sulphamate, although other structures are possible. The presence of two components with the same MS characteristics may be due to the presence of the sugar moiety as α - and β -anomeric forms.

The ion current trace for m/z 414 is shown in figure 1c, and the results for the MS experiments on this ion are given in table 2. Owing to the small quantities of material present, it was not possible to obtain further MS/MS data. The mass spectra for these components are illustrated in figure 3a, b.

The D_2O exchange experiments showed an isotopic cluster consistent with the presence of six exchangeable protons presumably four on the hydroxyl groups of the hexose moiety, one on the putative sulphamate group and one as the $(M+D)^+$.

Characterization of the ions of mass m/z 430/432

A group of three metabolites showing the characteristic loss of 162 Da were also observed with masses of 430/432 Da and as ammonium adducts with a corresponding mass of 447/449 Da eluting at about 26.3, 27.4 and 28.7 min, respectively (figure 1d and table 3a). The accurate masses for these putative glucosides ranged from 429.9811 to 429.9835 Da indicating an atomic composition of $C_{12}H_{17}NO_9S^{79}Br$ (calculated mass 429.9807 Da) (table 3a). MS/MS studies on the m/z 430 peaks showed loss of the putative glucose moiety to give an ion corresponding to the hydroxy-sulphated 4-BrA ($C_6H_7NO_4S^{79}Br$), followed by loss of SO_3 to give a fragment at 187.9711 Da (table 3b). There was also a weak peak at

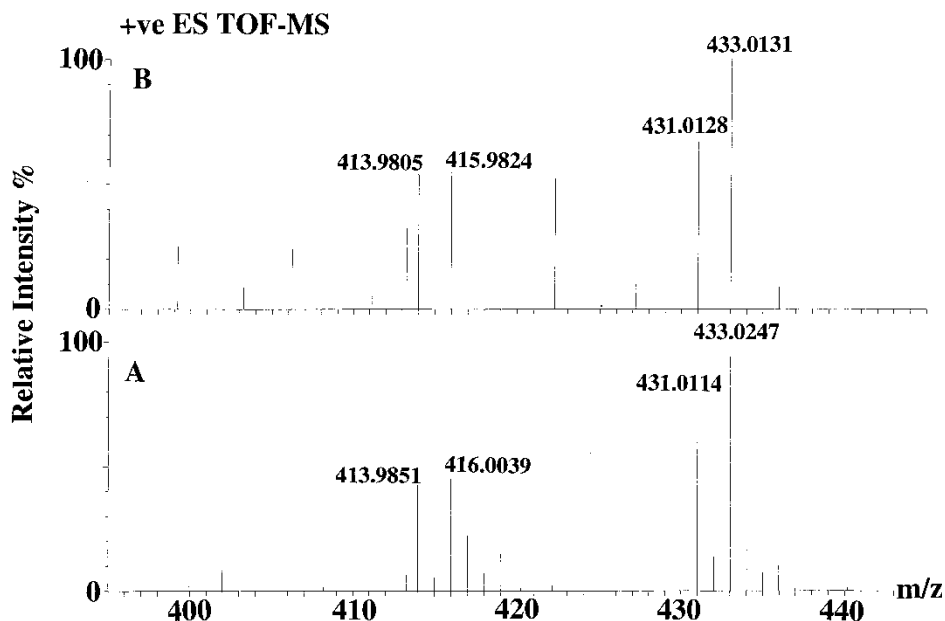


Figure 3. Positive ES-TOF-MS data obtained on the peaks giving ions at m/z 414 corresponding to the putative hexose-sulphamate conjugates of 4-BrA and eluting at 31.7 and 32.9 min (a and b, respectively).

m/z 350 corresponding to the loss of the sulphate directly from the protonated molecular ion. These spectra are shown in figure 4a–c.

A facile loss of sulphate via in source CID was also observed in positive-ion mode to give peaks at m/z 350/352 Da ($C_{12}H_{16}NO_6Br$) corresponding to the molecular formula that would be expected for a monohydroxylated-glucoside of 4-BrA. The MS and MS/MS data obtained on these metabolites support the identification of a hexose, with progressive losses of up to four molecules of water from the molecular ion. A further loss of 162 Da from the 350 Da fragment ion was also noted, which gave an ion of 188 Da corresponding to the presumed ring-hydroxylated metabolite of 4-BrA.

These studies were then repeated using D_2O in the mobile phase in order to determine the number of exchangeable protons present on the analytes. Although deuteration was not complete, the resulting data were consistent with the proposed structure. Thus, the resulting pseudomolecular ion for the ND_3 adduct was seen at 456/458 Da, with the nine observed deuteriums ($C_{12}H_{11}D_9N_2O_9S^{79}Br$) for the adduct indicating the presence of six exchangeable protons on the metabolite. Loss of SO_3 and ND_3 from these metabolites resulted in an ion at 356.0623 ($C_{12}H_{11}D_6NO_6^{79}Br$ calculated mass 356.0616) for the glucoside of the hydroxylated 4-BrA. In addition, an alternative loss of glucose from the molecular ion gave a fragment at 270.9491 amu ($C_6H_4D_3NO_4S^{79}Br$ calculated mass 270.9467) for the presumed hydroxysulphate.

The presence of a number of isomeric glucoside-sulphate conjugates of this monohydroxylated metabolite, which were indistinguishable by MS (figure 4a–c), is probably partly best explained by assuming that two of these peaks result from the formation of an *N*-glucoside of a ring hydroxylated sulphate metabolite comprised of a mixture of α - and β -anomers. The presence of a third glucoside

Table 3a. MS results from measurements on the putative hexose-hydroxysulphate conjugates of 4-BrA and their corresponding ammonium adducts (*m/z* 430 and 447).

RT (min)	Observed	Error (mDa)	Error (ppm)	Calculated	Formula
<i>m/z</i> 447					
26.3	447.0069	− 0.4	− 0.9	447.0073	C ₁₂ H ₂₀ N ₂ O ₉ S ⁷⁹ Br
27.4	447.0082	0.9	2.0		
28.7	447.0081	0.8	1.8		
<i>m/z</i> 430					
26.3	429.9835	2.8	6.4	429.9807	C ₁₂ H ₁₇ NO ₉ S ⁷⁹ Br
27.4	429.9811	0.4	0.8		
28.7	429.9826	1.9	4.3		
<i>m/z</i> 350					
26.3	350.0256	1.7	4.8	350.0239	C ₁₂ H ₁₇ NO ₆ ⁷⁹ Br
27.4	350.0263	2.4	6.8		
28.7	350.0262	2.3	6.5		

Table 3b. Results from MS/MS measurements on *m/z* 430.

RT (min)	Observed	Error (mDa)	Error (ppm)	Calculated	Formula
<i>m/z</i> 268					
26.3	267.9285	0.6	2.2	267.9279	C ₆ H ₇ NO ₄ S ⁷⁹ Br
27.4	267.9303	2.4	8.9		
28.7	267.9307	2.8	10.4		
<i>m/z</i> 188					
26.3	187.9746	3.5	18.6	187.9711	C ₆ H ₇ NO ⁷⁹ Br
27.4	187.9713	0.2	1.1		
28.7	187.9704	−0.7	−3.7		

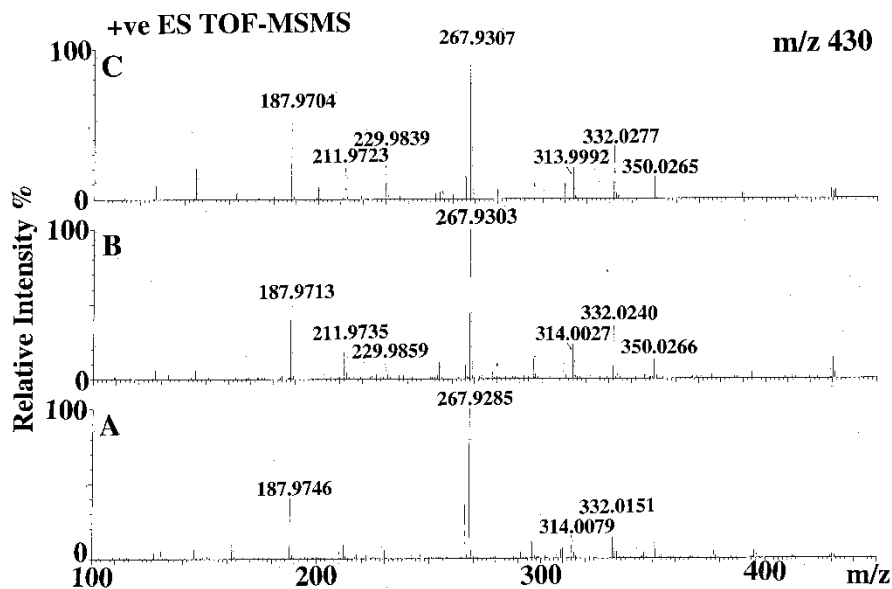


Figure 4. Positive ES-TOF-MS/MS data obtained on the peaks at *m/z* 430 corresponding to the putative hexose-hydroxysulphates of 4-BrA eluting at 26.3, 27.4 and 28.7 min (a–c, respectively).

might be explained in a variety of ways including conjugation of sulphate via a meta ring hydroxyl or, alternatively, an *O*-glucoside may have been formed combined with an *N*- or an *O*-sulphate.

Characterization of the metabolites at m/z 496/498

A loss of 162 Da was also observed from a group of four metabolites, eluting at 31.6, 32.3, 33.8 and 34.5 min, respectively, with a mass of 496/498 (figure 1e). The atomic composition of these compounds was established from the accurate mass of 496.0824 Da as $C_{18}H_{27}NO_{10}^{79}Br$ (calculated 496.0818 Da) for the component eluting at 31.6 min. The MS/MS spectra from all four metabolites were similar with an initial loss of 162 amu from the molecular ions to give a fragment at m/z 334. The accurate mass of the fragment ion (334.0320 Da) for the component at 31.6 min corresponded to an atomic composition of $C_{12}H_{17}NO_5^{79}Br$ (calculated 334.0290). The atomic composition and fragmentation of the molecular ion was thus consistent with the presence of two hexose moieties attached to 4-BrA. Further evidence for the presence of hexoses was provided by the observation of further losses of 18 (water) from these ions. In addition, the fragments at m/z 172, corresponding to 4-BrA itself, were also observed.

Spectra for these metabolites are shown in figure 5a–d, and the MS data are provided in table 4.

The deuteration experiment to determine the number of exchangeable protons was complicated by the incomplete exchange of the large number of exchangeable protons present in these putative di-hexoses. This resulted in a complex isotopic cluster that defied interpretation.

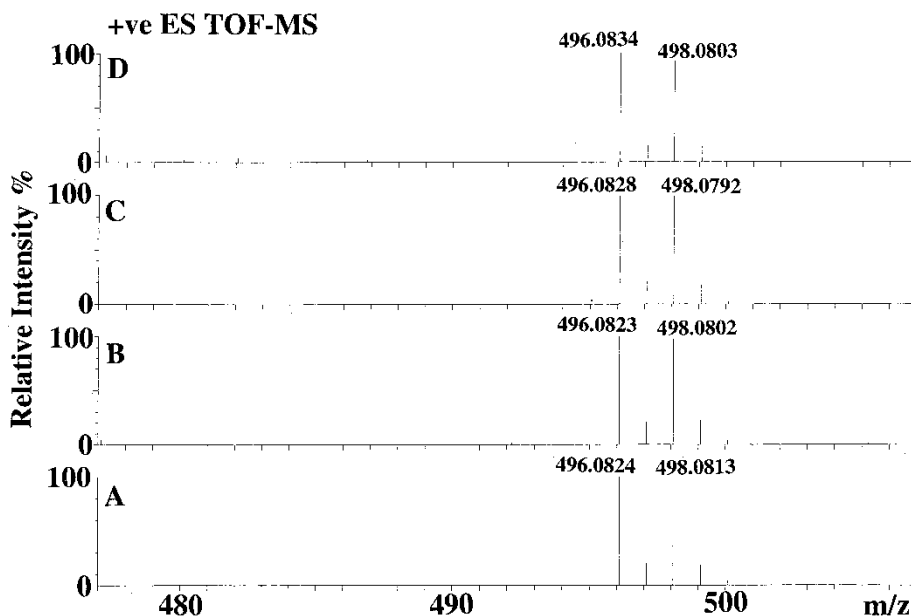


Figure 5. Positive ES-TOF-MS data obtained on the peaks giving ions at m/z 496 corresponding to the putative di-hexoses of 4-BrA eluting at 31.6, 32.3, 33.8 and 34.5 min (a–d, respectively).

Table 4. MS results from measurements on the putative di-hexose conjugates of 4-BrA (*m/z* 496).

RT (min)	<i>m/z</i> 496 observed	Error (mDa)	Error (ppm)
31.6	496.0824	0.6	1.1
32.3	496.0823	0.5	0.9
33.8	496.0828	1.0	2.0
34.5	496.0834	1.6	3.2
Calculated 496.0818		Formula C ₁₈ H ₂₇ NO ₁₀ ⁷⁹ Br	

Table 5. MS/MS results from experiments on the ammonium adducts of the putative di-hexose monohydroxysulphate conjugates of 4-BrA (*m/z* 609).

RT (min)	Observed	Error (mDa)	Error (ppm)	Calculated	Formula
<i>m/z</i> 609 ^a					
26.3	609.0602	0.1	0.2	609.0601	C ₁₈ H ₃₀ N ₂ O ₁₄ S ⁷⁹ Br
27.5	609.0618	1.7	2.7		
27.9	609.0594	− 0.7	− 1.2		
28.6	609.0604	0.3	0.5		
<i>m/z</i> 512					
26.3	512.0775	0.8	1.5	512.0767	C ₁₈ H ₂₇ NO ₁₁ ⁷⁹ Br
27.5	512.0767	0.0	0.0		
27.9	512.0778	1.1	2.1		
28.6	512.0762	− 0.5	− 1.0		
<i>m/z</i> 350					
26.3	350.0240	0.1	0.2	350.0239	C ₁₂ H ₁₇ NO ₆ ⁷⁹ Br
27.5	350.0215	− 2.4	− 6.8		
27.9	350.0247	0.8	2.2		
28.6	350.0258	1.9	5.4		

^a *m/z* 609 results taken from low-energy MS scan in neutral loss experiment.

Characterization of the metabolites at *m/z* 609/611

Four peaks showing neutral losses of 162 amu with masses of 609/611 Da were detected eluting at 26.3, 27.5, 27.9 and 28.6 min, respectively (figure 1f). The atomic composition of theses compounds, derived from the accurate mass data, was C₁₈H₃₀N₂O₁₄S⁷⁹Br and the accurate masses observed for all of these isomers were within 3 ppm of the calculated mass of 609.0601 (⁷⁹Br) for the postulated molecular formula (table 5). Further investigation of these metabolites revealed that the ions at *m/z* 609/611 were due to the formation of an ammonium adduct to the molecular ion as also seen for the metabolites of mass 414/416 and 428/430 Da described above. The actual molecular mass of these metabolites therefore corresponded to 592/594 Da. MS/MS studies suggested that loss of ammonia and sulphate from the ion at *m/z* 609/611 gave an ion corresponding to a di-hexose conjugate with a mass of 512.07 Da. These losses were followed by a further loss of 162 amu to form an ion at 350.02 amu (table 5). Further losses of 18 (water) from this ion were observed, together with another loss of 162 indicating the presence of a second hexose. In these MS/MS studies all four isomers gave very similar fragmentation patterns (figure 6a–d). Examination of the TOF-MS TIC showed that the ions at *m/z* 592/594 and 512/514 Da were present in addition to the

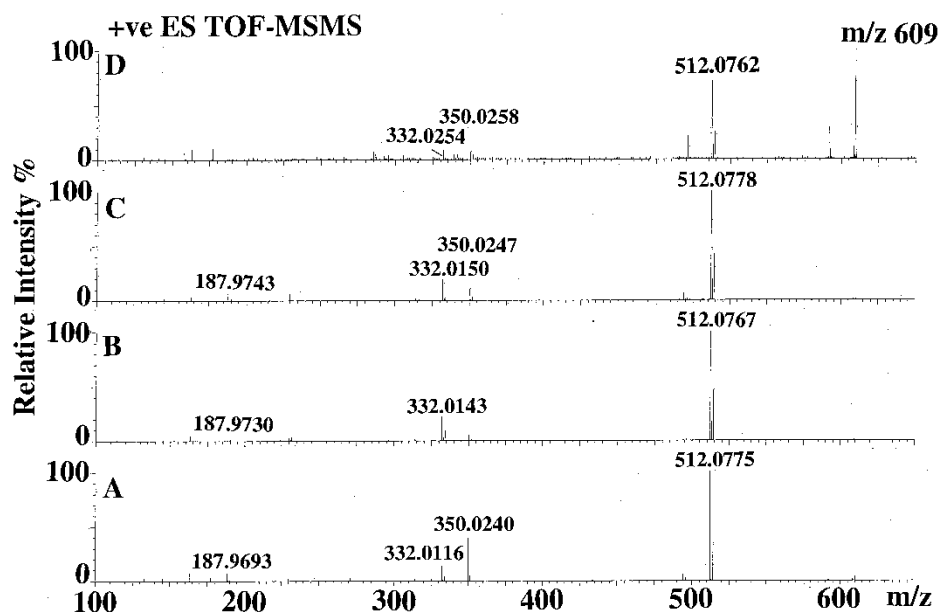


Figure 6. Positive ES-TOF-MS/MS data obtained on the peaks at m/z 609 corresponding to the ammonium adducts of the putative di-hexose hydroxysulphates of 4-BrA eluting at 26.3, 27.5, 27.9 and 28.6 min (a–d, respectively).

ammonium adduct at m/z 609/611 indicating that a degree of in source fragmentation was also occurring.

These studies were repeated with D_2O replacing the water in the mobile phase in order to determine the number of exchangeable protons on the putative di-hexoses, thereby providing further information on the structure. However, as a result of incomplete deuteration and the large number of exchangeable protons present on these metabolites the resulting spectra were complex. Thus, the ion previously observed at m/z 609/611 for the ammonium adduct was seen as a cluster of deuterated ions centred on m/z 622 (as an ND_3 adduct) ($C_{18}H_{17}D_{13}N_2O_{14}SBr$). MS/MS studies on this ion resulted in the detection of an ion at m/z 522.1373 for the first component eluting at 26.6 min (^{79}Br) following the loss of ND_3 and sulphate. This increase of 10 amu is consistent with a molecular composition of $C_{18}H_{17}D_{10}NO_{11}^{79}Br$ and implies the presence of 10 exchangeable protons on these metabolites. This would correspond to four on each of the presumed hexose units, one on the nitrogen, and one on the ring hydroxyl following the loss of SO_3 .

Discussion

The mass spectroscopic data obtained were consistent with the presence of a number of hexose conjugates (or adducts), most probably of glucose, to 4-BrA or its metabolites in rat urine. These compounds fall into two distinct groups, those containing only a sugar moiety and those which in addition are conjugated to sulphate, and are further subdivided into those possessing one hexose and those with two. Thus, we have detected at least four isomeric monohexoses, two

isomeric monohexose-sulphamates, three isomeric hexose sulphates, four isomeric di-hexoses and four isomeric di-hexose sulphates derived from 4-BrA.

In the case of the monohexoses of 4-BrA detected here, the point of attachment of the sugar moiety clearly has to be via the amino group. This is consistent with the literature, where *N*-glucosides have been detected for a number of compounds. Many of the *N*-glucosides detected in biological systems are found as both α and β forms and may owe more of their existence to chemistry rather than metabolism. Indeed, it is known from a number of studies that such conjugates can form spontaneously between glucose and suitable *N*-containing compounds such as, for example, sulphonamides and 5-aminosalicylic acid (Paulson *et al.* 1981, Giera *et al.* 1982, Parks 1984, Manis and Braselton 1986). However, even assuming both α - and β -anomeric forms are present the putative glucosides that are the most likely candidates for these hexose-containing compounds can only account for two of the four peaks detected. In the case of these other peaks, another hexose (or hexoses) must be invoked to explain their presence in the samples. Unfortunately, mass spectrometry cannot distinguish between the various possibilities and these components were present in such small amounts that NMR spectroscopy was not possible.

Using similar arguments, the two minor sulphamates detected here must also have been conjugated via the amino group, with the most likely candidate for the hexose moiety being glucose.

For the mixed hexose-sulphate conjugates, mass spectrometry provides good evidence for ring hydroxylation, and most likely this is the point of conjugation for the sulphate group. Given that the *ortho*-sulphate is the major urinary component observed following administration of 4-BrA to the rat (Scarfe *et al.* 2002b), it is not unreasonable to assume that this is also the position of ring hydroxylation conjugation here. However, the presence of at least three metabolites containing both sulphate and hexose moieties requires explanation. Possibly two of these metabolites correspond to α - and β -anomeric forms of an *N*-glucoside-2-sulphate metabolite of 4-BrA. The remaining metabolite could result from an *N*-sulphate-2-glucoside (either α - or β -anomers), from an *N*-glucoside 3-sulphate or indeed be formed from another hexose entirely. Once again mass spectrometry, whilst capable of detecting the various components making up these metabolites cannot distinguish between the various possible structures. Certainly *O*-glucosidation has been reported and unlike *N*-glucosidation is probably entirely, enzymatically mediated. As such these *O*-glucosides are not found as mixtures of anomers but as either α - or β -*O*-glucosides (e.g. Nakano *et al.* 1986, Kamimura *et al.* 1988, 1992, Tjornelund *et al.* 1989, Samara *et al.* 1990, Adams *et al.* 1996, Chemela *et al.* 2001).

Whilst the monohexoses described above are unusual, they are not without precedent. However, the metabolites displaying a molecular mass of 496/498 Da have been tentatively assigned to be a group of isomeric di-hexoses (possibly di-glucosides) of 4-BrA, and these would appear to be entirely novel. In the absence of a ring hydroxyl to act as a point of attachment on 4-BrA for these hexose moieties, both must be conjugated via the amino group. It is unclear whether or not the compounds detected here correspond to disaccharides such as maltose or to two monosaccharides (though the latter seems unlikely on steric grounds). Based on the mass spectra obtained here, we were unable to discriminate between these possibilities. However, the fragmentation pattern seen with plant flavonoid glycosides

shows a pattern of sequential cleavage of glycosidic bonds that is not dissimilar to that seen here (Stobiecki 2000) and a disaccharide seems most probable.

As with the putative diglucosides observed at m/z 496/498 discussed above, attachment of the hexose units for the 592/594 sulphate-containing metabolites was most likely to be via the amino function. Using the arguments deployed above for the likely site of hydroxylation in the monohexose sulphate metabolites it seems likely that the 2-position was also the site of hydroxylation (and therefore probably of sulphation) in these metabolites.

However, whilst HPLC-MS has revealed the presence of these compounds the actual structures of the putative di-glucoside and diglucoside-sulphate metabolites are less clear, as is the source of their diversity. Thus, the presence of groups of apparently identical conjugates that can be separated chromatographically, but which are indistinguishable by MS/MS, is a source of difficulty. Explanations that can be advanced include the possibility that in fact these losses of 162 amu are not all due to glucose, but to a variety of isobaric hexoses (e.g. xylose, fructose, galactose, etc.). An alternative and perhaps more likely possibility is that these chromatographic separations are the result of the presence of the glucose moieties in the metabolites being in various combinations of the α - and β -anomeric forms. So, for example, the α - α , α - β , β - α and β - β combinations of anomers of glucose would provide the necessary chemical variety needed for the formation of the four observed chromatographic peaks for the di-hexose and di-hexose-sulphate metabolites. In the case of the di-hexose-sulphate metabolites, another possibility is that there was a mixture of positional isomers due to ring hydroxylation/sulphation *ortho* and *meta* to the amino group which when combined with α - and β -glucose anomers could also produce the observed chromatographic result. To determine which of these possibilities is actually the case, it is probable that either ^1H -NMR spectroscopy or synthesis would be required. The trace quantities of these metabolites present in the samples suggest that obtaining sufficient material for unambiguous NMR spectra will present a not inconsiderable challenge. It is therefore likely that full identification of these substances will only be feasible by the full chemical synthesis of these putative structures. However, the difficulties of this approach should not be underestimated.

Irrespective of the actual structures of these metabolites, it would appear that the exposure of rats to 4-BrA has resulted in some extremely unusual isomeric conjugates that are present in the samples as minor components. It is probable that without the advances seen with modern MS instrumentation, combined with the characteristic (and readily identifiable) isotope pattern provided by the presence of bromine, that these unusual metabolites might well have gone unnoticed. It therefore seems likely that such compounds (together with the putative ribosides reported elsewhere; Major *et al.* 2003) are not unique to 4-BrA but may also be found to have been formed following the administration of other members of this class of compounds.

If these compounds were formed, at least in part, chemically rather than enzymatically, it also suggests that 4-BrA and its sulphated metabolites should be considered to be essentially reactive, with the potential to become associated with cellular macromolecules with all the potential for toxicological sequelae that this implies. As such, further investigation of the structure and mechanism of formation of these 4-BrA-related compounds to determine the nature of these minor metabolites would seem merited.

Conclusions

The application of diagnostic exact mass neutral loss acquisitions has resulted in the detection of a number of groups of minor metabolites of 4-BrA whose mass spectral data are consistent with the presence of mixtures of isomeric hexoses and di-hexoses (possibly glucosides and di-glucosides). Some of these metabolites also contain sulphate. Based on the available MS/MS data, it is not possible to advance definitive structures for these metabolites. However, these conjugates, particularly the putative diglucosides and diglucoside-hydroxysulphates, represent highly unusual metabolites without parallel in the literature.

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Mexiletine carbonyloxy β -D-glucuronide: a novel metabolite in human urine

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Received 29 January 2003

1. The study was performed to isolate and characterize a glucuronic acid conjugate of mexiletine that releases mexiletine on acid hydrolysis from urine samples obtained from healthy volunteers following a single oral dose of mexiletine.

2. The $[M - H]^-$ ion of the isolated metabolite was observed at m/z 398 in the negative electrospray ionization mass spectrum. This mass number was 44 higher than that of the product generated when mexiletine is subjected to direct glucuronidation. In positive-ion mode, collision-induced dissociation of the quasimolecular ion $[M + NH_4]^+$, m/z 417, gave product ions at m/z 224, 180 and 58. These mass spectral data indicated that the metabolite contained a carbonyloxy moiety in its structure in addition to mexiletine and a glucuronic acid moiety.

3. The presence of this carbonyloxy moiety was further supported by the following chemical reactions. When the metabolite was hydrolysed with an aqueous solution of 1 M sodium hydroxide at room temperature, mexiletine was released, whereas the *N*-methoxycarbonyl derivative of mexiletine was obtained after treatment of the metabolite with methanolic sodium hydroxide solution.

4. The results indicated that the structure of the isolated metabolite was the *N*-carbonyloxy β -D-glucuronic acid conjugate of mexiletine.

Introduction

Mexiletine (MX) [2-(2,6-dimethylphenoxy)-1-methylethylamine], an orally effective antiarrhythmic agent with sodium channel-blocking properties, is used for the treatment of ventricular arrhythmia (Campbell *et al.* 1978, Murray *et al.* 1989). After oral administration to man, MX is completely absorbed and more than 80% of the dose is recovered from urine (Haeselbarth *et al.* 1981). Since less than 10% of the dose is excreted in unchanged form in the urine (Pentikainen *et al.* 1983), MX is considered to be eliminated predominantly by metabolism. MX has various metabolic pathways, including oxidation of the carbon and nitrogen atoms, deamination, reduction, and conjugation (Beckett and Chidomere 1977). Hydroxylation is a major route of metabolism for MX in humans, resulting in the formation of 4-hydroxymexiletine (4HM) and 2-hydroxymexiletine (2HM). Because the polymorphic enzyme, CYP2D6, predominantly catalyses these latter hydroxylation pathways (Nakajima *et al.* 1998), several *in vivo* studies have been performed to define the role of CYP2D6 in MX pharmacokinetics (Broly *et al.* 1991,

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Turgeon *et al.* 1991, Lledo *et al.* 1993). Some reports, however, have indicated that total urinary recovery of these hydroxylated metabolites was less than 30% of the dose when MX was orally administered to healthy volunteers (Broly *et al.* 1991, Turgeon *et al.* 1991).

Glucuronidation is another major elimination pathway of MX. Approximately 30% of the dose is excreted as MX glucuronide, which is determined in terms of MX content released by acidic or enzymatic hydrolysis (Prescott *et al.* 1977, Abolfathi *et al.* 1993, Ueno *et al.* 2002), but little is known about the chemical structure of glucuronic acid conjugates of MX. Carbamoyl glucuronide conjugates of drugs containing a primary amino group (i.e. tocainide, rimantadine and mofegiline) have been identified as human urinary metabolites (Brown *et al.* 1990, Kwok *et al.* 1990, Dow *et al.* 1994). These metabolites are presumably formed in two steps: the amino group reacts with carbon dioxide to form a carbamic acid, and subsequently the carbamic acid moiety is conjugated with glucuronic acid. If the MX glucuronide has a carbamoyl moiety, the parent amine compound, MX, should be detected after chemical or enzymatic hydrolysis, because of the chemical instability of the carbamic acid intermediate.

The objectives of the current study were to isolate the MX glucuronide, to confirm the presence of a carbamoyl moiety and to quantify the urinary excretion ratio of the glucuronide. To avoid degradation of MX glucuronide due to the chemical instability of carbamic acid derivatives, all isolation and analytical processes were conducted under neutral conditions.

Materials and methods

Chemicals and reagents

MX, 4HM and 2HM were synthesized by Boehringer Ingelheim Pharma KG (Ingelheim, Germany) with >98% chemical purity as determined by high-performance liquid chromatography (HPLC) analysis. 1-(2',6'-Dimethylphenoxy)-2-propanol (DMPL), 1-(2',6'-dimethylphenoxy)-2-hydroxyaminopropane (*N*-hydroxymexiletine, NHM), 1-(2',6'-dimethylphenoxy)-2-propanone (DMPN), and 1-(2',6'-dimethylphenoxy)-2-propanone oxime (DMPNX) were synthesized from xlenol and chloroacetone. The purities of these compounds were confirmed by means of HPLC analysis and determined to be 98% pure. Xlenol, β -D-glucuronidase (from *Angullaria*, 25 000 unit ml⁻¹), and glucosaccharo 1,4-lactone were purchased from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals and reagents used were of analytical or HPLC grade and were obtained from local suppliers.

Human urine samples

Mexitile capsules[®] containing 100 mg MX hydrochloride were orally administered to healthy volunteers ($n=10$, male, 20–32 years of age and 51–71 kg body weight) at a single dose of two capsules (equivalent to 166 mg MX). The volunteers were fully informed about the experimental procedure and purpose of the study, and written consent was obtained from each subject. Approval for the study was given by the local Institutional Review Board. Urine samples were collected before administration and at scheduled intervals of 0–6, 6–12 and 12–24 h after the administration. All the samples were stored at –20°C until analysis.

Hydrolysis

Acid hydrolysis. A 5 M hydrochloric acid solution was added to samples to prepare a 1 M acidified reaction mixture, which was heated at 100°C for 30 min in a silicon-capped glass tube. The reaction mixture was then neutralized with sodium hydroxide and directly subjected to HPLC analysis.

Alkaline hydrolysis. The samples were exposed to 1 M aqueous or methanolic sodium hydroxide at room temperature. After the 30-min treatment, each reaction mixture was neutralized with hydrochloric acid and subjected to HPLC analysis.

β -Glucuronidase hydrolysis. The samples were incubated with 10 000 units ml^{-1} β -glucuronidase in acetate buffer (pH 5) at 37°C for 4 h, then the same volume of acetonitrile was added and the mixture was centrifuged. Identical samples were subjected to enzyme hydrolysis with 1 mg ml^{-1} glucosaccharo 1,4-lactone, a specific inhibitor of β -glucuronidase. The supernatants were individually subjected to HPLC analysis.

Isolation of conjugated MX from human urine

A portion of the urine was applied to an ion-exchange column, DIAION (HP-20, 3.0×15 cm, Mitsubishi Chemical Industry, Tokyo, Japan), and the column was washed with 0.5 litres water. Elution was done with a gradient of aqueous acetonitrile from 5 to 50% (v/v), and 100-ml fractions were collected. A portion (0.1 ml) of each fraction was treated with hydrochloric acid, followed by HPLC analysis to monitor the appearance of MX. The fractions containing conjugated MX were combined, evaporated *in vacuo* and lyophilized to give a semi-pure sample. The resultant sample was applied to a cartridge column packed with C18 (Megabond C18, Varian, CA, USA). The column was eluted with 3 vols water followed by elution with 3 vols 10% aqueous methanol and 6 vols 20% aqueous methanol in this order. The fractions containing conjugated MX were concentrated and lyophilized to yield the purified compound.

Synthesis of N-methoxycarbonylmexiletine (NCM)

MX hydrochloride (103 mg) solution in 50% aqueous acetonitrile (5 ml) containing triethylamine (175 μl) was treated with 1.1 equivalents of methyl chloroformate and the mixture was stirred overnight at room temperature. It was then thoroughly stirred with ethyl acetate and water, and the organic layer was separated and washed with diluted hydrochloric acid, water and saturated aqueous sodium chloride solution. After having been dried over sodium sulfate, the organic layer was evaporated *in vacuo* to give the desired product as crystals in 94% yield. Quasimolecular ions corresponding to $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{NH}_4]^+$ were observed at m/z 238 and 255, respectively, in positive ESI-liquid chromatography mass spectra (LC/MS). The chemical structure of NCM was determined by mass spectra and $^1\text{H-NMR}$.

HPLC analysis

MX and its metabolites in the urine samples, eluates from chromatography columns during purification and the hydrolysed reaction mixtures were analysed by HPLC by using a Waters HPLC system Alliance 2690 equipped with a photodiode array detector model 996, a fluorescence detector model 474 (Waters, Milford, MA, USA), and an analytical reverse-phase column Capcell Pak C18 (4.6 mm i.d. $\times$ 150 mm, 5 μm , Shiseido Co., Tokyo, Japan). The column temperature was maintained at 35°C and the eluate was monitored at 210 nm. Fluorescence conditions were set at 270 nm for excitation and at 312 nm for emission to confirm 4HM, 2HM and MX by the method previously described (Senda *et al.* 2001). An isocratic system was used for the separation of the analytes at a flow rate of 1.0 ml min^{-1} . The mobile phase was composed of acetonitrile and 44 mM phosphate buffer containing 0.5% triethylamine (pH 7.0) in a ratio of 35/65 (v/v). For the alkali hydrolysed samples, 44 mM ammonium acetate buffer containing 0.5% triethylamine (pH 7.0) was used in place of the phosphate buffer.

LC/MS/MS analysis

HPLC for LC/MS/MS was run on a Shiseido Nanospace SI-2 (Tokyo, Japan) equipped with a column of Inertsil ODS3 (2.1 mm i.d. $\times$ 150 mm, 5 μm , GL Science, Kyoto, Japan) at a flow rate of 0.2 ml min^{-1} . Two different isocratic solvent systems were used: acetonitrile-44 mM ammonium acetate (20/80 v/v) was used for analysis of the isolated MX metabolite and MX, whereas acetonitrile-44 mM ammonium acetate (50/50 v/v) was used for N-methoxycarbonylmexiletine. Mass spectra were obtained on a Finnigan quadrupole mass spectrometer (TSQ, ThermoQuest, San Francisco, CA, USA) fitted with an atmospheric pressure ionization source (API 2 Interface) using the following parameters: ESI voltage, 3.5 kV; sheath gas, 75 psi; auxiliary gas, 40 unit; capillary temperature, 350°C; collision-induced dissociation (CID) gas, Ar 2.0 mTorr.

Results

Identification and isolation of conjugated MX from urine sample

Human urine samples treated with hydrochloric acid were applied to the HPLC system to confirm the presence of conjugated MX. In this HPLC analysis, the retention times of MX and its metabolites, 4HM and 2HM, were 4.5, 2.1 and 2.3 min, respectively (figure 1A). Although the system could detect as little as 10 ng NHM, DMPL, DMPN and DMPNX at 6.5, 8.2, 10.5 and 10.7 min, respectively, these possible metabolites were not observed in any urine sample (data not shown). Figure 1B shows the chromatogram of an intact urine sample with a peak at 3.1 min and the MX peak at 4.5 min. After acid hydrolysis, the peak at 3.1 min disappeared, whereas the peak height at 4.5 min increased (figure 1C), indicating the presence of conjugated MX in the urine sample. Thus, the target peak (3.1 min) was carefully extracted by the method described above, with monitoring of MX production by using HPLC. Based on the HPLC analysis of the hydrolysed urine samples, the 0–6-h fraction was used for isolation of conjugated MX. The target peak was separated from the urine sample to give 3.5 mg purified compound after secondary purification with a packed column.

LC/MS/MS analysis of the isolated MX conjugate

The mass spectrum of the isolated MX conjugate is shown in figure 2. In the negative ESI mass spectrum, the $[M-H]^-$ ion appeared at m/z 398. This mass number is 44 mass units higher than that of the product expected when MX is subjected to direct glucuronidation (theoretical molecular mass, m/z 355). The isolated conjugate was analysed by positive ESI mass spectrometry to give a peak at m/z 417, corresponding to $[M+NH_4]^+$, and its CID mass spectrum showed m/z 224, 180 and 58 as product ions from the precursor ion $[M+NH_4]^+$ at m/z 417 (figure 3). These results indicate that the isolated conjugate is likely to contain a carbonyloxy moiety in addition to MX and glucuronic acid moieties.

Hydrolysis of the conjugated MX

Since the isolated conjugate was postulated to possess a carbamoyloxy moiety in the molecule, it was treated under a mild alkaline condition to confirm the structure. When ammonium acetate buffer was used as the mobile phase, the isolated MX conjugate emerged at 1.9 min (figure 4A) and the retention times of MX and its metabolites were slightly changed from those under the previous condition: MX = 3.0 min, NHM = 3.5 min, DMPL = 8.6 min, DMPN = 11.0 min and DMPNX = 11.5 min. When the isolated conjugate was treated with an aqueous solution of sodium hydroxide, a peak at the same retention time as that of MX (3.0 min) was observed in the HPLC chromatogram of the reaction mixture (figure 4B). The same product was obtained from reaction mixtures when the conjugate was hydrolysed with acid or β -glucuronidase (data not shown). When the conjugate was treated with alkaline methanolic solution, the reaction mixture gave two new peaks that emerged at 3.0 min (MX) and 8.8 min (unknown new peak) on HPLC analysis (figure 4C). This new peak at 8.8 min was considered an *N*-methoxycarbonyl derivative of MX, based on the chemical reaction mechanism.

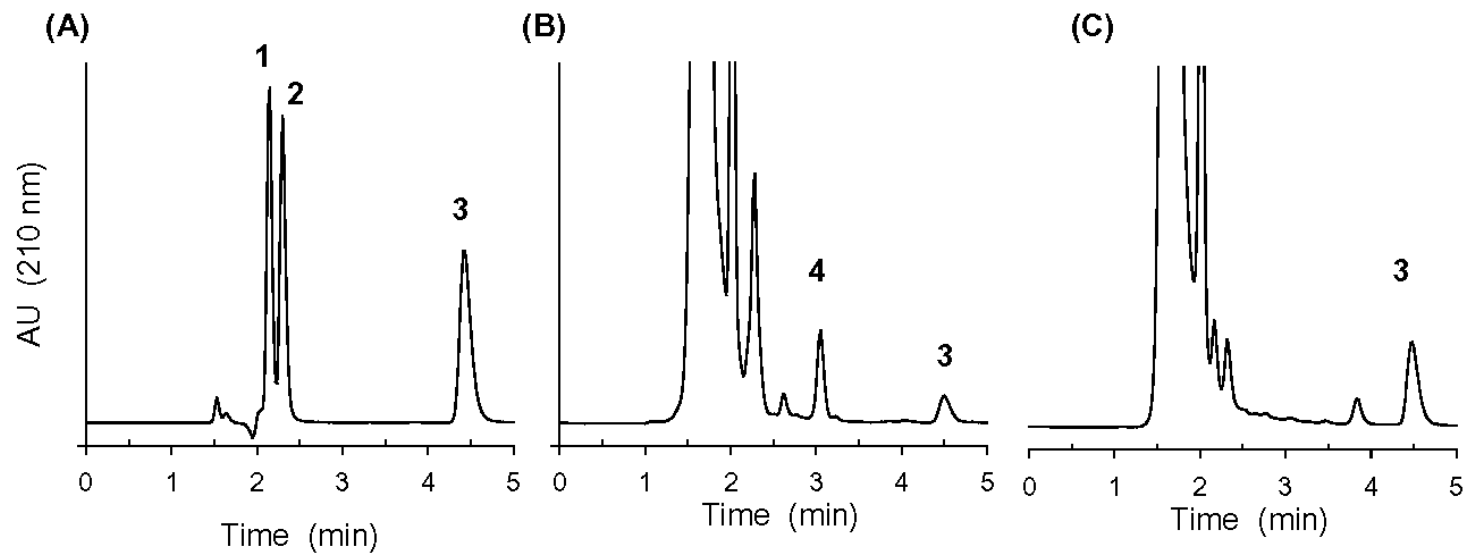


Figure 1. Typical chromatograms of authentic mexiletine (MX) and its metabolites (A) and of urine samples before (B) and after (C) acid hydrolysis. Peaks: 1, 4HM=2.1 min; 2, 2HM=2.3 min; 3, MX=4.5 min; 4, conjugated MX=3.1 min.

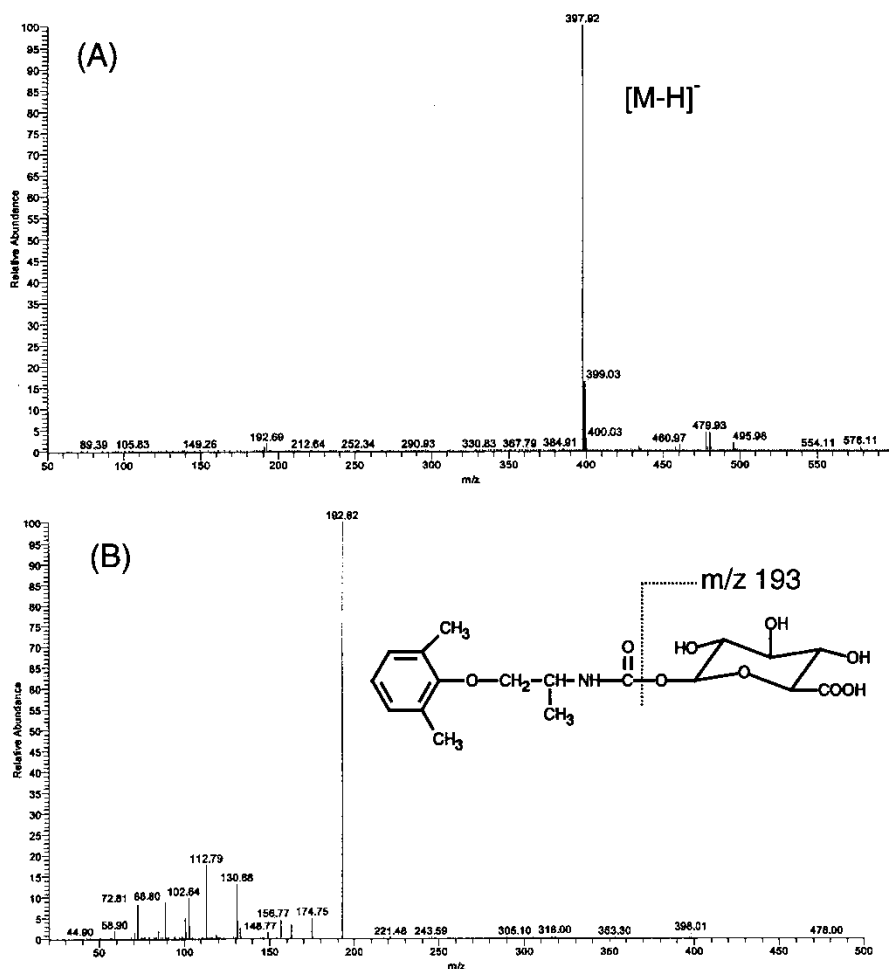


Figure 2. Negative electrospray ionization-liquid chromatogram mass spectrum (A) and collision-induced dissociation spectrum (B) from $[M - H]^-$ at m/z 398 of the metabolite isolated from human urine.

Authentic NCM was synthesized as a standard to compare its chromatogram and mass spectrum with those of the unknown product.

As shown in the ion chromatogram at m/z 238 (figure 5), synthetic NCM gave the same retention time as that of the product from the conjugate treated with an alkaline methanol solution. The MS/MS (CID spectrum) of m/z 238 derived from synthetic NCM was identical with that derived from the metabolite treated with sodium hydroxide in methanol (figure 6), indicating that the metabolite contains an $-NHCOO-$ group in its molecule. Thus, the structure of the isolated MX conjugate was concluded to be the *N*-carboxyloxy β -D-glucuronide of MX.

Semiquantification of MX carboxyloxy β -D-glucuronide in urine

Since enough authentic reference standard for MX carboxyloxy β -D-glucuronide was unavailable, each peak height at 3.1 min of collected urine samples

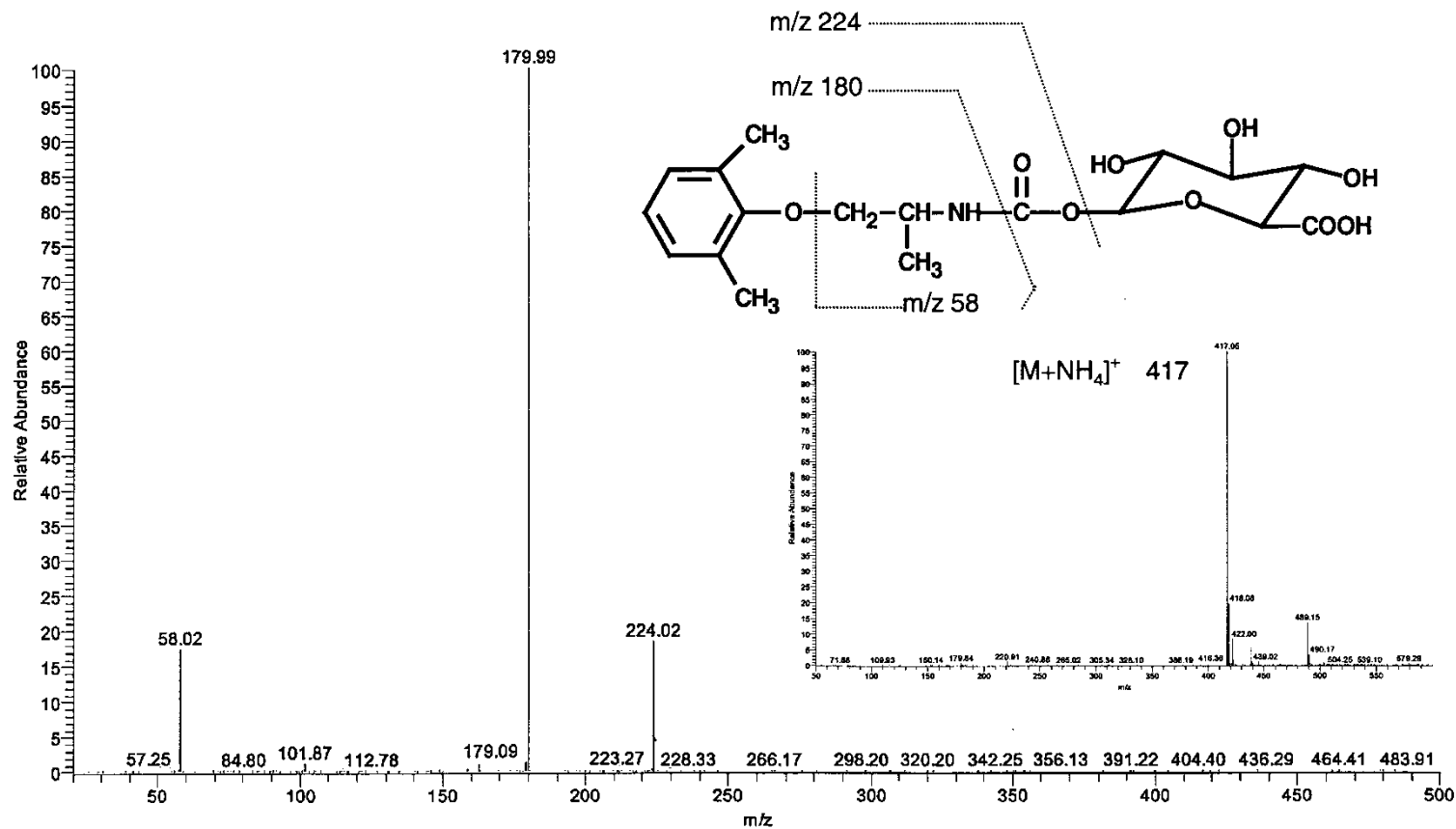


Figure 3. Precursor ion $[M+NH_4]^+$ at m/z 417 and collision-induced dissociation product ions of the metabolite isolated from human urine.

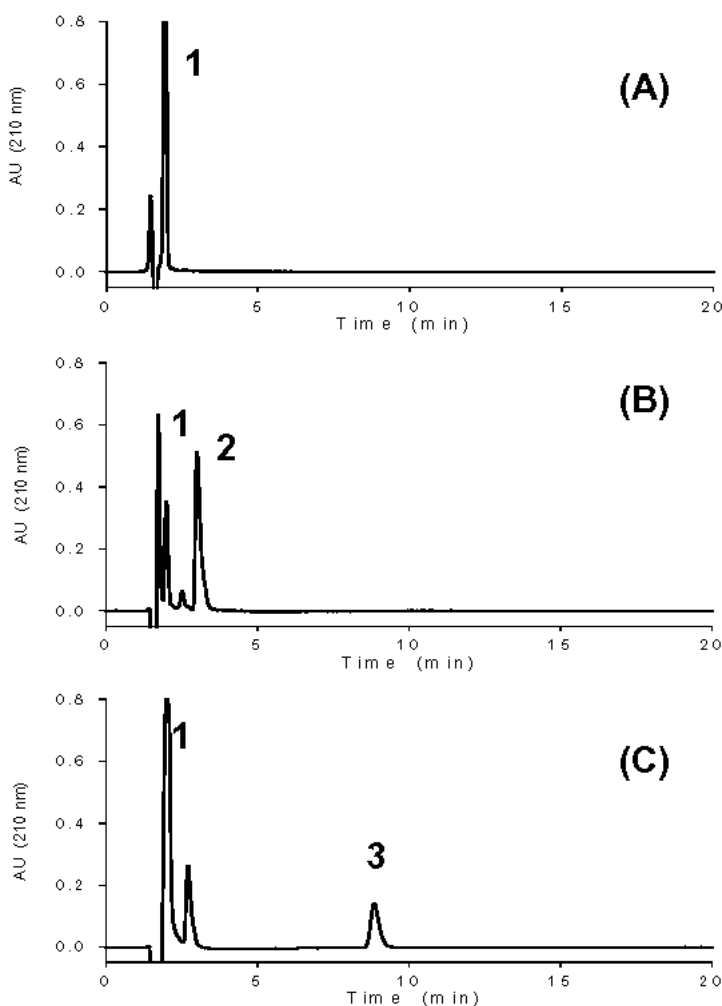


Figure 4. High-performance liquid chromatogram (HPLC) of the isolated metabolite (A). HPLC analysis of the product obtained after treatment of the isolated metabolite with 1 M sodium hydroxide solution (B) or 1 M sodium hydroxide methanolic solution (C) at room temperature. Peaks: 1, MX glucuronide = 1.9 min; 2, MX = 3.0 min; 3, new product = 8.8 min.

(0–6-h fraction, $n = 10$) was compared with that of MX carbonyloxy β -D-glucuronide ($1 \mu\text{g ml}^{-1}$) under the same HPLC condition. When the amount of the glucuronide in each urine sample was calculated by a peak height ratio, 17–31 mg MX carbonyloxy β -D-glucuronide was estimated to be present in each urine sample. On the basis of this semiquantitative data for each urine sample after acid hydrolysis, MX carbonyloxy β -D-glucuronide accounted for approximately 68–103% ($n = 10$) of the total amount of conjugated MX.

Discussion

From a portion of urine collected from subjects after MX administration, a new MX glucuronide was isolated. The use of soft ionization techniques in mass

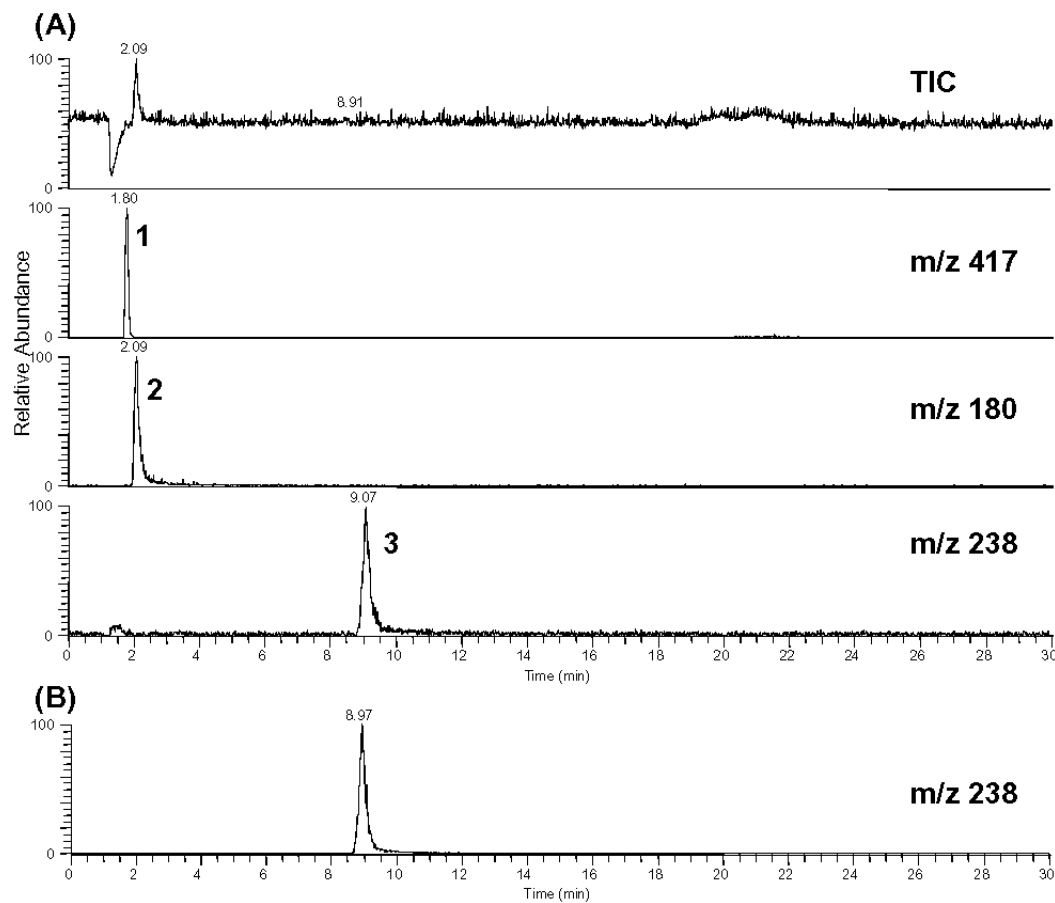


Figure 5. Total ion chromatogram (TIC) and extracted ion chromatograms of the reaction mixture after treatment of the isolated MX glucuronide with sodium hydroxide methanolic solution (A). Ion chromatogram of synthetic *N*-methoxycarbonylmexiletine (B). Peaks: 1, MX glucuronide; 2, MX; 3, unknown.

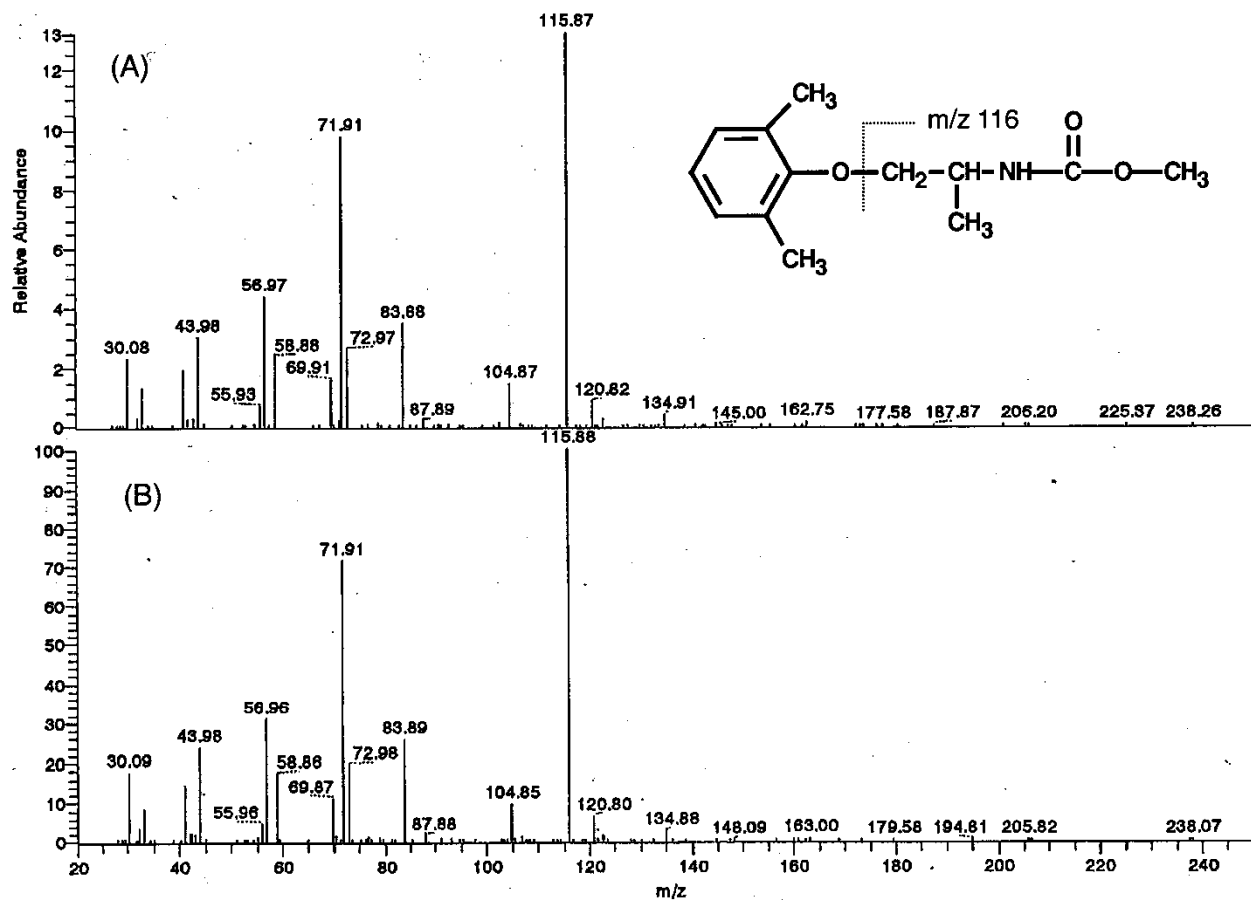


Figure 6. Collision-induced dissociation spectrum from $[M + H]^+$ at m/z 238 of synthetic *N*-methoxycarbonylmexiletine (A) and the product obtained from the urinary metabolite by treatment with sodium hydroxide methanolic solution (B).

spectrometry allows convenient characterization of intact conjugates and contributed significantly to the identification of this metabolite. MX and its major hydroxyl metabolites are usually extracted from biological samples under alkaline conditions, because of their pK_a values, and then quantified by HPLC analysis by using acidified eluents. In this study, the pH of the mobile phase for HPLC analysis and HPLC separation before the mass analysis was set at 7.0 to avoid degradation of the carbamoyl moiety in the analyte. Consequently, the $[M-H]^-$ ion of the isolated MX glucuronide was detected at m/z 398, equal to that of MX-glucuronide plus a carbonyloxy moiety. The CID product ion at m/z 193, derived from $[M-H]^-$ suggested the presence of a glucuronic acid moiety in the structure (figure 2B). The molecular mass of the conjugate was further confirmed in the positive-ion chromatogram by the presence of the ion adduct $[M+NH_4]^+$ at m/z 417. The observation of product ions at m/z 224 and 180 further supported the presence of a carbonyloxy moiety. The nucleophilic reaction of the methoxyl group with MX glucuronide resulted in the formation of *N*-methoxycarbonyl-mexiletine, which would not be expected from either *N*-hydroxyglucuronide or *N*-glucuronide. These results support our postulation that the isolated MX glucuronide has the *N*-carbonyloxy β -D-glucuronide structure. Although quantification by a validated analytical method has not yet been performed, the estimated amounts of the isolated glucuronide corresponded to about 80% of total MX glucuronide, as calculated from the increase of MX after acid hydrolysis of the urinary metabolite. Assuming that around 30% of orally administered MX is excreted as MX glucuronide in urine (Abolfathi *et al.* 1993), the urinary recovery of MX carbonyloxy- β -D-glucuronide is expected to account for over 25% of the dose. Therefore, this novel glucuronide appears to be one of the major metabolites of MX in human urine.

Crooks and Donnellan (1989) demonstrated that amine carbamates were formed in a single step from unprotonated primary or secondary aliphatic amines and CO_2 in the presence of H_2O as a catalyst. This reaction can readily occur in physiological type media, such as the carbon dioxide/bicarbonate buffer system (Myers and Nelson 1990) and also plays important roles in biological systems. For example, carbamic acid is formed during the transport of carbon dioxide by haemoglobin and is associated with the physiological reactions of a number of other proteins and peptides (Kilmartin and Rossi-Bernardi 1973, Gros *et al.* 1981). There are only a few reports on carbamate metabolites of drugs containing a primary amino group. *N*-Carbonyloxy- β -D-glucuronides of an antiarrhythmic agent, tocainide, and an antiviral agent, rimantadine, have been reported to be excreted in urine after oral administration of these drugs (Elvin *et al.* 1980, Brown *et al.* 1990, Kwok *et al.* 1990). The formation of the *N*-carbonyloxy- β -D-glucuronide conjugate of MX is likely to involve initial reversible addition of CO_2 to the primary amine function to form a carbamic acid intermediate, which is in turn glucuronidated to form a stable carbonyloxy- β -D-glucuronide conjugate. The formation of carbamoyl conjugates via carbamic acid intermediates may be quite common with amines, but would have been unnoticed because of the instability of the intermediates.

Regarding carvedilol and mofegilin, Schaefer (1992) and Dow *et al.* (1994) reported that the carbamate glucuronide was observed in urine and in a reaction mixture containing dog or human liver microsomes together with uridine diphosphoglucuronic acid (UDPGA) in carbonate buffer. They showed that incubation

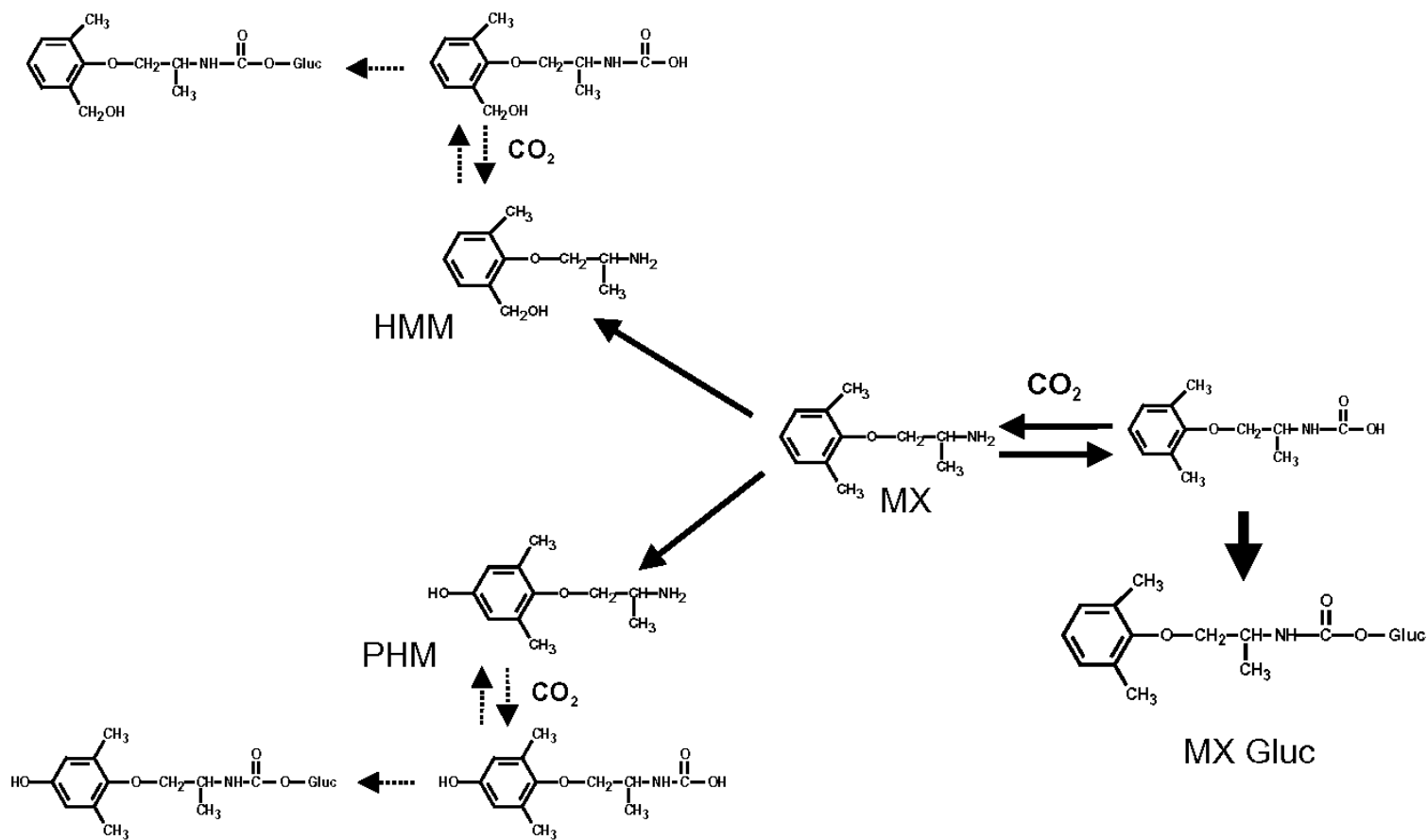


Figure 7. Proposed metabolic pathways of glucuronic acid conjugation of MX in man. Dotted lines express the possible metabolic pathways suggested by the results of this study.

in bicarbonate buffer spiked with UDPGA under a CO₂ atmosphere was necessary for the maximum formation of the carbamoyl glucuronide conjugate with hepatic microsomes. Interestingly, in our preliminary *in vitro* glucuronidated study of MX, 4HM, 2HM and NHM using a standard method without a CO₂ source, it was observed that NHM gave the *N*-hydroxyglucuronide, whereas the amounts of MX, 4HM and 2HM were unchanged after incubation, and no relevant product was detected on the chromatogram. Hence, it can be speculated that 4HM and 2HM are also conjugated with glucuronic acid following the formation of carbamic acid intermediates, because an amino moiety is present in both 4HM and 2HM, as well as MX. In summary, it is proposed that the metabolic pathways of glucuronic acid conjugation of MX in human are as shown in figure 7. The methanolysis reaction described here might be a generally useful method for the characterization of carbonyloxy glucuronides because the resulting methyl carbamate product was stable and could be determined by HPLC analysis.

The present results suggest that MX glucuronide was predominantly formed via non-enzymatic carboxylation followed by enzymatic glucuronidation. This compound had a different chemical structure to that proposed by Turgeon *et al.* (1992), who suggested that the major conjugated metabolite of MX observed in rabbits and humans was the conjugate of *N*-hydroxymexiletine. The latter authors proposed that this NHM glucuronide was hydrolysed to give MX *per se*. To test their hypothesis, NHM glucuronide was chemically synthesized in the authors' laboratory by the same method described in their paper, on a milligram scale. In the HPLC analysis of the reaction mixture after enzymatic hydrolysis of NHM glucuronide, NHM but not MX was detected.

In conclusion, a novel MX glucuronide was isolated from human urine and it was established that the metabolite contained a carboxyl moiety in its structure by using LC/MS/MS and HPLC under neutral conditions. The estimated recovery of mexiletine carbonyloxy β -D-glucuronide corresponded to over 25% of the dose of MX indicating that it is a major component of MX glucuronide in urine.

Acknowledgements

The authors thank Mr M. Kubota of ThermoQuest for the mass spectral analysis of the purified compound.

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Erratum

CYP2D6-mediated catalysis of tamoxifen aromatic hydroxylation with an NIH shift: similar hydroxylation mechanism in chicken, rat and human liver microsomes, 33, pp. 141–151.

Y. Hu, S.S. Dehal, G. Hynd, G.B. Jones and D. Kupfer

Figures 2 and 3 were incorrect at press and should have appeared as below:

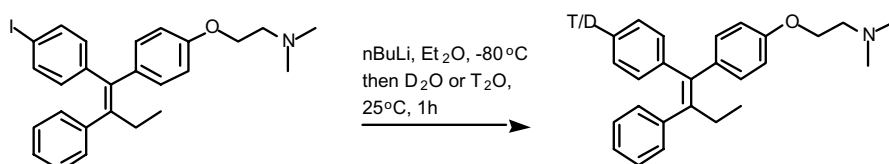


Figure 2.

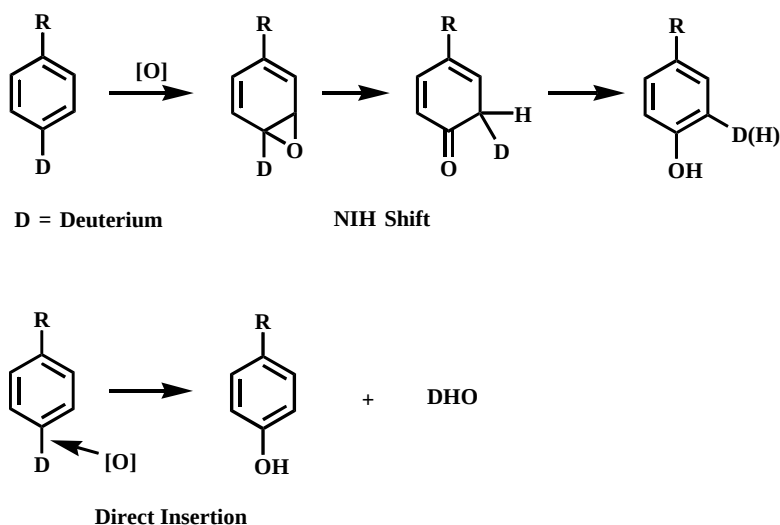


Figure 3.

Topics in Xenobiochemistry

Do metabolic pathways exist for xenobiotics? The micro-metabolism hypothesis

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Received 18 March 2003

1. The relevance of the concept of 'the metabolic pathway' for the understanding of xenobiotic metabolism is discussed in the light of advances in modern analytical methods that have enabled the detection and identification of minor metabolites present at ever lower concentrations.

2. A model is suggested where the overall metabolic fate of a xenobiotic is the sum of all the possible metabolic reactions permitted by the solution chemistry of the compound modulated by factors such as the metabolizing enzyme complement of the organism, the affinity of those enzymes for the xenobiotic substrates and the probabilities of all of these processes.

3. In this probabilistic, rather than deterministic, system, the resulting proportions of particular metabolites will, therefore, depend on the sums of the probabilities of particular biotransformation reactions occurring and the stability (chemical or metabolic) of the resulting metabolite.

4. In this model, all the potential metabolic possibilities that could result for any individual xenobiotic will occur to some extent. However, in actuality, many of the resulting metabolites will be produced or excreted in such small quantities as to defy ready detection with current methods.

Introduction

It is inevitable that at some stage in the development of any new field of research an attempt will be made to organize the observations derived from experimental data into some rational scheme. In this context, the publication of *Detoxication Mechanisms* by R. T. Williams in 1947 is considered by many to have been of seminal importance in the development of drug metabolism as a scientific discipline. This type of synthesis is valuable in enabling hypotheses to be generated that can subsequently be tested, and in this way, the science is advanced. Concepts such as phase I and II metabolism, and the idea of the metabolic pathway, where reactions occur in a particular sequence, have been valuable as a means of imposing some order by providing maps through metabolic space. However, when the terms 'phase I' and 'phase II' were proposed, there was, perhaps, a general assumption that these reactions were in some way ordered in a logical sequence with phase I reactions functionalizing the xenobiotic to make it into a suitable substrate for subsequent phase II conjugations. The latter thereby ensured that the metabolite was sufficiently polar and water soluble for excretion

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via the bile or kidney. It was also generally assumed that these various metabolic reactions had been 'designed' to be detoxification reactions, and that the production of active or toxic metabolites was an unfortunate metabolic 'accident'. Clearly, with the benefit of over half a century of hindsight in 'modern' drug metabolism, it is well understood that the concept of phase I metabolism being followed by phase II reactions and then excretion, and metabolism as always providing a detoxification mechanism, is far too simplistic a model. Such a view is hardly novel and the inadequacy of the terms 'phase I and II' to describe accurately xenobiotic metabolism have been pointed out by others (e.g. Testa and Jenner 1978). Indeed, there are many examples of phase II reactions that are performed directly on suitable molecules without the need for prior phase I metabolism because of the presence of a pre-existing functional group that is already suitable for conjugation. Examples would include the conjugation of phenol with sulphuric or glucuronic acids and salicylic acid with glycine, etc. Compounds can be eliminated as their phase I metabolites without the need for conjugation, e.g. practolol as its ring hydroxy-metabolite, salicylic acid as gentisic acid, etc. Furthermore, molecules on which phase II metabolism has occurred can still undergo further phase I and II reactions, and compounds can go down 'competing' pathways with combinations of phase I or II reactions, followed by excretion. Sometimes, phase I reactions introduce a functional group to a molecule, which is then excreted following conjugation, but with conjugation not to the newly introduced functional group but to a pre-existing one. Many of the features of this complexity can be illustrated by the example of ibuprofen (figure 1). In this case the carboxylic acid moiety of the parent drug is already a suitable substrate for glucuronidation and the compound is excreted as such, i.e. phase II in the absence of phase I. A major metabolic reaction for ibuprofen is hydroxylation of the side chain, and this is also followed by glucuronidation and excretion. However, perversely, glucuronidation is to the carboxylic acid moiety already present in the parent and not to the new hydroxyl group. As well as hydroxylation, phase I metabolism also proceeds to

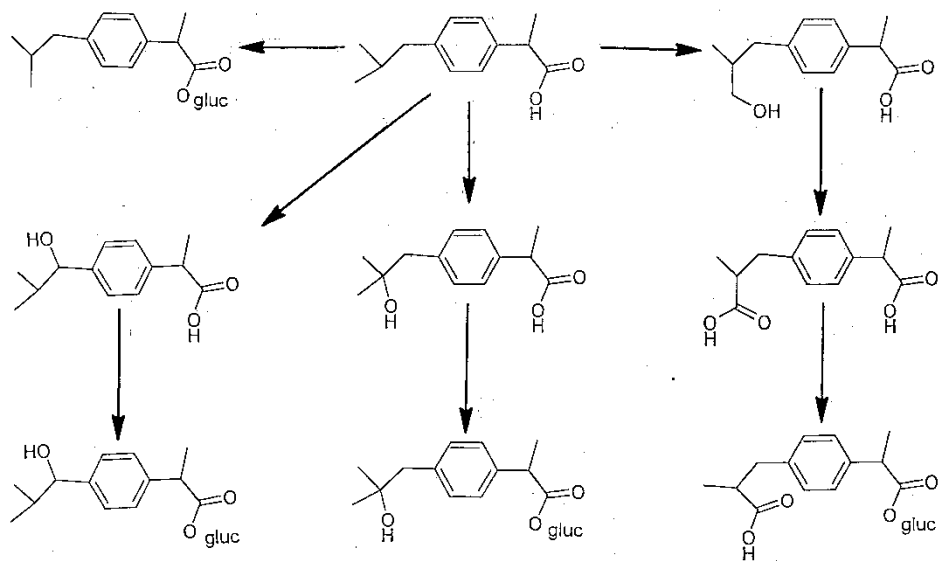


Figure 1. Conventional metabolic 'pathway' for ibuprofen.

introduce a second carboxylic acid group in place of one of the methyl groups on the side chain. There are also a number of more minor metabolites of ibuprofen (Hucker *et al.* 1980). Given that the parent compound can be metabolized directly to a glucuronide and excreted, there is no biological logic behind this sequence of competing metabolic pathways. As well as these 'detoxification' pathways, a major metabolic route for ibuprofen (and related compounds) involves chiral inversion, which produces no change in physicochemical properties, despite the effects of this transformation on pharmacological activity. It is also noteworthy that the production of some major metabolites, e.g. the dicarboxylic acid metabolite of ibuprofen requires several steps, and these intermediates are often either only seen in low concentrations or not at all. In the latter case they must be produced, but are simply not detected (see also mercapturate production below). One thing that is clear from the type of metabolic fate exemplified by ibuprofen is that there are many more routes of metabolism than are required to eliminate the compound from the body. Such complexity is obviously not restricted to ibuprofen and even a casual review of the literature provides many examples of multiple sites and routes of metabolism leading to myriad metabolites.

Xenobiotic metabolism considered as the product of probability not design

In a complex spatially resolved biochemical system such as a cell, all molecules behave as tumbling charged shapes (whether they are of endogenous or xenobiotic origin) and their interactions with cellular components are dependent purely on their molecular physicochemical properties, their molecular motions and their collisions with potentially reactive moieties. The outcome of such collisions, i.e. reactions, is dependent on the laws of thermodynamics and kinetics. In advanced organisms, most of the metabolic machinery is tuned by evolution to process endogenous metabolites but with extra capacity to deal with toxins in the diet. Many metabolic reactions (including non-enzyme-catalysed transformations) occurring in a series can be considered as based on conditional probabilities (i.e. A must go to B before B can go to C) and therefore follow Bayesian statistical rules. However, these are often complicated by displacing the 'sequential' processes in space and time as would occur in the case of initial metabolic activation in the liver, transport through the blood and further metabolism, say, in the kidney. The time displacement of these reactions significantly complicates attempts at metabolic modelling and introduces a series of non-linear convolutions that are hard to quantify (such as the presence or absence of a specific transporter for a metabolite or substrate in certain cell types).

As a result of metabolic investigations (both endogenous and xenobiotic), over many years we have begun to speculate that the concept of the metabolic pathway in the context of drug and xenobiotic metabolism is an oversimplification that reflects the very human desire for order rather than representing any underlying reality. There is also a subtle and very complex interaction between the metabolism of foreign compounds and the effects of these metabolites on endogenous metabolic pathways that can then feed back and alter the subsequent metabolism of the xenobiotic, thus further convolving the system (Nicholson *et al.* 2002). In this developing view of metabolism, all possible biotransformations will occur, providing that a metabolic reaction is feasible, i.e. if the potential is there because

the necessary enzymes exist and the reaction is chemically possible, then it will occur to some, possibly vanishingly small, extent. Thus, if the reaction has a high probability, either because it is thermodynamically or kinetically favourable or because there is a high level of expression of the particular enzyme system, or the compound has a high affinity for the metabolizing enzyme, then it will be a major 'route' of metabolism. Conversely, if very improbable, because the enzyme system is a minor one, the compound is a poor substrate, the enzyme is compartmentalized in such a way that the substrate cannot easily come in contact with it, or a large number of reactions are needed to produce a particularly unusual product, etc., the metabolite may well be produced, but be below the limits of detection of any currently available means of analysis. Also, it should not be forgotten that many metabolic reactions are essentially reversible, at least at the microscopic level (as dictated by the law of microreversibility in mechanistic organic chemistry) even if the equilibrium constant lies far to the right (in favour of products) for thermodynamic reasons. In addition, the body has many enzyme systems that can undo the effects of others, e.g. glucuronidases and esterases. If the rate of the reverse reaction is high, metabolites may be produced that never 'escape' into the excreta.

As an example of a Bayesian 'probabilistic' rather than a deterministic metabolic 'pathway', consider the well known and apparently comparatively simple example of the biotransformation of acetanilide to paracetamol (acetaminophen). This can be represented conveniently as a simple one-step biotransformation involving *p*-hydroxylation (figure 2a). The reality is much more complex than

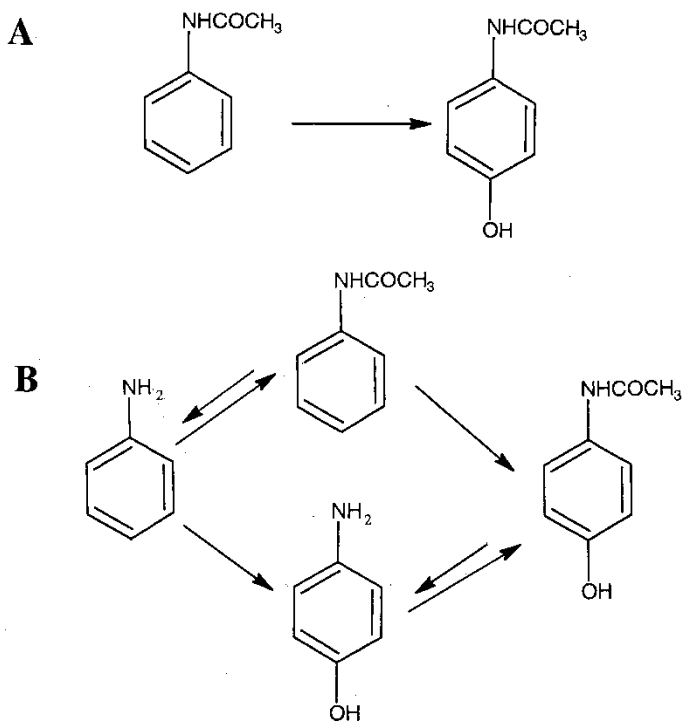


Figure 2. (a) Conventional scheme for the hydroxylation of acetanilide to acetaminophen; (b) same biotransformation represented as in the 'probabilistic' scheme.

this. So, whilst it is possible to go directly to paracetamol in a single step, the competing reaction of N-deacetylation to give aniline is also a highly probable metabolic fate. The production of aniline can then be followed by hydroxylation to *p*-aminophenol, and then N-acetylation to give paracetamol, or the aniline could be N-acetylated to give acetanilide once more allowing either *p*-hydroxylation to paracetamol or even further futile deacetylation to aniline (figure 2b). Even when paracetamol has been formed, it can still be deacetylated to give *p*-aminophenol, etc., only to be reacylated at some point (e.g. Nicholls *et al.* 1997). These 'futile acetylation' reactions are surprisingly common and extensive, but because the reaction is metabolically 'silent', i.e. an acetylated molecule is dosed and an acetylated molecule is excreted, it is rarely observed unless specifically investigated using appropriate labelling methods. Only after the removal of these compounds from the body does the potential for metabolism to occur cease. Now the sum of all these reactions is still that acetanilide will be excreted predominantly as paracetamol, and its further metabolites, but clearly the simple direct route exemplified by the conventional metabolic pathway is only an overall summary and provides no real description of the likely fate of any individual molecule of the dosed acetanilide at any stage in the process.

In this Bayesian view of metabolism, it is also necessary to consider each new metabolite as a new compound, with its own potential for metabolism, and not simply as an intermediate on a preordained metabolic pathway. So, consider again the case of acetanilide discussed above where N-deacetylation to aniline or *p*-hydroxylation to *p*-aminophenol are the first steps. A moment's reflection should be all that is needed to appreciate that the physicochemical properties of these two new compounds are very different indeed, and that they are both quite different in their properties to the parent. It would, therefore, be wholly remarkable if the potential of these new compounds to interact with the enzyme systems responsible for metabolism was either identical to each other or the same as the parent anilide.

Partly, this probabilistic view of metabolism has also come out of our experience of studying metabolism during a period where the development of increasingly powerful and sensitive analytical techniques has been very rapid. After all:

Nothing tends so much to the advancement of knowledge, as the application of a new instrument. The native intellectual powers of men in different times are not so much the causes of the different success of their labours, as the peculiar nature of the means and artificial resources in their possession. (Davy 1840, p. 37)

These new techniques, e.g. high field nuclear magnetic resonance spectroscopy, tandem and time-of-flight mass spectrometry, etc., enable the specific detection of metabolites at ever lower concentrations. As such, they have, therefore, allowed the detection and characterization of increasingly minor metabolites that would have gone undiscovered in earlier studies and, therefore, by definition, could not be included in any broader theories of drug metabolism.

A way of visualizing this model being proposed here, which is essentially a matrix of reactions occurring to produce each different metabolite, is shown in a highly simplified form in figure 3a. Here, the fate of a compound is considered at three different levels. In the first, the parent compound (A) is metabolized to eight metabolites, only two of which (B, C) are of high probability and, therefore, produced in any quantity. Although other metabolites are produced from the

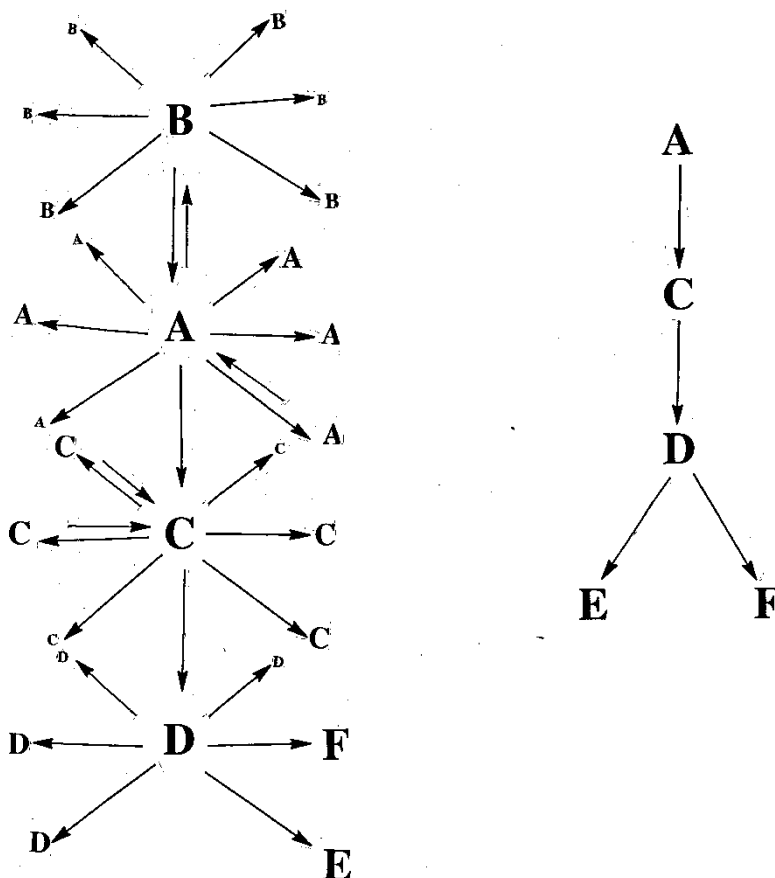


Figure 3. (a) Stylized representation of the complex metabolism of a xenobiotic; (b) reinterpretation of (a) as a conventional metabolic pathway.

parent compound, they are effectively invisible as they are below the limits of detection. In this scheme, metabolite B is rapidly converted back to parent compound and so, despite being produced in large amounts, is also effectively invisible. However, once formed, metabolite C, which effectively needs to be considered as a new chemical entity, is also available for metabolism to a range of products of which only D is detectable, etc. The biotransformations leading to detectable quantities of metabolites can then be rearranged in the form of a conventional 'metabolic pathway' (figure 3b). Of course, figure 3a is a highly stylized representation of the model as the same processes will also need to be considered for each metabolite produced, at each level.

Bromoaniline metabolism in rats

An illustrative example of how changing analytical techniques can change the perceived metabolic fate of a compound, which illustrates just how complex is the metabolic fate of even 'simple' compounds, is shown in our work on 4-bromoaniline. Our original studies were based on the use of HPLC-ultraviolet (UV) and HPLC-NMR-MS for the detection and identification of metabolites

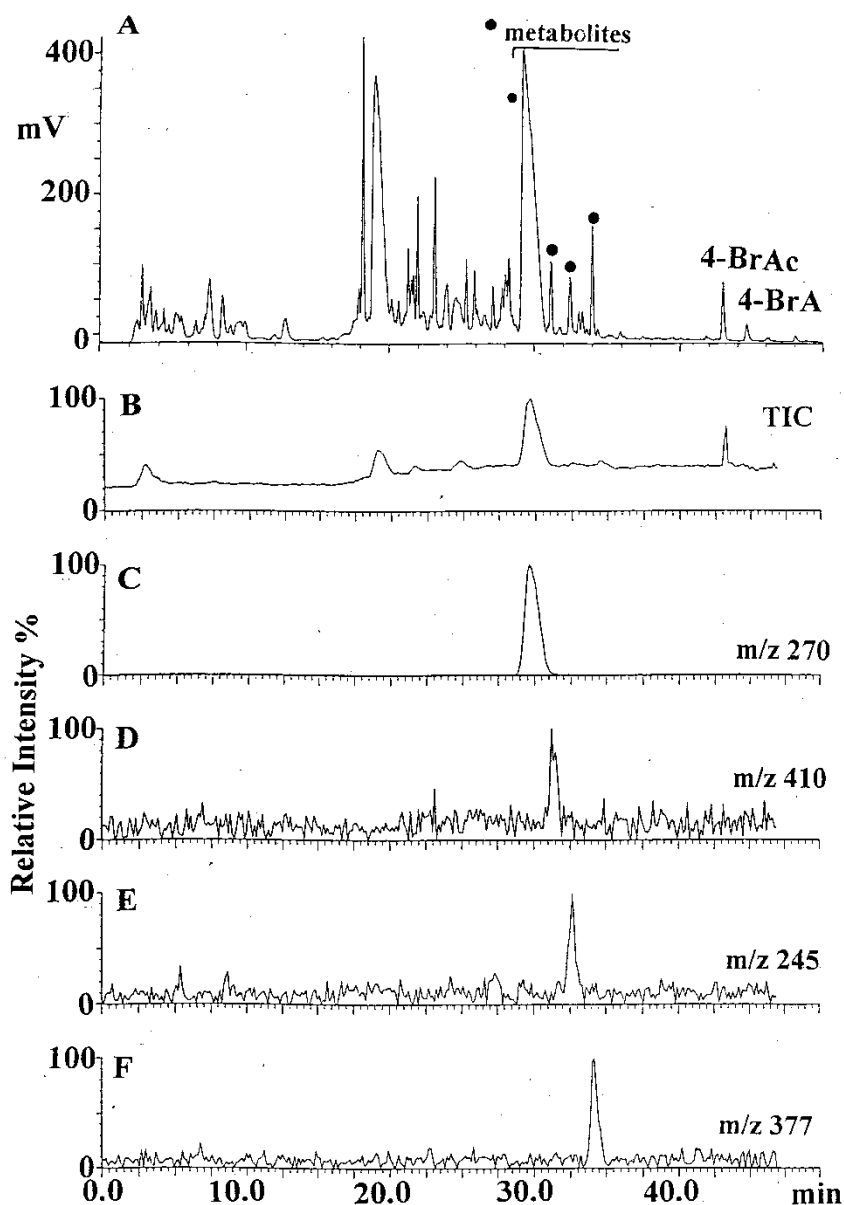


Figure 4. HPLC-UV and HPLC-MS chromatograms of an 0–8-h urine sample for a rat administered 50 mg kg^{-1} 4-BrA. Metabolite peaks are indicated by the solid circles.

(Scarfe *et al.* 2002). UV and total ion-current mass chromatograms are shown in figure 4. These studies resulted in the identification of a number of structures including a novel mercapturate, but not the original glutathione conjugate nor any of the intermediates on the pathway from it to the mercapturate, indeed these types of intermediate are rarely detected in urine. These reactions are illustrated in the metabolic summary shown in figure 5. Interestingly, the conversion of glutathione conjugates to mercapturates can still be considered to be a true metabolic pathway, but it represents a special case as it is actually a pathway of intermediary

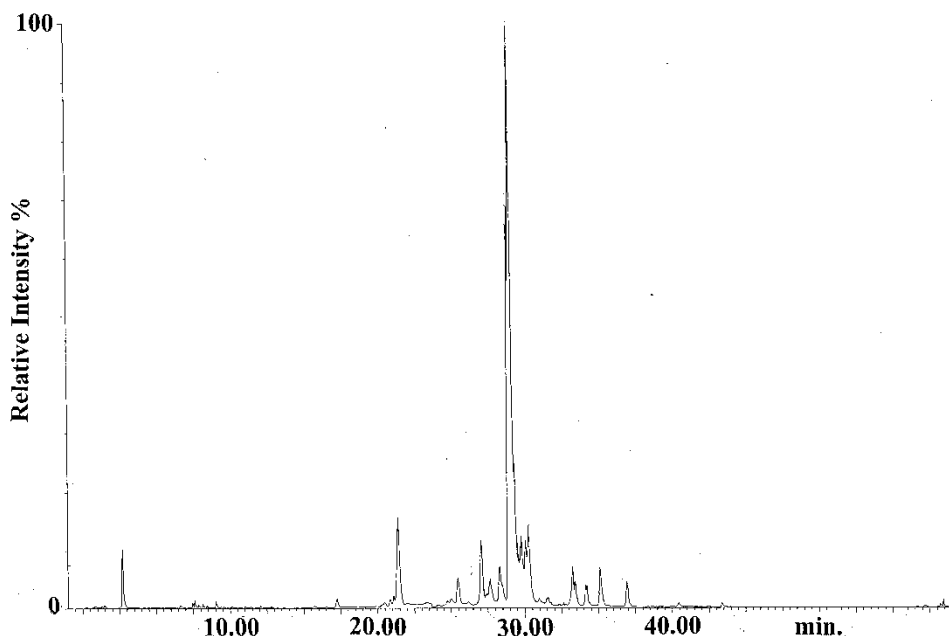


Figure 6. 'Bromatogram' obtained for the sample shown in figure 4 showing the distribution of bromine-containing peaks present in rat urine after dosing with 4-BrA (Nicholson *et al.* 2000).

metabolism that has evolved to deal in a general way with the glutathione moiety rather than the attached xenobiotic.

However, re-examination of the metabolic fate of 4-BrA with the application of a more specific Br-selective detector via the use of HPLC-ICPMS revealed the presence of a large number of previously undetected bromine-containing minor metabolites (Nicholson *et al.* 2000). The 'bromatogram' obtained for a typical 0–8-h sample is shown in figure 6. Further investigation of these urine samples using HPLC-time-of-flight mass spectrometry (TOF-MS) confirmed the presence of a large number of metabolites that were undetected in our first study, whilst at the same time showing that many of the peaks in the bromatogram were made up of several components (Abou-Shakra *et al.* 2002). We have still to assign structures to many of them, partly because the MS data suggest some very unusual structures and high-field NMR studies will also be needed to confirm identity, and also because many of them have different retention times but exactly the same molecular mass and therefore the same atomic composition.

Therefore, for example, the mass 403.999 corresponds to a metabolite of 4-BrA that has been acetylated, hydroxylated and glucuronidated (Abou-Shakra *et al.* 2002). The mass chromatogram (figure 7) for this ion shows four distinct resolved chromatographic peaks. One of these presumably corresponds to the previously identified glucuronide of the glycolanilide (Scarfe *et al.* 2002), and there are clearly two possible ring hydroxyglucuronide isomers. The identity of the remaining peak could either be an N-glucuronide, with a free ring hydroxyl, or a glucuronide of an N-hydroxylated metabolite. Probably both are formed and the use of an even more sensitive mass spectrometer would find even more isobaric species corresponding to further, increasingly unlikely, combinations of the possibilities of glucuronidation, hydroxylation and acetylation.

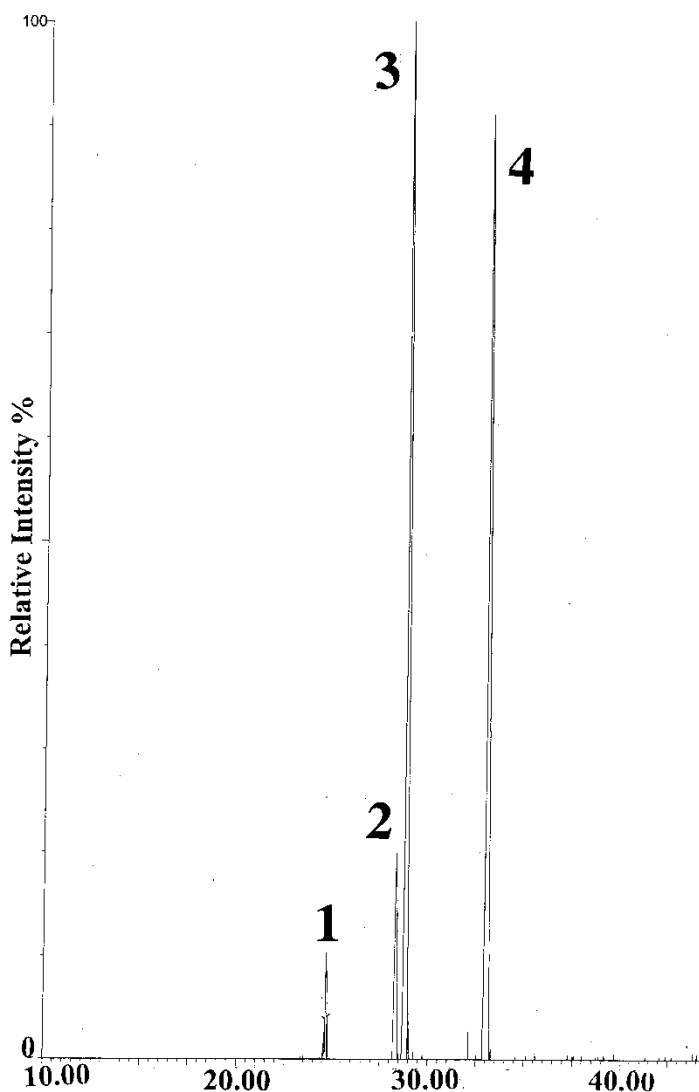


Figure 7. Mass chromatogram obtained for the urine sample shown in figure 5 for ions of mass 403.999 corresponding to acetyl-glucuronides of 4-BrA (Abou-Shakra *et al.* 2002).

The point being made here is that a very simple molecule, examined for common metabolic reactions such as hydroxylation, acetylation, sulphation and glucuronidation, etc., gives rise to very complex pattern of metabolism, and that not all of the metabolites are present in similar quantities. In fact, the overall metabolic fate of 4-bromoaniline is very complex with over 100 bromine-containing metabolites detected to date, and probably many more debrominated metabolites (unpublished data) and remains to be fully elucidated even to the extent that current 'state-of-the-art' methods will allow. Whilst such diversity at first sight appears excessive for such a simple molecule, it is easily achieved if all of the various combinations of phase I and II metabolic reactions are considered. As an example of the complexities that result from such 'combinatorial metabolism', we have recently partially characterized some of these very minor metabolites as a

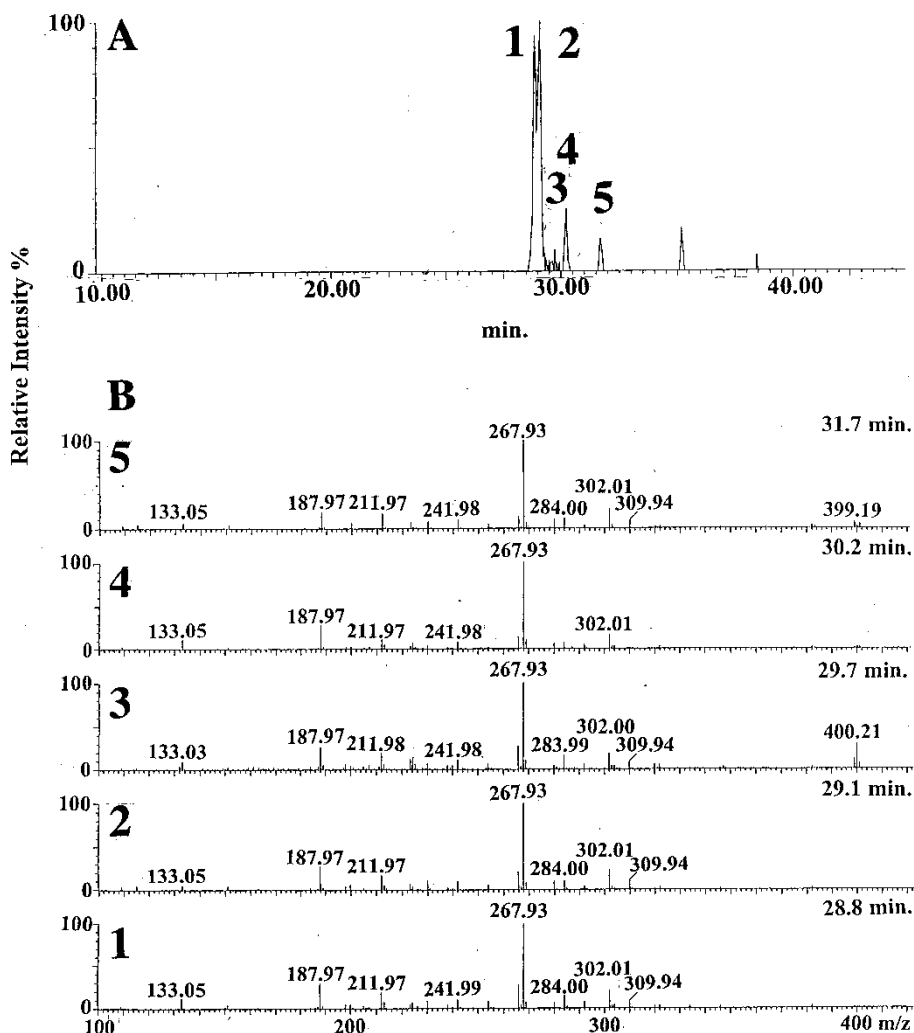


Figure 8. Mass chromatogram for the sample shown in figure 5 showing (a) the ions at m/z 399.97 corresponding to the five putative ribosidehydroxysulphates of 4-BrA and (b) the mass spectra obtained for these metabolites (Major *et al.* 2003).

group of five putative double conjugates of 4-bromoaniline containing a sulphate moiety and a pentose, possibly ribose (Major *et al.* 2002). The single ion chromatogram for this group of compounds is shown in figure 8a, with the identical spectra obtained for each peak shown in figure 8b. As well as these minor pentose-containing metabolites, we have also detected a large number of minor hexose and di-hexose conjugates of 4-BrA and 4-BrA-hydroxysulphates or sulphamates (Major *et al.* in press).

Whilst the fact that we have detected such a large range of metabolites for 4-bromoaniline is a result of the greatly increased sensitivity of time-of-flight mass spectrometers, we have also been aided by the presence in the detected metabolites of the bromine isotope pattern. This isotope pattern has provided us with a 'handle' that has allowed us to pick out compound-related peaks even when these were present in very small amounts. Without such a handle, it is very unlikely,

given the range of biotransformations seen, that we would have observed these unusual minor metabolites or, had we detected them, they might well have been misinterpreted as endogenous interferences. Clearly, the loss of bromine as a result of metabolism would make the subsequent products very difficult to find and there may be many more metabolites of 4-BrA still undetected as a result.

Discussion

It seems, therefore, based on results such as those described above for 4-BrA, that in addition to major metabolites, there will always be a large number micro-metabolites produced as the result of the exposure of an organism to a xenobiotic. Such data are only explicable in terms of many Bayesian events giving rise to an extensive series of 'low probability' metabolic outcomes (products). Many of these low-probability metabolites will be devoid of pharmacological or toxicological properties, or even if they possess them, will be present in such trivial amounts and provide such low exposures as to be essentially irrelevant. In considering such compounds, the very pressing need, for example, to deliver new therapies to the patient requires a pragmatic approach be taken to safety evaluation, such as that outlined in the recent discussion of drug metabolites in safety testing (MIST) by Baillie *et al.* (2002). However, just because a metabolite is present at low concentration does not mean it is always without biological significance, and whilst the approach outlined for MIST places a clear emphasis on examining the role of major metabolites, a case-by-case approach is advocated even for minor components. With this in mind, it is useful to consider the possible influence of this hypothesis on 'idiosyncratic toxicity' of the type that may occur rarely in man due to unusual combinations of toxic metabolites in individuals for many, often unexplained, reasons (see also Li 2000). It is certainly possible that some classes of idiosyncratic toxicity result from rare combinations of unusual metabolic polymorphisms (not all of which may be in classical 'drug metabolism pathways') coupled with some dietary metabolic interactions that affect the production of toxic metabolites that are normally present in undetectable concentrations because the probabilistic conditions for their production in significant amounts are not satisfied in animal models. Almost by definition, such toxic events in man will not be predictable from any type of *in vitro* or *in vivo* animal studies.

We offer a definition of the micro-metabolism concept being advanced here:

The fate of a xenobiotic in any biological system will be the sum of all the possible combinations of metabolic and chemical reactions that can occur in solution, with the extent to which metabolites are produced based only on the conditional probability that they can be formed.

The extent to which these metabolites will be observed obviously depends not only on the fact of their production, but also on their stability and propensity for further biotransformation. If this view of xenobiotic metabolism is accepted, the next question to be asked is what is the significance of this model, given that it does not change the overall day-to-day metabolic result (in the same way that Newtonian physics still provides a very good local approximation even in an Einsteinian universe)? This view of metabolism clearly implies that it is axiomatic that it will not be possible to characterize *fully* the metabolic fate of any compound. Indeed, it is quite likely that even 'unmetabolized' compounds might actually

undergo a wide range of biotransformations, but at a level below our current detection ability. This model, however, might have implications where toxicity is observed or the relationship between pharmacokinetics/dynamics or toxicokinetics/dynamics is unclear. There is always a tendency to ascribe these effects to a metabolite(s), but which one(s)?: major-, micro- or nanometabolites that have been detected and identified or putative, but potentially potent, femtometabolites that have not even been detected let alone characterized. Also, it should not be forgotten that a drug or pollutant present at some level over the course of a lifetime might produce significant exposure to a long-lived/accumulating active 'femtometabolite' (or a cocktail of such minor metabolites), even when present at very low concentrations, with consequent toxicological sequelae.

A useful thought experiment that illustrates the relationship between our understanding of metabolic fates and the ability to determine them is to consider the sort of analytical sensitivity required to elucidate fully the metabolism of one mole of a compound dosed to a suitable test organism. For the most abundant metabolites excreted at the fractions of a mole level, detection and structure determination is unlikely to pose any difficulty to modern NMR- or MS-based methods. Indeed, even micromolar metabolites pose no particular difficulties. However, with metabolites accounting for increasingly smaller proportions of the dose such as nano-, pico- and femtomolar metabolite detection, identification will begin to pose enormous difficulties. At the zeptomolar level, i.e. a metabolite that accounts for perhaps a few molecules out of the original 6.0225×10^{23} administered, the difficulties will probably be insurmountable. However, our inability to detect this, presumably highly improbable, zeptomolar metabolite does not mean that it has not been produced. There is at present a large gap between our current analytical capabilities, and therefore the quantities of metabolites that we can reasonably expect to identify, and the zeptomole where many more metabolites may remain uncharacterized. Currently the state of analytical instrumentation is such that detection of a compound present at concentrations of 1 pg ml^{-1} is challenging, and to characterize and identify an unknown metabolite at this level would be impressive. However, for a metabolite with a molecular mass in the region of 500 Da, 1 pg represents about 10^9 molecules in a given sample. From a pragmatic point of view, of course, the existence of zeptomolar, or even femtomolar, metabolites is probably only of academic interest as a single exposure of an organism to such substances is extremely unlikely to be associated with a measurable effect.

The relationship between the metabolism of xenobiotics and general intermediary metabolism in such a model also needs to be briefly considered. In normal cell metabolism, the same general arguments can be applied with respect to the fate of a particular endogenous molecule being a mixture of propensity and probability. However, the major difference between intermediary and xenobiotic metabolism is that, by definition, xenobiotics are not used by the cell for any of its normal functions, whilst intermediary metabolism deals with compounds that have definite functions. In this respect, xenobiotic-metabolizing enzymes such as CYP3A4, which are capable of dealing with a broad array of substrates, can be considered to be quite promiscuous compared with the enzymes involved in intermediary metabolism. Indeed, it is evident that cells over the course of evolution have optimized the metabolic process to ensure that for endogenous compounds the probability of the 'right' reaction occurring is very high. This can be done, for example, by regulating the number of enzymes available for a particular metabolic

step and ensuring appropriate compartmentalization of enzymes and substrates and optimizing the specificity of the enzyme for its substrate to a high degree. Even so, some small portion of the substrates will still probably 'escape' and form different products to those required of them. However, if the system is properly optimized, this portion will be small and the mechanisms undoubtedly exist either to dispose of these small quantities of unwanted biomolecules or to redirect them back into the productive flow. As alluded to above, xenobiotic metabolites might occasionally enter into what are effectively endogenous pathways as illustrated by the efficient conversion of glutathione conjugates to mercapturates, but these represent the exception and not the rule.

Conclusion

It is our view that xenobiotic metabolism cannot, and should not, be considered as an ordered process of the type exemplified by the reductionist idea of the metabolic pathway as a sort of industrial production line. We do not doubt, however, the value of the pathway as a useful diagrammatic summary. Instead, as indicated, metabolism has to be considered as the sum of the probabilities of all the various possible metabolic reactions that could take place and that some of these probabilities rely on Bayesian events, i.e. are also conditional on previous metabolic or chemical occurrences. The point is that there is no logical sequence of reactions, but simply a collection of, often, unrelated reactions that lead to an overall end point. In addition, many (all?) of the common metabolic reactions are reversible, and only become fixed when the next reaction removes the product or if the metabolite is eliminated in the excreta.

Useful as these 'metabolic pathways' have been as a 'shorthand' for tabulating and organizing the metabolism of a xenobiotic, the fact that it seems logical to connect these various reactions into an apparently ordered sequence does not make it so, and many of the connections are dispersed in physical space (e.g. liver metabolism followed by metabolism in the kidney) as well as time. This is not to say that there is no pattern to metabolism, and, indeed, it is becoming increasingly possible to predict the major, i.e. most probable, metabolites of many classes compounds. However, despite this level of success, it is probable, because of limitations in analytical methodology, that it will never be possible completely to define the metabolic fate of an individual xenobiotic and, therefore, never completely predict its toxicity in man or animals.

It might be wise, therefore, to exercise caution when ascribing some pharmacological or toxicological response to a particular xenobiotic metabolite, as in fact such effects might result instead from an 'invisible' but highly potent micrometabolite (or group of micrometabolites). In the future, even if we cannot predict or explain idiosyncratic toxicity of drugs, a greater understanding of micrometabolic processes might enable us to predict when a drug or drug class is likely to be toxicologically unpredictable!

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Actions of quercetin on gluconeogenesis and glycolysis in rat liver

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Received 13 February 2003

1. The action of quercetin on glucose catabolism and production was investigated in the perfused rat liver.
2. Quercetin inhibited lactate production from glucose: 80% inhibition was found at a quercetin concentration of 100 μM , and at higher concentrations inhibition was complete.
3. Pyruvate production from glucose presented a complex pattern, but stimulation was evident at 100 and 300 μM quercetin. Oxygen uptake tended to be increased.
4. Glucose synthesis from lactate and pyruvate was inhibited. Inhibition was already evident at 50 μM quercetin and almost complete at 300 μM . Concomitantly, the increment in oxygen uptake caused by lactate plus pyruvate was stimulated by 50 μM quercetin, but clearly inhibited by higher concentrations (100–500 μM).
5. Glucose phosphorylation in the high-speed supernatant fractions of liver homogenates was inhibited by quercetin, but only at concentrations above 150 μM .
6. It is concluded that quercetin can inhibit both glucose degradation and production and increase the cytosolic NAD^+/NADH ratio.
7. These effects are likely to arise from many causes. Reduction of oxidative phosphorylation, inhibition of Na^+/K^+ -ATPase, inhibition of glucokinase and inhibition of glucose 6-phosphatase could all contribute to the overall action of quercetin.

Introduction

It is well established that quercetin as well as other flavonoids can inhibit glycolysis in tumour cells (Suolinna *et al.* 1973, 1975) and rat testis (Trejo *et al.* 1995). This action has been related to a highly effective inhibition of Na^+/K^+ -adenosine triphosphatase, a phenomenon which leads to low cellular concentrations of ADP and inorganic phosphate. These events, in turn, are likely to lead to a strong limitation of glycolysis, contributing to a reduction of tumour cell growth (Suolinna *et al.* 1975).

It was reported that quercetin exerts several effects on glycogen catabolism: it increases glycogenolysis, and consequently glucose release, it reduces lactate production, derived from the glycolytic chain, and it increases oxygen uptake (Gasparin *et al.* 2003). Quercetin also increases the cytosolic NAD^+/NADH ratio, as indicated by the reduced lactate/pyruvate ratio (Sies 1982, Gasparin *et al.* 2003). Furthermore, it was also found that quercetin inhibits sequestration of $[5\text{-}^3\text{H}]\text{glucose}$ by a mechanism that does not include inhibition of glucose transport across the plasma membrane. The mechanisms underlying these effects seem to be very complex and they include inhibition of enzymatic systems (e.g.

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glucose 6-phosphatase and several ATPases) as well as impairment of mitochondrial energy transduction. Impairment of mitochondrial energy transduction should reduce biosynthetic processes. One such process is gluconeogenesis, which is strictly dependent on mitochondrial ATP production. The fact that quercetin increases the cytosolic NAD^+/NADH ratio (Gasparin *et al.* 2003) is likely to represent an additional burden for glucose synthesis in the presence of the flavonoid because reducing power in the form of NADH is required for gluconeogenesis. Furthermore, quercetin inhibits glucose 6-phosphatase, the last enzyme in the long chain of events which lead to the release of *de novo* synthesized glucose.

All these previous reports of experiments with liver, tumour and other cells strongly suggest that quercetin may decrease two opposing metabolic routes: glucose synthesis and glucose degradation. The present work represents an attempt to test this hypothesis in the isolated perfused rat liver. The latter is an appropriate system for measuring both glycolysis and gluconeogenesis provided that glycogen degradation is minimized and that substrates for each metabolic route are supplied. Furthermore, quercetin, as possibly most natural flavonoids, is partly transformed in the liver (Manach *et al.* 1995, Morand *et al.* 1998). The relation between exposure to foreign compounds and liver metabolism inevitably enhances the interest in these studies.

Materials and methods

Material

The liver perfusion apparatus was built in the workshops of the University of Maringá. The glucose, enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were from the best available grade (98–99.8% purity) and purchased from Merck (Darmstadt, Germany), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

Liver perfusion

Male Wistar rats weighing 200–280 g were used in all experiments. They were fed a standard laboratory diet (Purina®), but food was withdrawn 24 h before the perfusion experiments. All experiments were conducted in strict adherence with the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá. For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). Haemoglobin-free, non-recirculating perfusion was done according to the technique described by Scholz and Bücher (1965). After cannulation of the portal and cava veins, the liver was positioned in a Plexiglas chamber. Flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted to between 30 and 35 ml min^{-1} , depending on the liver weight. The perfusion fluid was Krebs/Henseleit bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C . The composition of the Krebs/Henseleit bicarbonate buffer was as follows: 115 mM NaCl, 25 mM NaHCO_3 , 5.8 mM KCl, 1.2 mM Na_2SO_4 , 1.18 mM MgCl_2 , 1.2 mM NaH_2PO_4 and 2.5 mM CaCl_2 . Quercetin was dissolved in the perfusion fluid. Solubilization was achieved by the simultaneous addition of an equivalent amount of NaOH.

Samples of the effluent perfusion fluid were collected at 2-min intervals and analysed for their metabolite contents. Lactate and pyruvate were assayed by means of standard enzymatic procedures using L-lactate dehydrogenase (Czok and Lamprecht 1974, Gutman and Wahlefeld 1974). Interference by quercetin (absorbance at 340 nm) was excluded by performing control reactions in the presence or absence of quercetin. The oxygen concentration in the outflowing perfusate was monitored polarographically using a Teflon-shielded platinum electrode adequately positioned in a Plexiglas chamber at the exit of the perfusate. Quercetin interferes with the glucose oxidase reaction and, for this reason, glucose was measured colorimetrically by means of an *o*-toluidine method (Dubowski 1962). Lactate dehydrogenase was assayed in a medium containing 80 mM phosphate buffer (pH 7.4), $80 \mu\text{M}$ NADH and 0.5 mM pyruvate (Ishii and Bracht 1985). The decrease in absorbance at 340 nm due to NADH oxidation was measured during 5 min.

Hepatic glucose phosphorylation capacity (glucokinase)

One of the spectrophotometric methods described by Kuwajima *et al.* (1986) was used. Rats were decapitated and the liver was homogenized at 4°C in 5 vols 50 mM triethanolamine, 0.3 M sucrose, 1 mM EDTA, pH 7.2. After centrifugation for 1 h at 100 000g, the supernatants were used for the assays. The assay system was contained in a final volume of 1 ml: 100 mM Tris-HCl, pH 7.2, 5 mM ATP, 10 mM MgCl₂, 1 mM NAD⁺, 5 units glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and 20 µl liver extract. The increase in absorbance at 340 nm, resulting from the production of NADH (Kuwajima *et al.* 1986), was measured over 5–20 min. Rates were evaluated from the slope of the traces and expressed as nmol min⁻¹ mg protein⁻¹. Protein content was measured according to Lowry *et al.* (1955).

Data analysis and calculations

Metabolic rates were calculated from input–output differences and the total flow and were referred to the wet weight of the liver. Statistical analysis (Student's *t*-test) was done by means of the Primer program; *p* < 0.05 was adopted as a criterion of significance.

Results

Glycolysis was measured in livers from 24-h fasted rats using lactate and pyruvate productions as indicators, according to Kimmig *et al.* (1983). The glycogen content of livers from fasted rats was very low, so that interference by glycogen catabolism was minimal. Figure 1 shows the mean results obtained with four different quercetin concentrations. Glucose (20 mM) was infused after a short pre-perfusion of 10 min. After 30 min, the quercetin infusion was initiated and continued during the last 20 min. The production of lactate and pyruvate increased in response to glucose infusion and reached steady levels within the 10 min that preceded quercetin infusion. These steady-state levels were maintained for at least 1 h, as revealed by control experiments (data not shown). The addition of 50 µM quercetin did not produce significant changes, but higher concentrations produced changes in both lactate and pyruvate production. Lactate production gradually decreased after the onset of quercetin infusion. At 100 µM quercetin, the reduction was around 80% at the end of the infusion, and with higher quercetin concentrations lactate production was completely inhibited at the end of the infusion. Pyruvate production, on the other hand, tended to be increased by 100 and 300 µM quercetin, with somewhat complex kinetics. At 500 µM quercetin, pyruvate production was decreased initially, but at the end of the infusion it had already returned to the values found before quercetin infusion. Taken as a whole, the results in figure 1 reveal that quercetin inhibits glycolysis from exogenous glucose. Oxygen uptake, which was followed polarographically in all experiments (figure 1), was increased by 0.23 µmol min⁻¹ g⁻¹ with 100 µM quercetin. This increase was stable. Higher quercetin concentrations produced similar increments, but they were only transient.

The possibility that the decreases in lactate production due to quercetin could be caused by loss of cytosolic lactate dehydrogenase was investigated by measuring the activity of the latter enzyme in the outflowing perfusate. Lactate dehydrogenase release before quercetin infusion was equal to 0.030 ± 0.001 units min⁻¹ (g liver wet weight)⁻¹ and it increased to 0.037 units min⁻¹ g⁻¹ in the presence of 500 µM quercetin.

It has been reported that glycolysis from endogenous sources (glycogen) is also inhibited by quercetin, but to a somewhat smaller extent (Gasparin *et al.* 2003). Glycolysis from glycogen does not depend on the glucokinase reaction, but for glycolysis from exogenous glucose this is the first and, partly at least, the

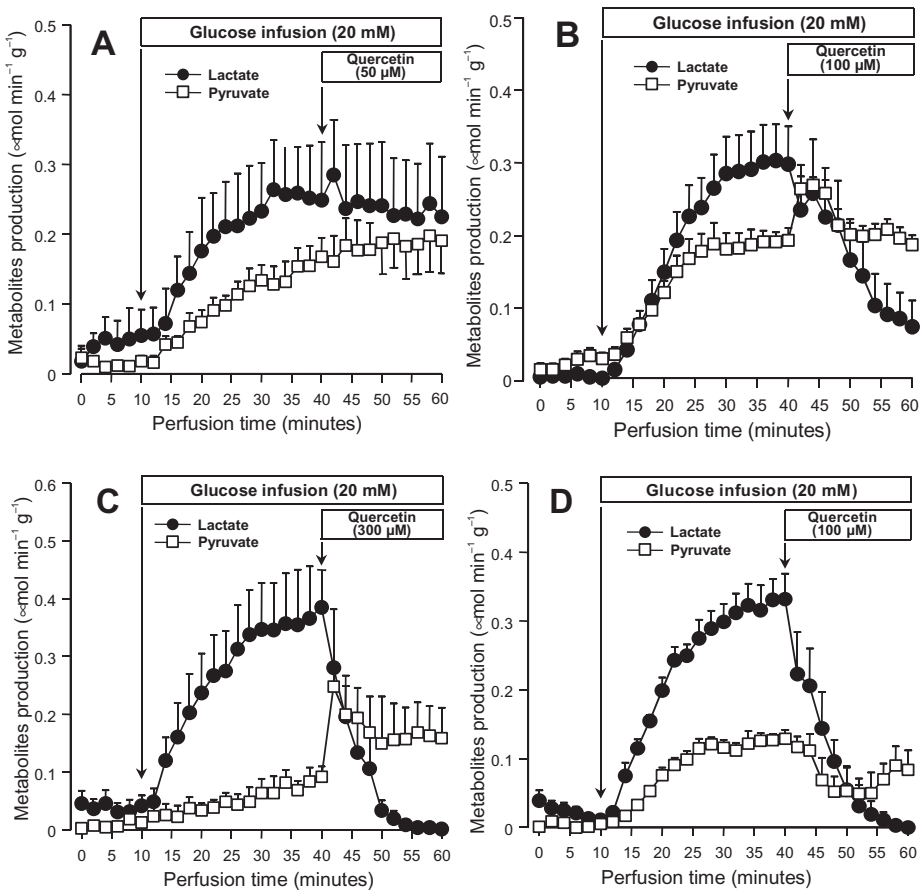


Figure 1. Rates of lactate and pyruvate production due to glucose and the influence of quercetin in the perfused rat liver. Livers from fasted rats were perfused as described in Materials and methods. Glucose infusion (20mM) was initiated at 10 min. Quercetin was infused at the indicated concentrations during the last 20 min. Data are the means of four to seven liver perfusion experiments. Vertical bars are mean standard errors.

rate-limiting step (Ku wajima *et al.* 1986). A possible action of quercetin on this step was investigated using high-speed supernatants of liver homogenates, as originally proposed by Ku wajima *et al.* (1986). In figure 2, initial rates of glucose phosphorylation were measured as a function of quercetin concentration in the range up to 250 μM. The indicator reaction of the assay system is catalysed by glucose 6-phosphate dehydrogenase, which reduces NAD⁺ to NADH, the latter being measured at 340 nm. Quercetin strongly absorbs at this wavelength and the maximal concentration that can be used is 250 μM. Above this concentration, linearity between NADH concentration and absorbance no longer exists (data not shown). Figure 2 reveals that quercetin inhibits glucose phosphorylation, but the effect was significant only at a concentration of 150 μM. At 250 μM quercetin, the degree of inhibition was around 62%.

Glucose production from lactate and pyruvate was investigated according to the protocol illustrated by figure 3, using livers from 24-h fasted rats. After a pre-perfusion period, 2 mM lactate and 0.2 mM pyruvate were infused. As expected from previous reports (Constantin *et al.* 1994, Marques da Silva *et al.* 1997), rapid

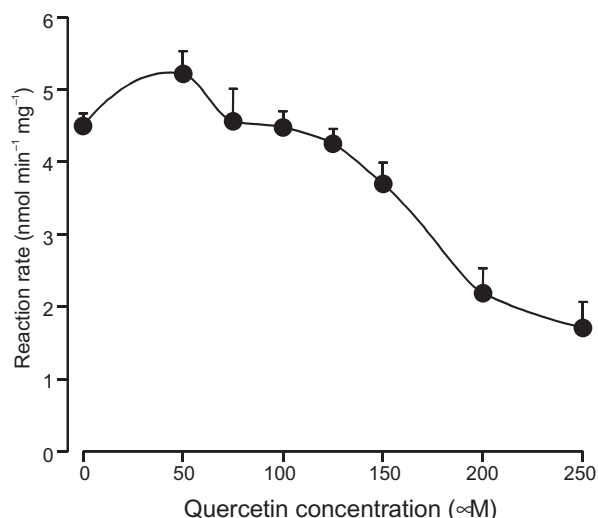


Figure 2. Effect of quercetin on the activity of glucokinase. Livers from fasted rats were homogenized and subjected to differential centrifugation as described in Materials and methods. The high-speed supernatant was used for the glucokinase assay. Initial rates were measured at various quercetin concentrations. The data points represent the means of five determinations. Bars are standard errors of the mean.

increases in both glucose production and oxygen uptake were observed. The addition of 200 μM quercetin produced decreases in both glucose production and oxygen uptake. Termination of quercetin infusion generated a tendency toward recovery, but was still incomplete at the end of the perfusion. The effects of 200 μM quercetin were quite pronounced: gluconeogenesis was extensively inhibited and the increase in oxygen uptake produced by lactate plus pyruvate infusion was almost abolished. Figure 4 allows an evaluation of the effects of quercetin concentrations in the range 50–500 μM . Inhibition of gluconeogenesis was already evident at 50 μM quercetin, but increased considerably when the concentration was raised to 100 μM . Oxygen uptake was actually increased by quercetin 50 μM , but all concentrations above this were inhibitory. Clearly, there is at least a partial correlation between the actions of quercetin on both oxygen uptake and gluconeogenesis.

Discussion

The results obtained confirm the central hypothesis raised above, namely that quercetin inhibits both glycolysis and gluconeogenesis in the liver. Glycolysis inhibition has already been reported when glycogen catabolism was measured (Gasparin *et al.* 2003). The glucosyl units came from endogenous glycogen and inhibition was not as pronounced as that found here, where the substrate was exogenously supplied glucose. The characteristics, however, were similar: essentially, no inhibition of pyruvate production, sometimes even stimulation, but a clear inhibition of lactate production, resulting in a substantial reduction in the lactate-to-pyruvate ratio.

Glycolysis inhibition may partly be due to inhibition of glucose phosphorylation (glucokinase). Inhibition of this enzymatic activity, however, cannot be the

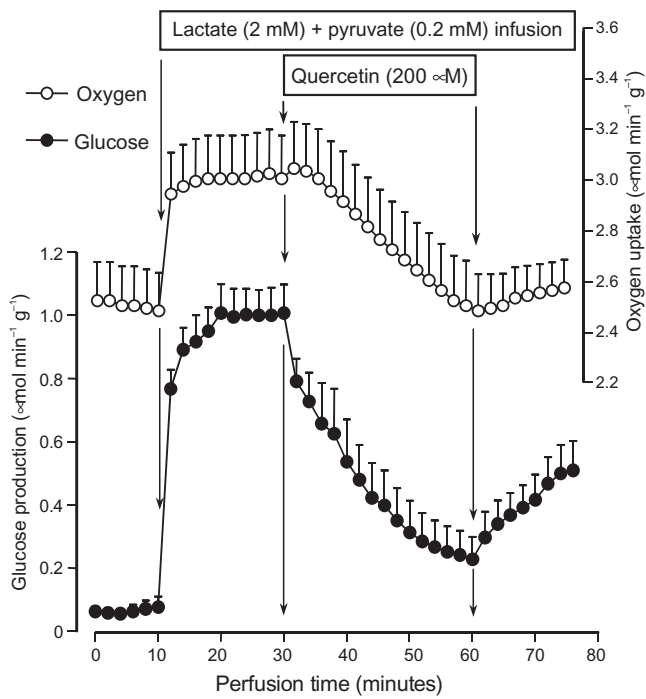


Figure 3. Time-course of the changes caused by 200 μM quercetin in glucose production and oxygen uptake in the perfused rat liver. Livers from fasted rats were perfused as described in Materials and methods. Lactate (2 mM) and pyruvate (0.2 mM) was simultaneously infused during 66 min (10–76 min of perfusion time). Quercetin was introduced at 30 min. Samples were taken for the measurement of glucose production by means of a colorimetric technique, as described in Materials and methods. Oxygen uptake was followed polarographically. The data are the means of five liver perfusion experiments. Vertical bars are mean standard errors.

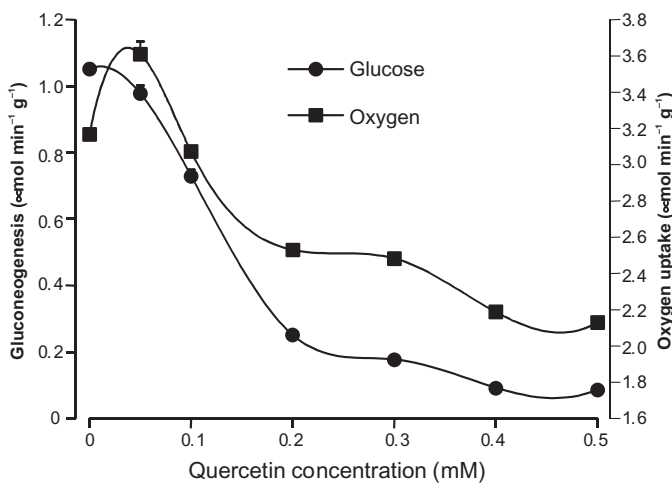


Figure 4. Effect of quercetin on oxygen uptake and glucose release: concentration–effect relationships. Changes in glucose release and oxygen uptake were calculated from experiments shown in figure 3. Each datum point is the mean of five to eight liver perfusion experiments. Error bars are standard errors of the mean.

only factor determining glycolysis inhibition because the phenomenon was significant only at concentrations above 150 μM . However, note that inhibition of glucokinase could be the factor that makes glycolysis from exogenous glucose more sensitive to quercetin in comparison with glycolysis from endogenous glycogen (Gasparin *et al.* 2003) because the latter does not depend on the phosphorylation reaction. The second factor in addition to glucokinase inhibition that probably contributes to glycolysis inhibition is most probably also present when glycolysis from endogenous glycogen occurs. This factor comprises the sum of limitations and alterations generated by inhibition of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (Gasparin *et al.* 2003). In tumour cells, inhibition of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ causes a substantial reduction in the ADP concentration (Suolinna *et al.* 1973, 1975), an event likely to limit glycolysis. This seems not to occur in the liver, however, because no clear reduction of cellular ADP was observed (Gasparin *et al.* 2003). Possibly, the ADP concentration does not decrease in liver cells because diminution of the efficiency of energy transduction in the mitochondria is a factor that operates in the opposite direction (Gasparin *et al.* 2003). Inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and other ATPases, however, can lead to substantial alterations in the concentration of several regulatory substances and ions, including K^+ , for example. For the latter, a reduction in its cytosolic concentration can be expected, a phenomenon which should lead to a reduction in pyruvate kinase activity, which is strictly dependent on K^+ (Ramirez-Silva *et al.* 2001). Furthermore, a direct inhibitory effect on pyruvate kinase seems equally possible (Grisolia *et al.* 1975). A reduction in pyruvate kinase activity, in turn, should also lead to a slowing down of glycolysis.

The fact that lactate production was substantially reduced by quercetin without a corresponding reduction in pyruvate production results in a reduction in the lactate to pyruvate ratio. The latter, in turn, yields an increase in the cytosolic $\text{NAD}^+\text{-to-NADH}$ ratio, because lactate dehydrogenase, the enzyme that interconverts lactate and pyruvate, is a near-equilibrium enzyme (Sies 1982). This effect has already been observed in livers from fed rats that were doing glycolysis at the expense of endogenous glycogen (Gasparin *et al.* 2003), but in the present experiments the effect of quercetin in reducing the lactate to pyruvate ratio was more pronounced. As shown by Gasparin *et al.* (2003), lactate dehydrogenase is not inhibited by quercetin. Furthermore, the action of quercetin on lactate dehydrogenase release in the perfused liver was very small (1.2-fold). Respiratory inhibitors like steviol can increase lactate production several fold (Kelmer-Bracht *et al.* 1985) in spite of a simultaneous 15-fold increment in lactate dehydrogenase release (Ishii and Bracht 1985). Therefore, lack of enzyme activity cannot be responsible for the reduced lactate-to-pyruvate ratio. The cause of this phenomenon cannot be inferred from the available data, but its consequences can be partly predicted. An increased cytosolic $\text{NAD}^+\text{-to-NADH}$ ratio, for example, is likely to exert a strong negative effect on gluconeogenesis, which is dependent on reducing power in order to occur, especially at the glyceraldehyde 3-phosphate dehydrogenase step. Limitation of gluconeogenesis by a decreased availability of reducing power could indeed be the main cause for the inhibitory action of quercetin, an interpretation that explains the different patterns of oxygen uptake in substrate-free perfused livers from fed rats (Gasparin *et al.* 2003) and in livers from fasted rats in which gluconeogenic substrates were added to the perfusion fluid. In the former condition, stimulation of oxygen uptake predominated in the range up to

400 μM (Gasparin *et al.* 2003) and is probably the consequence of an uncoupling action (Constantin *et al.* 1995, Lopez *et al.* 1998). In the latter condition, as already described, inhibition predominated and stimulation was substantially reduced and restricted to a quercetin concentration of 50 μM . Lactate and pyruvate infusion produced an increment in oxygen uptake. This increment is proportional to the extra ATP necessary for the transformation of these substrates into glucose. If this metabolic route is decreased as a consequence of an inhibition of some rate-limiting step, the energy demands are evidently equally reduced, i.e. oxygen uptake will be reduced. Consequently, it could be that the dependence of oxygen uptake on the quercetin concentration in the presence of gluconeogenic substrates results from the interplay of two different forces: the reduced energy demands due to gluconeogenesis inhibition by the reduced availability of reducing equivalents in the cytosol and a direct action on mitochondria. The former tends to inhibit, whereas the latter, at low concentrations at least, tends to increase oxygen uptake.

In conclusion, the results further corroborate the conclusions derived from Gasparin *et al.* (2003) that quercetin is a compound that can change liver metabolism in substantial and several ways. In this sense, the present study fully confirms previous investigations with other experimental systems (Suolinna *et al.* 1973, 1975, Murakami *et al.* 1992, Trejo *et al.* 1995, Santos *et al.* 1997). The quercetin concentrations employed in the current study were those used in most other studies. Such concentrations, however, are impossible to reach when quercetin is ingested in a normal diet (Peterson and Dwyer 1998). Consequently, effects like those reported here and in many other studies can only be expected under *in vivo* conditions if considerable high doses of quercetin are ingested or injected.

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Antioxidant and free radical scavenging activity of isoflavone metabolites

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Received 20 March 2003

1. Soy isoflavones have been extensively studied because of their possible health-promoting effects. Genistein and daidzein, the major isoflavone aglycones, have received most attention; however, they undergo extensive metabolism in the gut and liver, which might affect their biological properties.

2. The antioxidant activity, free radical-scavenging properties and selected cellular effects of the isoflavone metabolites equol, 8-hydroxydaidzein, O-desmethyllangiolensin, and 1,3,5 trihydroxybenzene were investigated in comparison with their parent aglycones, genistein and daidzein.

3. Electron spin resonance spectroscopy indicated that 8-hydroxydaidzein was the most potent scavenger of hydroxyl and superoxide anion radicals. Isoflavone metabolites also exhibited higher antioxidant activity than parent compounds in standard antioxidant (FRAP and TEAC) assays. However, for the suppression of nitric oxide production by activated macrophages, genistein showed the highest potency, followed by equol and daidzein.

4. The metabolism of isoflavones affects their free radical scavenging and antioxidant properties, and their cellular activity, but the effects are complex.

Introduction

Soy isoflavones have been extensively studied because of their potential beneficial effects in a number of disease processes (Bingham *et al.* 2003). Genistein and daidzein, the aglycones of the major soy isoflavones, have received considerably more attention than their metabolites, yet metabolism might be the key to understanding the beneficial effects of isoflavones since it is the metabolites, rather than the parent compounds, to which cells are predominantly exposed (Hendrich 2002). Isoflavones are ingested mainly in the conjugated form and undergo extensive hydrolysis by intestinal (jejunum) and bacterial (distal gut) β -glucosidases that release the principal aglycones, genistein and daidzein (Hur *et al.* 2002, Setchell *et al.* 2002). The aglycones can be absorbed directly from the small intestine by passive diffusion or can first undergo further biotransformation to a range of metabolites. Genistein is transformed to dihydrogenistein, which is

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further metabolized to 6'-hydroxy-*O*-desmethylangolensin. Daidzein is metabolized to dihydrodaidzein and then transformed to both *O*-demethylangolensin (*O*-DMA) and equol (Joannou *et al.* 1995, Setchell *et al.* 1998). The formation of equol from daidzein is thought to be a particularly significant step, as the oestrogenic potency of equol is substantially higher than its precursor (Shutt and Cox 1972, Setchell 1998).

Specific enzymes produced by a limited number of as yet largely unknown bacterial species in the colon appear to account for the above biotransformations (Bingham *et al.* 2003). Lampe *et al.* (2001) reported that faecal inoculums from equol-producing individuals caused a significant conversion of daidzein to equol and dihydrodaidzein *in vitro*. Antibiotics could inhibit equol and dihydrodaidzein production, clearly indicating the significance of the gut microflora. Coldham *et al.* (2002) reported the intermediate products of genistein incubated with faeces as dihydrogenistein and *O*-DMA, and the end products as 2-(4-hydroxyphenyl)-propionic acid and 1,3,5-trihydroxybenzene (1,3,5-THB).

Biotransformations occur in the liver as well as in the gut. The oxidative metabolism of genistein and daidzein was recently investigated in rat liver microsomes by Kulling *et al.* (2000). Both genistein and daidzein were extensively metabolized to monohydroxylated and dihydroxylated products by cytochrome P450-dependent pathways. A major metabolite of daidzein was identified as 8-hydroxydaidzein (8-OH-daidzein). In both isoflavones, the additional hydroxyl groups were exclusively introduced into the *ortho* position of the existing phenolic hydroxyl groups.

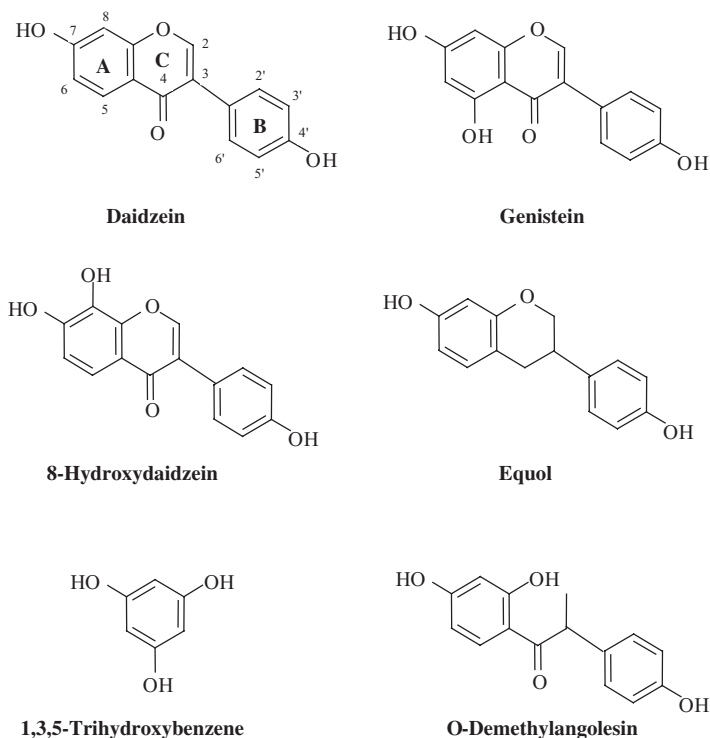


Figure 1. Structure of the isoflavone test compounds. (Adapted from Bingham *et al.* 2003.)

Gut and liver isoflavone metabolites are found in plasma and urine after soy consumption (Kulling *et al.* 2001). However, data about their chemical and biological properties are scarce. Although it is believed that many of the beneficial effects of isoflavones might be related, at least partially, to their antioxidant properties (Guo *et al.* 2002), there has been no comprehensive evaluation of the free-radical scavenging properties of isoflavone metabolites towards reactive oxygen species. Furthermore, little is known about the cellular activity of isoflavone metabolites. The aims of the present study were twofold: (1) to analyse the free-radical scavenging and antioxidant properties of isoflavone metabolites, compared with their parent isoflavones, using electron spin resonance spectroscopy (ESR), as well as the ferric reducing ability of plasma (FRAP) and trolox equivalent antioxidant capacity (TEAC) assays; and (2) to determine if isoflavones affect the production of nitric oxide (NO) by activated RAW 264.7 macrophages. This murine cell line was chosen since it was an established model for examining determinants of NO production and other inflammatory processes (Guo *et al.* 2001).

Materials and methods

Chemicals and reagents

Potassium persulfate and neutral red were obtained from Fisher Scientific (Loughborough, UK). Daidzein, genistein, 1,3,5 THB, equol, 8-hydroxydaidzein and O-DMA were obtained from Plantech (Reading, UK). Purities of the isoflavone test components were 97–98%. 5,5-Dimethyl-1-pyrroline-N-oxide was purchased from Labotec Ltd (Japan) and used without further purification. Hydrogen peroxide (H_2O_2 , 31% w/v) was purchased from Mitasubishi Gas (Japan). Ferrous chloride, diethylenetriaminepentaacetic acid (DTPA) and hypoxanthine were purchased from Kanto Chemicals (Japan). Xanthine oxidase from buttermilk was purchased from Nacalai Tesque (Japan). All other chemical reagents used for ESR studies were purchased from Wako Chemicals (Japan). Rabbit polyclonal anti-iNOS type II was purchased from BD Transduction Laboratories (San Diego, CA, USA). All other chemicals were obtained from Sigma-Aldrich (Poole, UK).

Electron spin resonance spectroscopy

Hydroxyl and superoxide anion radicals were detected by electron spin resonance spectroscopy (ESR). ESR experiments were carried out on a JEOL JES-TE 200 ESR spectrometer (X-Band microwave unit). A total of 50 μl of the reaction mixtures described below were introduced into a disposal capillary tube, and ESR spectra were recorded 3 min after starting the reaction. All ESR experiments were done in triplicate at room temperature.

Assay for hydroxyl radical in the Fenton system

For the ESR spin trapping study, a Fenton reaction was carried out in 100 μl aqueous solution in a micro test tube (1 ml) containing 10 μl 5 mM H_2O_2 , 10 μl 100 mM DMPO, 20 μl re-distilled water, 25 μl 50 mM phosphate buffer, 25 μl 0.4–4.0 mM of the test compounds (final concentration 0.1–1 mM) and 10 μl 0.5 mM freshly prepared ferrous chloride solution. Metal free water was added instead of the test compounds for the control experiment. The Fenton reaction was initiated by adding an aliquot of FeCl_2 solution finally. After stirring for 5 s, 50 μl of the reaction mixture were transferred into a 100- μl disposal capillary tube. The bottom of the capillary was sealed by a small amount of clay and ESR spectra were measured 3 min after the addition of FeCl_2 . ESR spectrometer settings were: central field 327.5 ± 5 mT, microwave frequency 9.18 GHz; modulation amplitude 0.1 mT; microwave power 8 mW; time constant 0.1 s, gain 8.0×10^5 , sweep time 2 min, scan width 100 G.

Assay for superoxide radical generated in the xanthine/xanthine oxidase system

For the ESR spin-trapping study, superoxide scavenging activity of antioxidants was carried out in 300 μl aqueous solution in a micro test tube (3 ml), containing 80 μl 2 mM hypoxanthine solution in 50 mM phosphate buffer solution, 10 μl 10.5 M DMPO, 80 μl 0.4 U ml^{-1} xanthine oxidase solution in 50 mM phosphate buffer, 100 μl 0.3–3.0 mM antioxidants (final concentration 0.1–1 mM) and 30 μl

10 mM DTPA solution. The reaction was initiated by adding an xanthine oxidase solution finally. After stirring for 5 s, 50 μ l of the reaction mixture were transferred into a 100- μ l disposal capillary tube. ESR spectra were measured 3 min after the addition of xanthine oxidase solution. ESR spectrometer settings were same as for the hydroxyl radical assay except gain, which was 5.0×10^5 .

Ferric reducing ability of plasma (FRAP)

The FRAP assay was performed as described by Benzie and Strain (1996). FRAP reagent was freshly prepared each day by mixing together 10 mM 2,4,6-tripridyl-s-triazine (TPTZ) and 20 mM iron(III) chloride in 0.25 M acetate buffer, pH 3.6. The absorbance of the test components as compared with ascorbic acid was read at 593 nm (Perkin Elmer UV/Vis Lambda Bio 20) 6 min after incubation at room temperature against a blank of FRAP reagent and distilled water. Quercetin dihydrate, dissolved in ethanol was used as a positive control for the TEAC and FRAP assay.

Trolox equivalent antioxidant capacity (TEAC)

Sixteen hours before the assay being performed, the ABTS radical was prepared by adding 5 ml 4.9 mM potassium persulfate solution to 5 ml 14 mM ABTS solution (Re *et al.* 1999). This solution was diluted in distilled water to yield an absorbance of 0.70 at 734 nm (Perkin Elmer UV/Vis Lambda Bio 20). The final reaction mixture contained 10 μ l standard or test compound in 1 ml ABTS solution. The samples were vortexed for 10 s, and 6 min after addition the absorbance was recorded and compared with ABTS radical solution plus distilled water. A standard curve was obtained by using Trolox as an internal standard (range 0–100 μ M). TEAC values expressed the μ mol of Trolox having the antioxidant capacity corresponding to 1.0 μ mol of the test substance (Betancor-Fernandez *et al.* 2002).

Cell culture

RAW 264.7 cells, a murine cell line of monocyte macrophages (obtained from the European Collection of Cell Culture (ECACC), Salisbury, UK), were maintained at 37°C in 5% CO₂ according to standard protocols (Rimbach *et al.* 2000a, b). The medium consisted of DMEM containing 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin. Cells were stimulated with 500 ng ml⁻¹ lipopolysaccharide (LPS) and 100 U ml⁻¹ interferon gamma (IFN- γ). For all experiments, isoflavones were dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C until required. When added to medium, the final DMSO concentration was $\leq 0.1\%$ (v/v).

Cell viability

The uptake of the neutral red dye was used to measure cell viability as described by Valacchi *et al.* (2001). Briefly, after treatment of cells with isoflavones, the culture medium was replaced with fresh medium containing 60 μ g ml⁻¹ neutral red. Following incubation for 3 h at 37°C, the medium was removed and the cells extracted using a solution comprising 50 : 49 : 1 (v/v/v) ethanol, water and glacial acetic acid. Absorbance at 540 nm was recorded using a microplate reader.

NO production

NO production was assessed by measurement of nitrite (NO₂⁻) concentration in the medium using the Griess reaction. Supernatants from cultured macrophages were deproteinized with 0.3 M NaOH and 0.3 M ZnSO₄. Equal volumes of Griess reagent (1% sulphanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄) and deproteinized samples were incubated for 10 min at room temperature in the dark. Nitrite concentration was determined from absorbance at 548 nm using a standard curve for sodium nitrite (Park *et al.* 2000).

Western blotting

Protein from 10⁶ RAW 264.7 cells was extracted in ice-cold lysis buffer (7 M urea, 2 M thio-urea, 1% dithiothreitol (DTT), 2% 3-[(3-deoxycholic acid (cholamido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.8% pharmlayte (pH 3–10) and protease inhibitor cocktail from Sigma-Aldrich (according to the manufacturer's instructions). The supernatant was used for protein determination using the Bradford assay. Solubilized extracts (70 μ g) were electrophoresed on 4–20% Tris-glycine SDS gel and transferred electrophoretically to nitrocellulose membranes. After blocking for 2 h with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated overnight at 4°C with rabbit polyclonal anti-iNOS type II antibodies (1 : 10000) in 1% non-fat dried milk in TBS-T. The membranes were washed, incubated for 2 h with peroxidase-labelled secondary antibodies (1 : 3000). Bands were visualized by an Amplified Opti-4CN Substrate Kit from Bio-Rad (UK).

Statistical analyses

Data are presented as mean $\pm$ standard error of the mean (SEM) of between three and five independent experiments performed in triplicate. The data were checked for normality and log transformed where appropriate. Statistical analyses were carried out using ANOVA and a Student's *t*-test, and the Kruskal–Wallis and Mann–Whitney *U*-test (SPSS for Windows version 10.0). Differences were considered significant if $p < 0.05$.

Results

Electron spin resonance spectroscopy and spin trapping were used to measure scavenging of hydroxyl and superoxide anion radicals by the test components. In the model Fenton system containing H_2O_2 and Fe^{2+} , the typical ESR signal of the $\text{DMPO}/\bullet\text{OH}$ adduct, consisting of a 1 : 2 : 2 : 1 quartet with splitting constants of $a\text{N} = a\text{N} = 14.9$ G, was observed in the presence of DMPO (figure 2A). 1,3,5-THB

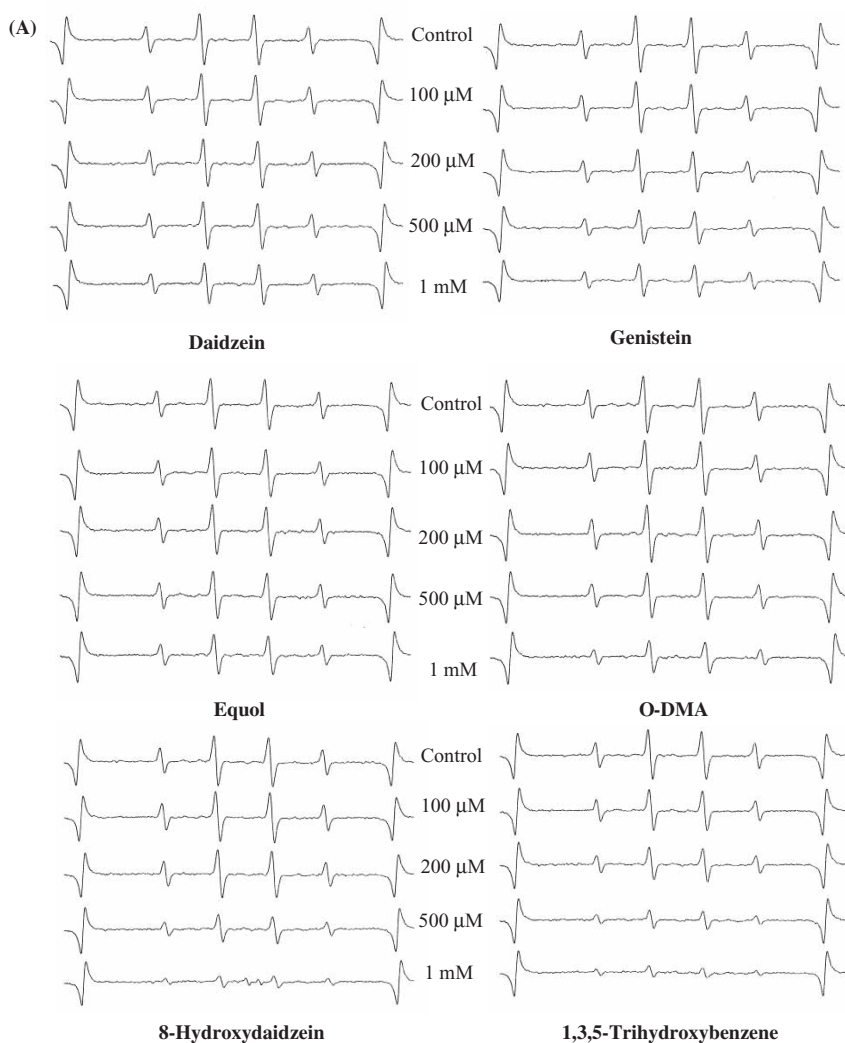


Figure 2. Hydroxyl (A) and superoxide anion (B) scavenging activity of isoflavone test components. ESR spectra of one of three independent experiments, performed in triplicate, are shown.

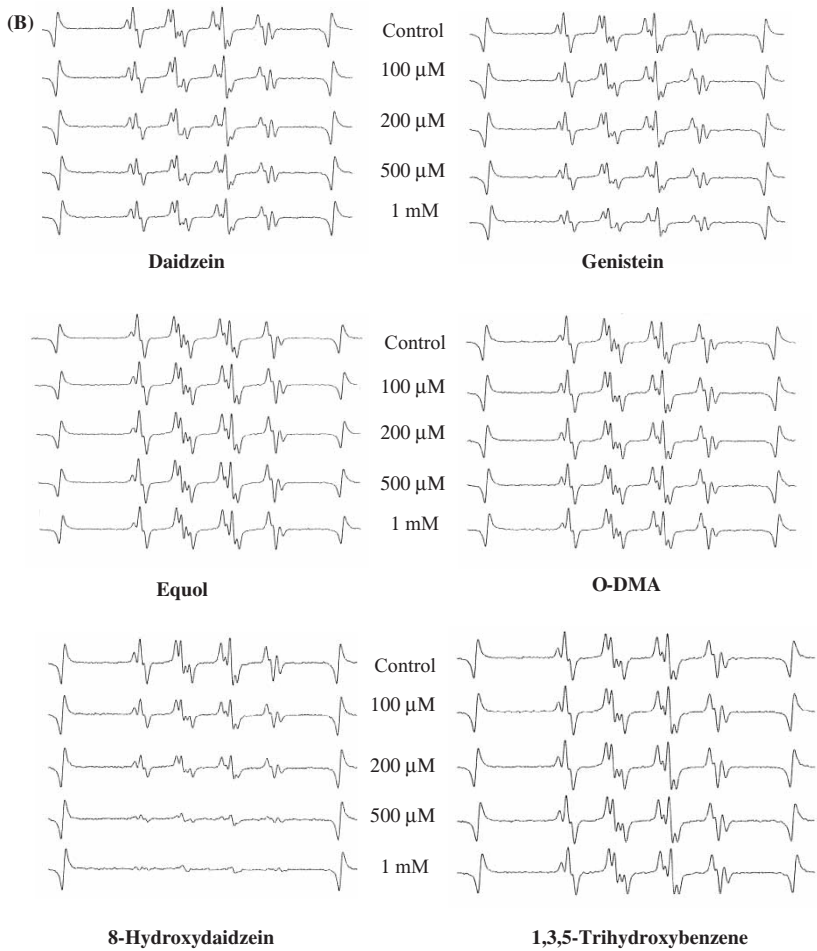


Figure 2. Continued.

and 8-OH-daidzein at 1 mM concentrations caused almost a complete quenching of the ESR signal. O-DMA, equol, as well genistein and daidzein, were less potent than 1,3,5 THB and 8-OH-daidzein in scavenging hydroxyl radicals.

A typical ESR spectrum of DMPO/•OOH is given in figure 2B. 8-OH-daidzein dose-dependently scavenged superoxide anion radicals generated by the xanthine/xanthine oxidase reaction. However, no superoxide anion radical-scavenging activity was observed for any of the other test components.

The TEAC assay measures the ability of an antioxidant to scavenge the ABTS free radical. The results were compared with scavenging by Trolox, giving a Trolox equivalence value. O-DMA, 1,3,5-THB and equol were the most potent scavengers of the ABTS radical, followed by 8-OH-daidzein (figure 3A), having approximately twice the antioxidant capacity of Trolox, daidzein and genistein. The TEAC value of the positive control, quercetin, was twofold higher than that of the isoflavone metabolites.

FRAP values indicate the ability of the isoflavones to reduce Fe^{3+} to Fe^{2+} . As summarized in figure 3B, the gut metabolites had higher FRAP values than the

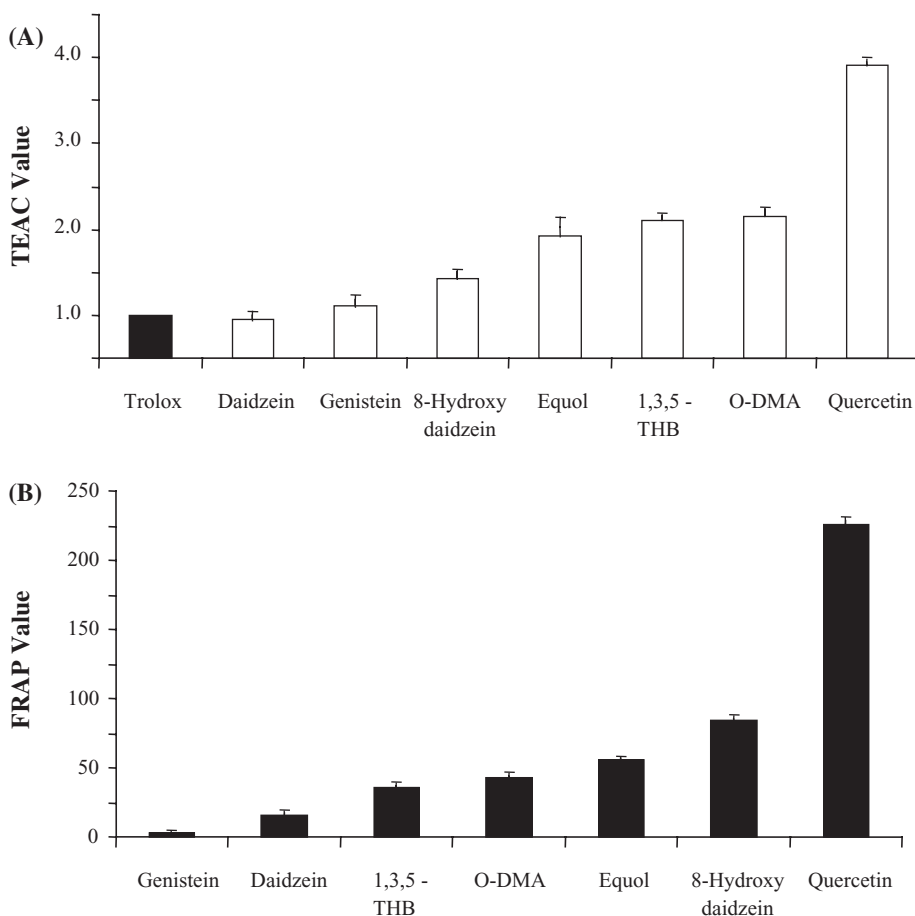


Figure 3. TEAC (A) and FRAP (B) values for the isoflavone test components. Data are the mean $\pm$ SEM of three independent experiments performed in triplicate.

parent isoflavones. 8-OH-daidzein showed the highest potency, followed by equol, O-DMA and 1,3,5-THB. The FRAP value of 8-OH-daidzein was about 40% of that for ascorbic acid, and about five times greater than its parent isoflavone, daidzein. The FRAP values of equol, O-DMA and 1,3,5-THB were between two and three times larger than their parent isoflavones.

Pretreatment of RAW 264.7 macrophages with up to 100 μ M genistein, daidzein and 1,3,5-THB did not affect cell viability (figure 4A). However, cell viability was significantly affected by equol, O-DMA and 8-OH-daidzein at concentrations of 75 and 100 μ M, respectively.

Twenty-four hours after treatment with IFN- γ plus LPS, the NO $_2^-$ concentration in the culture medium increased from 5 to about 100 μ M/10 ^{6} cells. Pretreatment of RAW 264.7 with genistein, daidzein and equol for 24 h was associated with a concentration-dependent decrease in nitrite production (figure 4B). Genistein showed the highest potency followed by equol and daidzein. The isoflavone metabolites 1,3,5-THB, 8-OH-daidzein and O-DMA did not affect NO production in activated macrophages at concentrations below that at which cytotoxicity was observed.

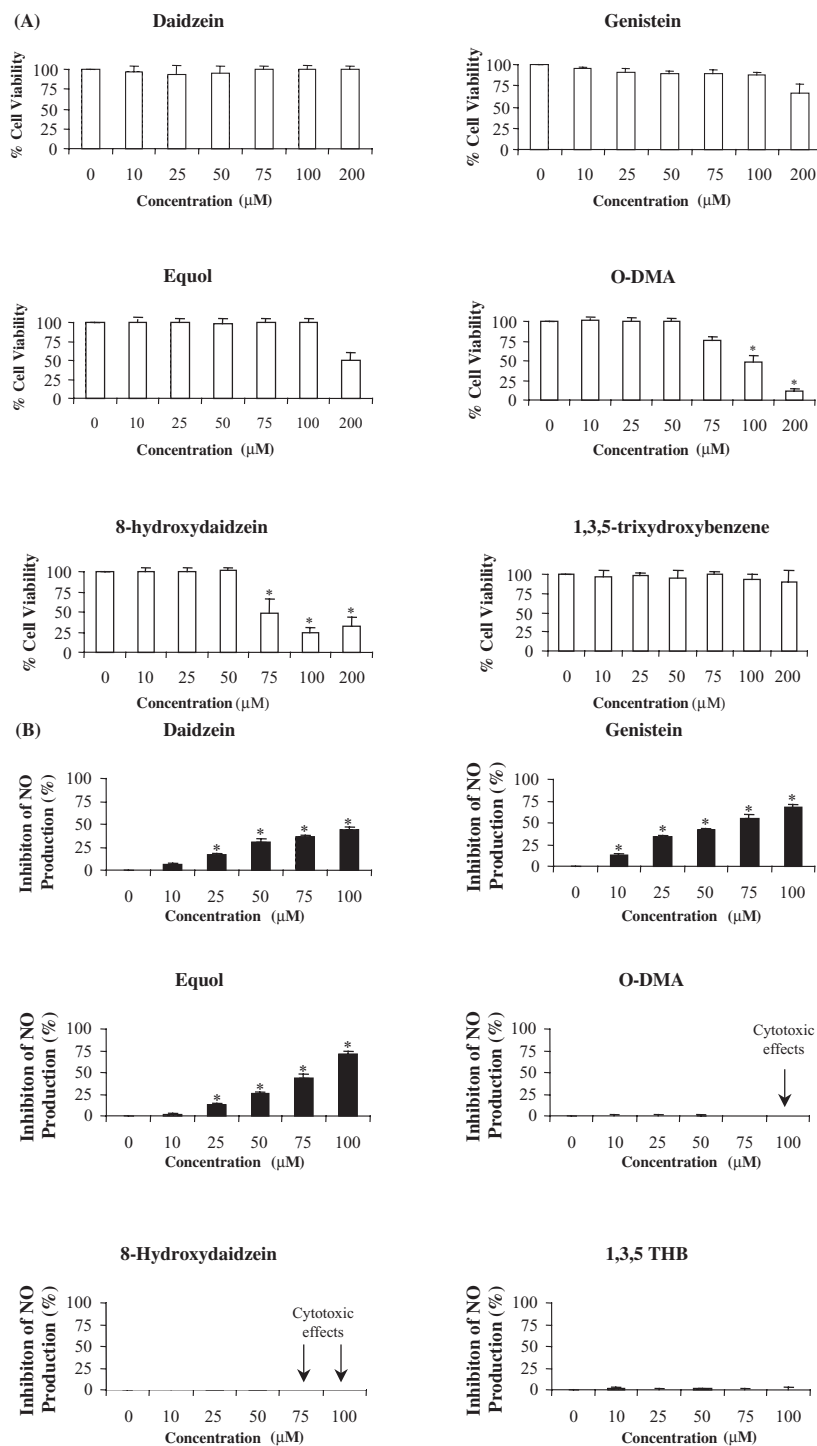


Figure 4. Effect of isoflavones and their metabolites on cell viability (A) and nitric oxide production (B) in RAW 264.7 macrophages activated with IFN- γ and LPS. Data are the mean $\pm$ SEM of five independent experiments performed in triplicate. Data were compared with activated controls that were not treated with isoflavones (100% reference). *Significant differences ($p < 0.05$).

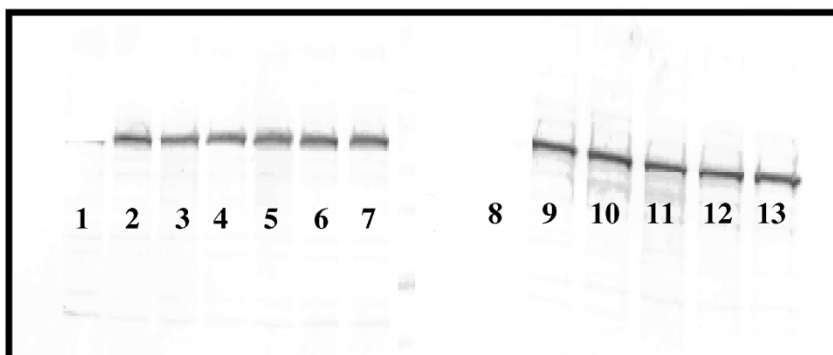


Figure 5. Effect of isoflavones and their metabolites on iNOS protein levels in RAW 264.7 macrophages. Non-activated macrophages (lanes 1 and 9), macrophages activated with IFN- γ plus LPS (2), macrophages activated with IFN- γ plus LPS and pretreated for 24 h with 50 μ M genistein (3), 100 μ M genistein (4), 50 μ M daidzein (5), 100 μ M daidzein (6), 50 μ M equol (10) 50 μ M O-DMA (11), 50 μ M 8-OH daidzein (12) and 50 μ M 1,3,5 THB (13). The same amount of protein (70 μ g) was added in all lanes.

As shown in figure 5, non-activated RAW 264.7 did not express detectable levels of iNOS protein (lanes 1–8). However, treatment with IFN- γ plus LPS induced a substantial increase in iNOS protein expression (lanes 2 and 9). Under these experimental conditions, neither the isoflavone metabolites (lanes 10–13) nor their parent compounds (lanes 3–7) had an effect on iNOS protein levels in the activated macrophages.

Discussion

Soy isoflavones are thought to play a role in the prevention of coronary heart disease (Tikkanen and Adlercreutz 2000) and breast, prostate and colon cancer (Sarkar and Li 2002), but the mechanisms by which they and their metabolites mediate these beneficial effects have not been fully established. Antioxidant properties are an obvious candidate. However, although antioxidant parameters, such as oxidative damage to lipids, proteins and DNA have been investigated in several studies (Ruiz-Larrea *et al.* 1997, Harper *et al.* 1999), the direct scavenging activity of reactive oxygen species by isoflavone metabolites is largely unknown. Furthermore, little is known about the extent to which gut and liver metabolism of isoflavones affects their chemical and biological properties. The antioxidant, free radical scavenging and cellular properties of the aglycones, genistein and daidzein were investigated here, as well as their major gut and liver metabolites.

ESR studies indicated that 8-OH-daidzein was the most potent scavenger of hydroxyl and superoxide anion radicals; its parent isoflavone, daidzein, was much less potent. 8-OH-daidzein also exhibited higher antioxidant activity than daidzein in the FRAP and TEAC assays. Thus, the introduction of a hydroxyl group at the C8 position significantly increases the antioxidant activity of daidzein. The FRAP and TEAC assays also showed that genistein was more potent than daidzein, indicating that the loss of the hydroxyl group in the C5 position diminishes the ability of isoflavones to reduce Fe^{3+} to Fe^{2+} and to scavenge the ABTS radical. Equol and O-DMA exhibited higher potencies than

their parent isoflavones, suggesting that the absence of the 2,3-double bond enhances the antioxidant activity of these compounds. Since equol exhibited slightly higher FRAP and TEAC values than O-DMA, it seems likely that the loss of the 4-oxo group might also enhance the antioxidant activity of isoflavones.

1,3,5-THB has recently been reported as a novel gut metabolite of genistein (Coldham *et al.* 2002), but its antioxidant activity has not been investigated. The ESR data indicated that it scavenges superoxide anion radicals with a potency comparable with that of 8-OH-daidsen. 1,3,5-THB has a similar chemical structure to pyrogallol. The pyrogallol moiety is known to be an active component of flavonoids displaying high superoxide anion radical scavenging activity (Furuno *et al.* 2002).

All the isoflavones tested exhibited a lower antioxidant activity than the flavonol quercetin, which has been demonstrated by ESR and spin-trapping techniques to be an efficient scavenger of hydroxyl and superoxide anion radicals (Guo *et al.* 2001). The high antioxidant activity of quercetin relates to its O-dihydroxy structure in the B-ring and the unsaturated C-ring with its 3-hydroxyl group, linking with the 5,7-dihydroxy structure in the A-ring (Rice-Evans *et al.* 1995).

To determine whether the cellular activity of isoflavones can be related to their antioxidant properties, their influence on NO production by murine macrophages that had previously been stimulated IFN- γ plus LPS was investigated. This particular model was chosen because it had been used in previous studies that had demonstrated an influence of the parent aglycones (Gottstein *et al.* 2003). Furthermore, the model has intrinsic importance since overproduction of NO has been associated with oxidative stress and chronic inflammation (Moncada *et al.* 1991, Beckman and Koppenol 1996) and may play a key role in a number of diseases, particularly atherosclerosis. Genistein, daidsen and equol significantly and concentration-dependently inhibited NO production induced by IFN- γ plus LPS. Although this result implies that genistein, daidsen and equol might have anti-inflammatory properties, the concentrations used in the cell culture experiments were higher than those physiologically achievable in plasma (Adlercreutz *et al.* 1993). The isoflavone metabolites O-DMA, 8-OH-daidsen and 1,3,5-THB did not suppress NO production despite their higher antioxidant activity. These findings suggest that the suppression of NO production by genistein, daidsen and equol was independent of their intrinsic antioxidant activity. Alternatively, the lack of activity of O-DMA, 8-OH-daidsen and 1,3,5-THB might be related to a different cellular uptake, which is currently under investigation.

Under the conditions investigated, the iNOS protein levels determined by Western blotting remained unchanged by the isoflavones, suggesting that the inhibitory effect of genistein, daidsen and equol occurs at a post-transcriptional level. In contrast to our findings, Sheu *et al.* (2001) reported an inhibitory effect of genistein and daidsen on LPS-induced expression of the iNOS gene in macrophages. However, their co-incubation of LPS and isoflavones might have given rise to this effect since polyphenols suppress LPS activity (Azumi *et al.* 1997); inhibition of iNOS expression might have been caused by a direct interaction of these compounds with LPS rather than by a direct effect on the cell. In the present investigation, macrophages were pre-incubated with isoflavones for 24 h and were

then washed twice with PBS before the addition of IFN- γ and LPS in order to avoid any direct chemical interaction.

In conclusion, the current data indicated that the metabolism of daidzein and genistein to equol, 8-OH-daidzein, O-DMA and 1,3,5-TBH enhanced their antioxidant properties. However, the increased antioxidant activity of isoflavone metabolites was not accompanied by an increased suppression of NO production in activated macrophages, indicating that isoflavones might inhibit NO production via an antioxidant-independent mechanism. However, it is important to note that antioxidant influences of isoflavones are not exclusively mediated by their intrinsic free radical-scavenging properties. Previous work has indicated that isoflavones might also act by increasing the activity of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase (Wei *et al.* 1995). Furthermore, it has been shown that isoflavones at physiological achievable concentrations induce cellular glutathione, which might be important for their action in biological systems (Guo *et al.* 2001). The present investigations have been carried out *in vitro*; they might have more direct relevance than other *in vitro* experiments to dietary intervention studies examining possible antioxidant and anti-inflammatory effects, since cells are exposed to isoflavone metabolites rather than the aglycones in such studies. However, the circulating forms are often glucuronidated and sulphated on the 7 and 4' position, which removes their hydroxyl groups and is likely to affect their antioxidant and cellular activities (Mitchell *et al.* 1998). The properties of such compounds are currently under investigation.

Acknowledgements

G. R. was supported by a grant from the BBSRC. The PhD studentship of B. A. E. was supported by grants from the Gen Foundation (UK Registered Charity No. 1071026) and Unilever R&D, Colworth, UK.

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Expression of drug-metabolizing enzymes, nuclear transcription factors and ABC transporters in Caco-2 cells

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Received 17 February 2003

1. Caco-2 cells are frequently used in intestinal drug absorption and metabolism studies, but little is known about the effects of drugs on the simultaneous expression of genes coding for drug-metabolizing enzymes (DMEs), nuclear transcription factors and ABC transporters.

2. The gene expression and enzyme activities of control and Aroclor 1254-treated cultures were therefore explored, the latter being a powerful inducer of DMEs. Fourteen- and 80-fold induction of *CYP1A1* and *CYP1A2* mRNA were shown, whereas expression of other DMEs was either increased (*CYP2C8-2C19*, 10-fold; *CYP3A5*, twofold; *FMO1*, 2 and 5, twofold; epoxide hydrolase, threefold) or repressed (*CYP2D6* and *CYP2E1* to 75% of control values).

3. Notably, gene copies of *CYP3A4* and *CYP2B6/7* were below the limit of detection, but a three- and 10-fold induction of HNF 1 α + β , HNF-4 α 4 and a similar 10-fold increase in STAT 3 and 4 was observed.

4. Similarly, c/EBP transcripts were only detected in treated cell cultures, but MRP1, its isoforms 3–5 as well as MDR-1 were increased threefold after dosing with Aroclor 1254.

5. Overall, CYP gene expression correlated well with the cognate enzyme activity using testosterone as a marker substrate.

Introduction

The Caco-2 cell line is widely used in intestinal drug absorption studies (Artursson 1990, Hilgers *et al.* 1990, Ranaldi *et al.* 1992, Rubas *et al.* 1996) and although derived from a human colon adenocarcinoma, the cells spontaneously differentiate into monolayers of polarized enterocytes under standardized cell culture conditions (Hidalgo *et al.* 1989). The cells exhibit well-developed microvilli, junctional complexes and a polarized expression of several brush border hydrolases. Many active transport systems located in small intestinal enterocytes are also present in Caco-2 cells (Hidalgo and Borchardt 1990, Riley *et al.* 1991, Hu and Borchardt 1992, Ma *et al.* 1994, Mesonero *et al.* 1995) and certain drug-metabolizing enzymes (DMEs), including CYP (cytochrome P450) monooxygenases and phase II enzymes, i.e. glucuronosyl transferases and sulfotransferases, are also expressed (Baranczyk-Kuzma *et al.* 1991, Bjorge *et al.* 1991, Boulenc *et al.* 1992, Rosenberg and Leff 1993). Despite their frequent use in drug transport and metabolism studies, the mRNA expression of an array of DMEs, nuclear transcription factors and ABC transporters has not been

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extensively studied. Furthermore, certain drugs and other xenobiotics can alter mRNA and protein expression of the above-named genes, but once again little is known about these events in cultured Caco-2 cells. This information, however, is of importance for use of these cells in experimental pharmacology and thus knowledge of its ability to respond to treatment with a wide range of chemicals and drugs is desirable.

The mRNA expressions of DMEs, hepatic nuclear transcription factors (HNFs) and ABC transporters were studied in cultures of Caco-2 cells and after treatment with Aroclor 1254. This mixture of polychlorinated biphenyls is well known for its ability to induce CYP monooxygenases and liver enriched transcription factors (Borlak and Thum 2001). Total RNA extracts of human liver were used as a positive control for the abundant expression of CYP monooxygenases and liver-enriched transcription factors, although no direct comparison is suggested, as the Caco-2 cell line is derived from a colon adenocarcinoma. Gene expression of the DMEs was also correlated with enzymatic activity using testosterone as a substrate. Overall, we aimed to typify further the Caco-2 cell line for its better use in experimental and molecular pharmacology investigations and to reduce the variability of results across laboratories based on poor characterization of the cell line.

Materials and methods

Human liver

Ethical approval was obtained from the Ethics Committee of the Medical School of Hannover, Germany. Liver tissue was used from male donors, aged 58, 63 and 67 years, with secondary liver due to colorectal carcinoma. Macroscopic healthy tissue was pooled and further processed for RNA isolation, as described below.

Cell culture

The Caco-2 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Caco-2 cells were grown in 25-cm tissue culture flasks (Costar, Bodenheim, Germany) at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity. Cells were passaged every 5–6 days at a split ratio of 1:4 using a trypsin/EDTA solution (0.25% trypsin, 0.2% EDTA). Cells developed well and were free of contaminating cells. After the fifth passage, cells were used for experimentation.

Culture medium

Dulbecco's modified Eagle's medium (DMEM, 4.5 g l⁻¹ glucose) was purchased from Gibco-BRL (Eggenstein, Germany). The culture medium was supplemented with 10% foetal calf serum (Cytogen, Berlin, Germany), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (all Gibco) and changed every second day.

Experimental design

For each experimental group and for each time point, three independent flasks were used and studied separately. Cells were treated with 10 µM Aroclor 1254 and harvested after 24 or 72 h of treatment. Four hours before harvest, 100 µM testosterone and 25 µM finasteride (see below) were added to each culture flask. The medium was collected for testosterone assay whereas cells were frozen at -80°C to await further analysis.

RNA and cDNA

Total RNA was isolated from Caco-2 cells and a pool of human liver donors using the NucleoSpin RNA kit from Macherey & Nagel (Düren, Germany), according to the manufacturer's recommendation. The integrity of isolated RNA was verified by staining of 18S and 28S ribosomal bands with ethidium bromide and separation on a 1.0% agarose gel.

A total of 4 µg total RNA was used for reverse transcription. RNA and random primer (Promega, Mannheim, Germany) were preheated for 10 min at 70°C and then chilled on ice for 2 min. A total of 5 × RT-avian myoblastosis virus (AMV) buffer (Promega), dNTP's (10 mM), RNAsin, AMV-RT (avian myeloblastosis virus-reverse transcriptase) (all Promega) and DEPC-H₂O were added to a final volume of 20 µl. Reverse transcription was carried out for 60 min at 42°C and was stopped by heating to 95°C for 5 min. The resulting cDNA was diluted with 60 µl DEPC and frozen at -20°C to await further analysis.

Semiquantitative thermocycler RT-PCR

Primer design was done with the program Primer 3 (<http://www.genome.wi.unit.edu/cgi-bin/primer/prime3.cgi>). Cross-reaction of primers with the genes was excluded by comparison of the sequence of interest with a databank (Blast 2.0, US National Centre for Biotechnology Information).

As shown in table 1, polymerase chain reaction (PCR) reactions for transcription factor and transporter genes were undertaken with a 20-µl reaction mixture containing HotStarTaq Master Mix (Qiagen, Hilden, Germany), DEPC, 1 µl cDNA and 1.0 µM concentration of the 3'- and 5'-specific oligomers (synthesized by Invitrogen, Karlsruhe, Germany). In the case of cytochrome P450 genes, PCR amplification was done with a reaction mixture containing 25 µl supermix (Invitrogen), 1.0 µM concentration of the 3'- and 5'-specific oligomers and 1 µl cDNA as template. PCR reactions were carried out in a thermal cycler (T3, Biometra, Göttingen, Germany) using the following melting and annealing conditions: 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min using specific oligonucleotides for the cytochrome P450 genes (table 1). PCR conditions for the transcription factor and transporter genes were 95°C for 15 min, 94°C for 45 s and then followed by the individual conditions for each gene (table 1).

PCR reactions were done within the linear range of amplification, e.g. after having established that 35 cycles were in the mid-exponential phase for every primer set used. Amplification products were separated on a 1.5% agarose gel and visualized by ethidium bromide under UV transillumination.

Quantitation of PCR products was done with the programme NIH Image V.1.62 and Kodak Image Station version ID 3.5 software. Examples of ethidium bromide-stained agarose gels are shown in figures 1–3. Data are expressed as the ratio of the gene of interest (nominator) versus the housekeeping gene GAPDH (figures 4–6). Aroclor 1254 treatment did not change GAPDH expression. Note that ethidium bromide-stained bands for low transcript copy genes such as *CYP2D6*, *CYP2E1* and *CYP2C8-19* were faint. Consequently, the bands of these CYPs can hardly be seen on hard copy. Nevertheless, the amplification products of these low transcript copy genes were visible on the computer screen and consequently could be used in the computation of gene expression.

Testosterone assay

To estimate the activity of individual CYP enzymes, Caco-2 cells cultures were treated with culture medium containing 100 µM testosterone. A total of 25 µM finasteride was added as a 5α-reductase inhibitor because of its contribution to the production of certain testosterone metabolites, e.g. dihydrotestosterone. Testosterone and its metabolites were analysed by HPLC according to Arlotto *et al.* (1991) with slight modifications, as described below. 11-α-Hydroxyprogesterone was used as an internal standard for the quantitative determination of testosterone and its metabolites using the following procedures: 1 µg 11-α-hydroxyprogesterone was added to 1 ml cell culture supernatant. Following addition of 100 µl isopropanol, the samples were extracted with 5 ml ethyl acetate by gentle shaking for 20 min. Extracts were evaporated to dryness and the residues reconstituted in 100 µl of the mobile HPLC phase (water/methanol/acetonitrile 60/25/15 v/v/v) and 80 µl sample were injected onto the HPLC system (Hewlett Packard HP1100 Agilent, Waldbronn, Germany). The mobile phase was delivered at a flow rate of 1 ml min⁻¹ using the HP 1100 Quaternary Pump. Chromatographic separation of cytochrome P450 produced testosterone metabolites was achieved on a C18 Nucleosil column, 250 × 4 mm and a particle size of 5 µm (Macherey & Nagel). Chromatographic separation was undertaken at 30°C and testosterone and metabolites were detected by UV absorption at 238 nm using synthetic reference standards. The mobile phase consisted of water (solvent A), methanol (B) and acetonitrile (C) and chromatographic separation was achieved with an isocratic elution of 60% A, 25% B and 15% C for 12 min, followed by 45% A, 40% B and 15% C for 3 min and 45% A, 45% B and 10% C thereafter. The total run time was 30 min per sample.

Statistical analysis

Hottelings T² test was used with the SPSS software package (version 10.0). Whenever significant results were computed, further pair wise *t*-tests were done. With this approach, computing errors as a result of multiple testing could be controlled. Results were considered significant at *p* < 0.05.

Table 1. Oligonucleotide sequences for PCR assays.

Gene (accession no.)	Forward primer (5'–3')	Reverse primer (5'–3')	Product length (bp)	Annealing temperature, CaCo	Annealing temperature, liver
CYP 1A1 (D01150)	gctcttaggtgcttgaga gcc	ctgtgtctctggagggt gtg	432	55°C, 1 min	55°C, 1 min
CYP 1A2 (M38504)	accattctgaggccagg gag	tagagacagggtccca ctatg	308	55°C, 1 min	55°C, 1 min
CYP 2B6/7 (X13494)	ccatacacagaggcagt cat	ggtgtcagatcgtgtct tc	357	55°C, 1 min	55°C, 1 min
CYP 2C8-19 (Y00498)	gctaaagtccaggaag gaattga	tcctgctgagaaggca tgaagt	332	55°C, 1 min	55°C, 1 min
CYP 2D6 (M33189)	cgtgtccaacaggagat cgac	gcctcatccttcagcac cga	332	55°C, 1 min	55°C, 1 min
CYP 2E1 (AF084225)	acctgccccatgaagca acc	gaaacaactccatcgga gcc	309	55°C, 1 min	55°C, 1 min
CYP 3A3 (X12387)	ccaagctatgtctttcac cg	tcaggctccacttacggt gc	323	55°C, 1 min	55°C, 1 min
CYP 3A5 (L26985)	tgtccagcagaaactgc aaa	ttgaagaagtcttgcgt gtc	472	55°C, 1 min	55°C, 1 min
EH (Y00424)	tgatgaggggagagcgg gtac	tcagcagggtcgctccagg gag	396	55°C, 1 min	55°C, 1 min
FMO1 (NM_002021)	gctgttcgagtcctgaaa gg	gccaaagaagacggtc agag	234	60°C, 1 min	60°C, 1 min
FMO2 (NM 0001460.1)	ctcctattagtatgcctg gtgg	tactggatcctgacaag ataataaagcccaaag	250	55°C, 1 min	55°C, 1 min
FMO5 (L37080)	ctctcagtttcatattgcc cag	acattatttcttctatct cagg	400	55°C, 1 min	55°C, 1 min
GAPDH (J02624)	ggccaaggtcatccatg a	tcagtgagcccaggatg a	353	55°C, 1 min	55°C, 1 min
HNF-1alpha + beta (X71346.1)	tagtggaggagtgcaa cagggc	tgggagaactggacgg gctg	702	64°C, 1 min	64°C, 60 sec.
HNF-4 (NM 000457.1)	ctgctcggagccacaa agagatccatg	atcatctgccacgtgatg ctctgca	370	60°C, 1 min	55°C, 1 min
HNF-4alpha2 + 4 (Z49825.1)	tggcagatgatcgagca gatc	ggtcggggccactcac acatc	223	54°C, 1 min	55°C, 1 min
HNF-4alpha3 (U72967)	cgtggatcctggcagat gatcgagcagatc	gacggatcctctagaca ggttaagcaactgc	218	62°C, 1 min	55°C, 1 min
HNF-4alpha4 (z49825)	catggacatggccgact acagtgt	acggagctcgtctccct gtggagaactgtc	196	63°C, 1 min	no signal
HNF-4gamma (NM 004133.2)	gccacactgcagagc atcac	gactgatgcttggtttgc agcta	312	54°C, 1min	no signal
STAT-3 (NM 003150.1)	acattctgggcacaaaac aca	atgttggagatcaccac aactg	247	53°C, 1 min	55°C, 1 min
STAT-4 (NM 003152.1)	agctgaagtttcaactcg ga	caaaccagatcagctttt accc	628	53°C, 1 min	53°C, 1 min
CEBP alpha (NM004364.1)	gaatctctagtctctggc tc	gatgagaacagcaacg agtac	353	60°C, 1 min	55°C, 1 min
MDR-1 (M14758.11)	aaaaagatcaactcgta ggagtg	gcacaaaatacaccaa caa	161	60°C, 1 min	60°C, 1 min
MRP-1 (L05628.1)	agtccccacagaagga gtgg	tcccccaccgtggagg attt	358	60°C, 1 min	no signal
MRP 3 (AF154001.1)	ctcaatgtggcagacat cgg	gggagctcacaaacgt gtgc	177	68°C, 1 min	55°C, 1 min
MRP-4 (U83660)	ccattgaagatcttctcg g	ggtgttcaatctgtgtgc g	238	53°C, 1 min	53°C, 1 min
MRP-5 (AB019002.1)	ctctgttgggaaggaat atga	gggtcgtccaggatgta gat	203	55°C, 2 min	55°C, 2 min

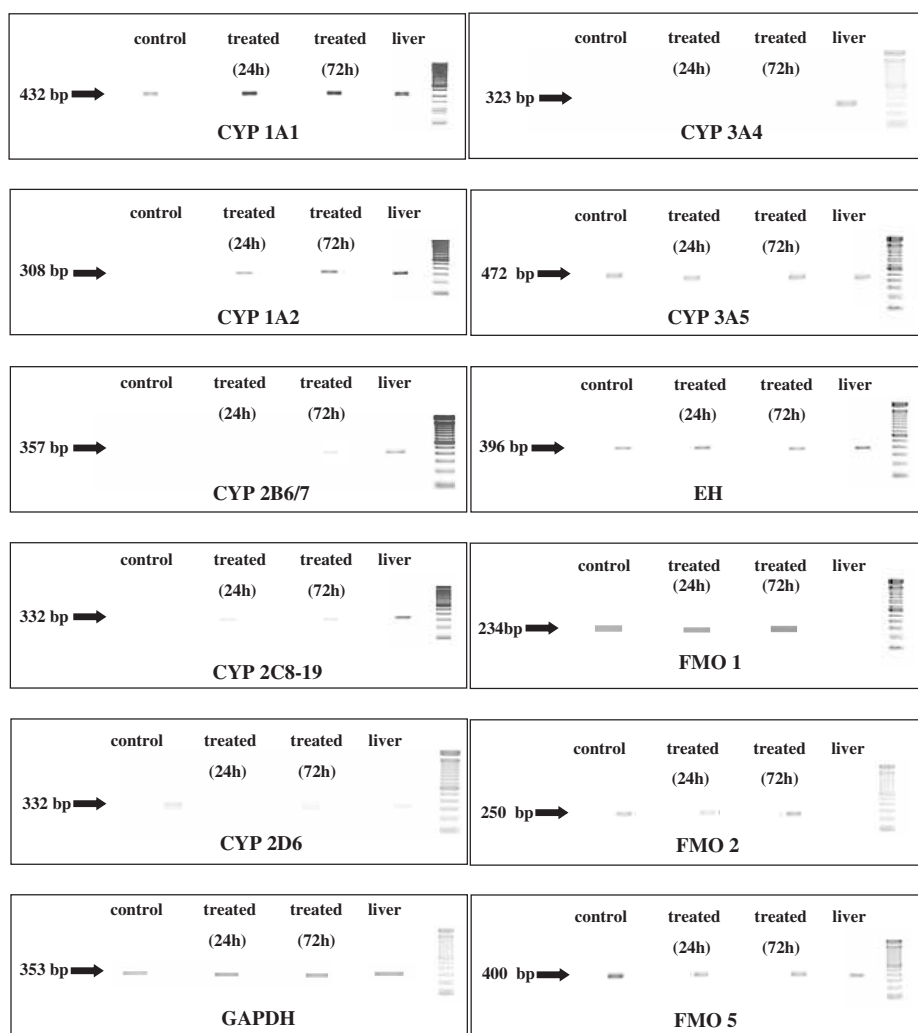


Figure 1. Examples of ethidium bromide-stained PCR amplification products of CYP monooxygenases, flavine-containing monooxygenases, epoxide hydrolase and GAPDH in control and Aroclor 1254-treated *Caco-2* cell cultures. Pooled human liver from $n = 3$ donors served as a positive control.

Results

Morphology

The purity and quality of cultured *Caco-2* cells were examined by phase-contrast light microscopy. Cells were fully confluent and their appearance did not indicate an unusual morphology.

Semiquantitative gene expression of drug- and xenobiotic-metabolizing enzymes (DMEs)

Figures 1–3 show typical examples of ethidium bromide-stained amplification products of six CYP monooxygenases (*CYP1A1*, *CYP1A2*, *CYP2C8-19*, *CYP2D6*, *CYP2E1*, *CYP3A5*), three flavine-containing monooxygenases

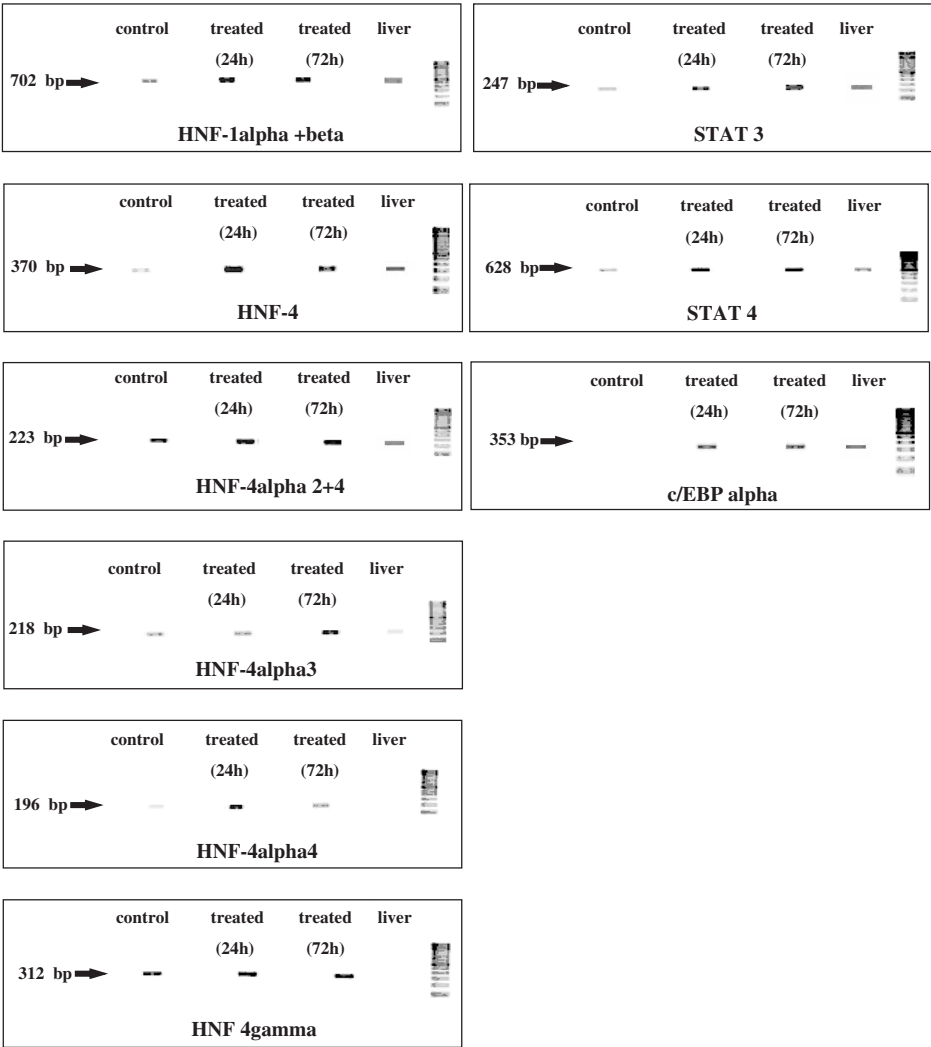


Figure 2. Examples of ethidium bromide-stained PCR amplification products of nuclear transcription factors in control and Aroclor 1254-treated Caco-2 cell cultures. Pooled human liver from $n = 3$ donors served as a positive control.

(FMO1, 2, 5), epoxide hydrolase (EH) and the housekeeping gene GAPDH (glyceraldehyd-3-phosphat-dehydrogenase) in control and Aroclor 1254-treated Caco-2 cells. For comparison, the results from the pooled human liver total RNA extract are also shown. For *CYP1A1*, Aroclor 1254 treatment resulted in an approximately 14-fold mRNA induction (figure 4), but this treatment produced a substantial 50- and 80-fold increase in *CYP1A2* mRNA transcripts after 24 and 72 h of treatment, respectively. In the case of *CYP2C8-2C19*, treatment of cultured cells with Aroclor 1254 resulted in a 10-fold increase in mRNA expression, but with *CYP2D6* the mRNA expression fell to 14 and 74% of control values, 24 and 72 h post-treatment, respectively. A small 2.4-fold increase in *CYP2E1* mRNA expression was observed 24 h after treatment, but declined thereafter to 75% of control values. With *CYP3A5*, Aroclor 1254 treatment resulted in a similar

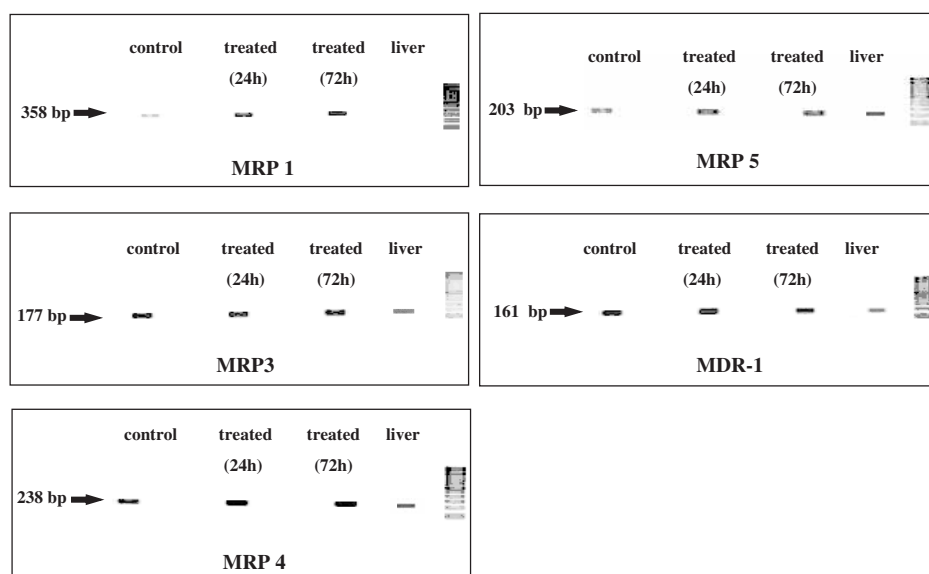


Figure 3. Examples of ethidium bromide-stained PCR amplification products of ABC transporters in control and Aroclor 1254-treated Caco-2 cell cultures. Pooled human liver from $n = 3$ donors served as a positive control.

2.5-fold increased expression. With the exception of *FMO1* and 2, expression of all DMEs was significantly higher in human liver (up to 80-fold).

The time scale of gene expression differed among the flavine-containing monooxygenases with *FMO1* expression remaining constant and *FMO2* levelling from 60% to near twofold, whereas *FMO5* expression declined by approximately 30% at the 72-h point (figure 4).

Note that with the pool of human livers, no transcript copies for *FM1* and 2 could be identified, whereas expression of *FMO5* was similar between untreated cultures and the pooled human liver RNA extract.

Epoxide hydrolase responded to Aroclor 1254 treatment with an approximately threefold increase in expression. The mRNA level of *EH* in control cultures was twice (figure 4) of the human liver pool.

Note that transcripts for *CYP3A4* and *CYP2B6/7* were not detected in Caco-2 cells and, hence, are not shown in figure 4, but transcripts were detected in extracts of human liver total RNA. When compared with Caco-2 cell cultures, that were 11.7-, 28-, 77.6-, 71-, 3.7-, 3.3- and 2.4-fold increased expression levels of *CYP1A1*, *CYP1A2*, *CYP2C8-19*, *CYP2E1*, *CYP3A5*, *CYP2D6* and epoxide hydrolase, respectively, in the pooled human liver sample.

Semiquantitative gene expression of transcription factors

Typical examples of PCR amplification products are shown in figure 2, and a summary of the gene expression data is given in figure 5. Treatment of Caco-2 cells with Aroclor 1254 resulted in a threefold induction of HNF-1 α + β mRNA 24 and 72 h post-treatment. In the case of HNF-4, the expression of several splice variants was investigated. As shown in figure 5, expression of HNF-4, HNF-4 α 2 + 4 and HNF-4 α 3 differed after treatment with Aroclor 1254, with HNF4 and the splice variant HNF-4 α 4 being increased 10-fold 24h post-treatment. This induction

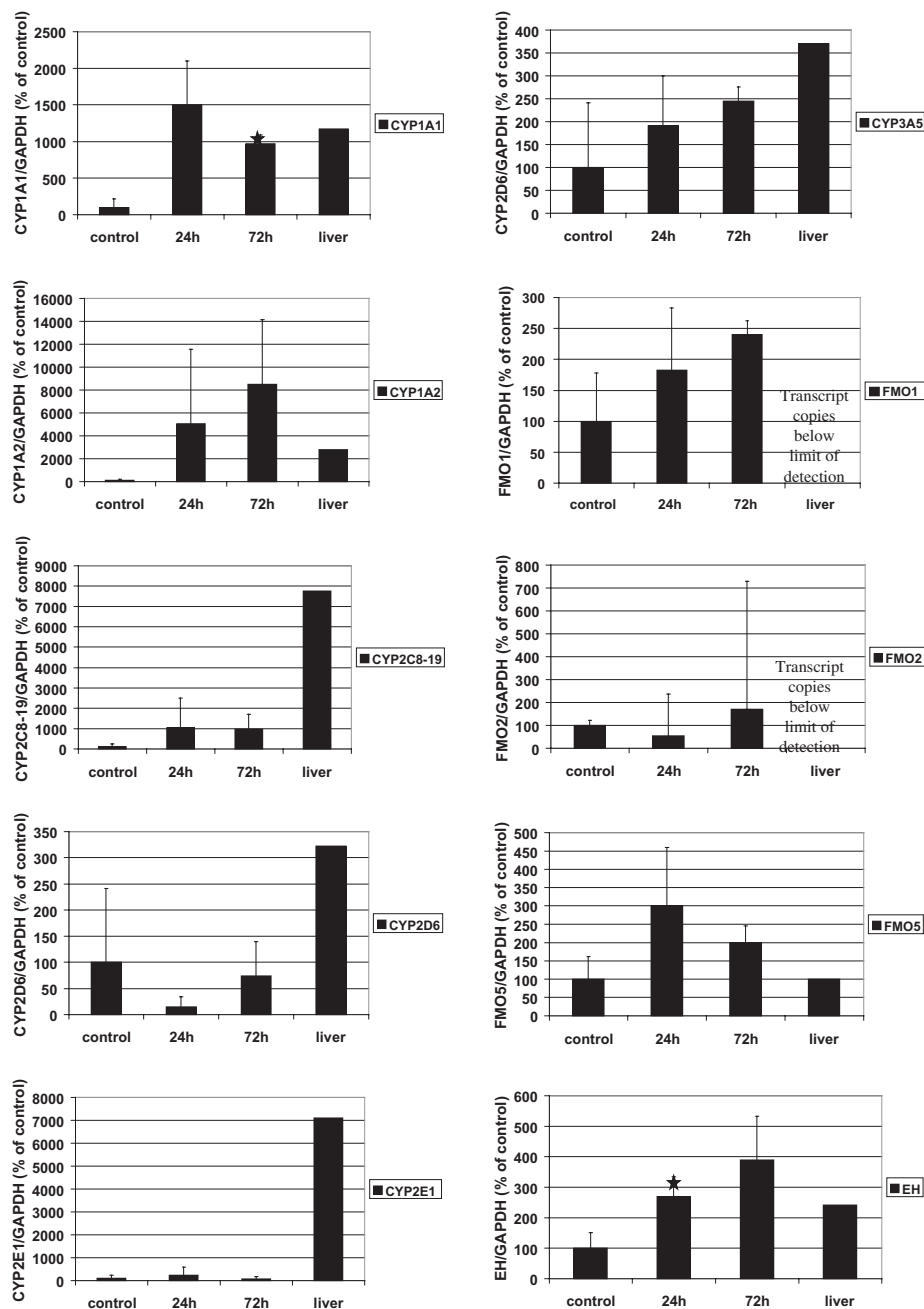


Figure 4. mRNA expression of CYP monooxygenases, flavine-containing monooxygenases and epoxide hydrolase in control and Aroclor 1254-treated Caco-2 cell cultures. Pooled human liver from $n = 3$ donors served as positive control.

declined to a six- and twofold increase after 72 h in culture. In the case of HNF-4 γ , a three- and twofold increase in transcript level after 24 and 72 h of treatment, respectively, was observed. The gene expression of STAT (signal transducer and activator of transcription) 3 and 4 was also investigated and eight- and 10-fold

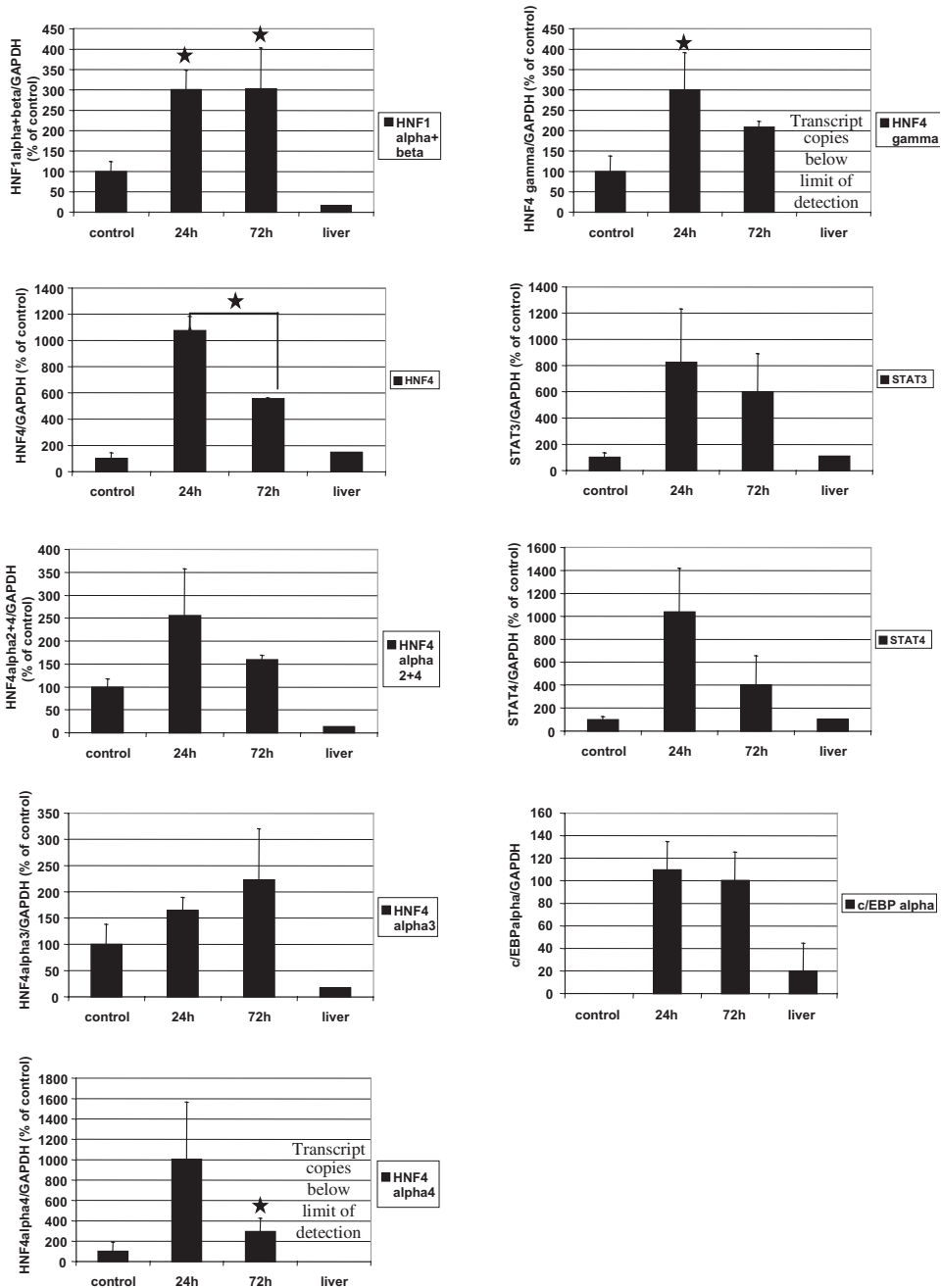


Figure 5. mRNA expression of nuclear transcription factors in control and Aroclor 1254-treated Caco-2 cell cultures. Pooled human liver from $n = 3$ donors served as positive control.

increases were found after 24 h, and six- and twofold increase after 72 h of treatment, respectively. Further transcript copies of c/EBP α (CCAAT/enhancer binding protein) were identified in Aroclor 1254-treated Caco-2 cells and human liver only, but not in extracts of untreated cultures. Thus, the level of induction could not be determined, but it is obvious that Aroclor 1254 elicited an extensive induction

of c/EBP α . Furthermore, expression of c/EBP α mRNA was fourfold higher in Aroclor 1254-treated Caco-2 cell cultures when compared with extracts of pooled human liver samples (see 24-h point). Significantly lower levels of expression were also found, i.e. 16, 14 and 17% for HNF-1 α + β , HNF-4 α 2 + 4 and HNF-4 α 3, respectively, while the expression of HNF-4 was increased 1.5-fold in human liver RNA extracts, but once again this comparison was based on Aroclor 1254-treated Caco-2 cell cultures. Interestingly, with the pooled human liver sample, the expressions of HNF-4 α 4 and HNF-4 γ were below the limit of detection.

Semiquantitative gene expression of multidrug resistance proteins of the ATP binding cassette (ABC)

The expression of genes coding for MDR (multidrug resistance)-1 and MRP (multidrug-resistant related protein) 1 and 3–5 was investigated. Typical examples of PCR amplification products are shown in figure 3 and a summary of the gene expression data is given in figure 6. As shown in figure 6, expression of MRPs differed slightly when the various isoforms were compared. Indeed, MRP1 and some of its isoforms were moderately increased (threefold) upon Aroclor 1254 treatment. A similar increase in gene expression was observed for MDR-1. Significantly lower expression levels were found in the pooled human liver RNA extract, i.e. 7, 9, 9 and 47% for MRP3, MRP4, MRP5 and MDR-1, respectively, when compared with Caco-2 cell cultures.

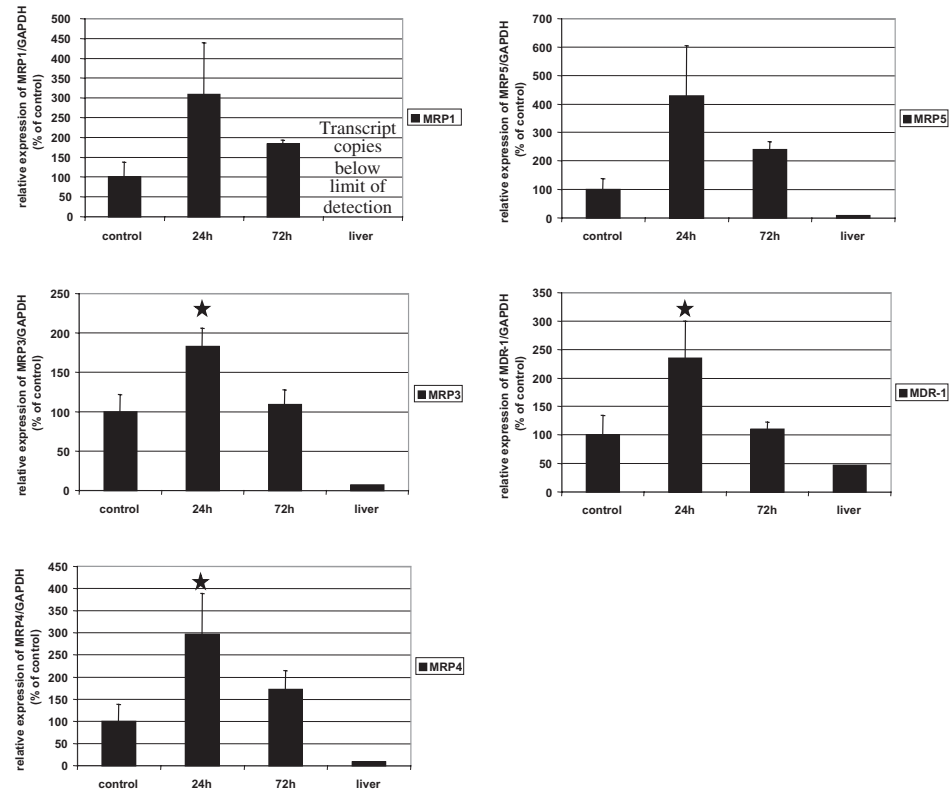


Figure 6. mRNA expression of ABC transporters in control and Aroclor 1254-treated Caco-2 cell cultures. Pooled human liver from $n = 3$ donors served as positive control.

CYP monooxygenase enzyme activity of cultured Caco-2 cells

Testosterone was used as a substrate to determine CYP monooxygenase activities, and figure 7 shows the production of extractable metabolites from culture media of Caco-2 cells. As described in the Materials and methods, testosterone was added to control and Aroclor 1254-treated cell cultures to allow production of metabolites during a 4-h incubation assay. In previous studies, the 4-h incubation was within the linear range. There was approximately a threefold increased production of 6 β -hydroxytestosterone ($p < 0.05$) in cultures treated with Aroclor 1254 for 72 h. The production of 6 α -hydroxytestosterone was basically unchanged, e.g. from 15 to 18 ng ml⁻¹ culture media after 24 and 72 h, respectively. Notably, Aroclor 1254 treatment resulted in a reduced production ($p < 0.05$) of 16 α -hydroxytestosterone, e.g. from 90 to 70 ng ml⁻¹, in cultures treated for 72 h. Similarly, the production of androstendione ($p < 0.05$) declined in the 24-h treatment group to about 2400 ng ml⁻¹,

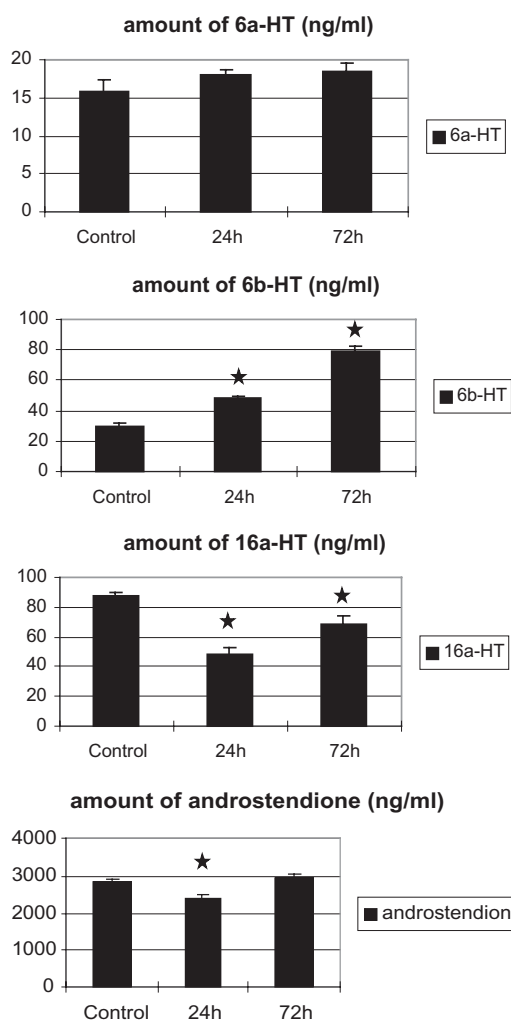


Figure 7. Metabolism of testosterone in control and Aroclor 1254-treated Caco-2 cell cultures.

but returned thereafter to control values (figure 7). Overall, the order of testosterone metabolite production was androstendione > 16 α -hydroxytestosterone > 6 β -hydroxytestosterone > 6 α -hydroxytestosterone.

Discussion

The expression of drug-metabolizing enzymes, nuclear transcription factors and genes coding for the multidrug resistance proteins of the ATP binding cassette (ABC) has been reported in cultures of Caco-2 cells. The gene expression and enzyme activities of DMEs were also correlated and the responsiveness of Caco-2 cells upon treatment with Aroclor 1254 was demonstrated.

Evidence is thus presented for the expression of a number of important drug-metabolizing genes including *CYP1A1*, *CYP1A2*, *CYP2C8-19*, *CYP2D6*, *CYP2E1*, *CYP3A5*, *FMO1*, 2 and 5, and epoxide hydrolase, whereas gene expression of *CYP3A4* and *CYP2B6/7* were below the limit of detection in RNA extracts of cultured Caco-2 cells. The significant increase in *CYP1A1* mRNA expression implies an Ah-receptor-mediated transcriptional regulation and several investigators have reported *CYP1A1* mRNA expression and its induction by various chemicals including β -naphthoflavone (Carriere *et al.* 1998), benzimidazole derivatives (Daujat *et al.* 1996), and 3-methylcholanthrene, benz[a]anthracene, benzo[a]pyrene, benzo[e]pyrene and phenobarbital (Rosenberg and Leff 1993). With a particular Caco-2 cell clone, termed TC7, Pandrea *et al.* (2000) reported that confluency of cell culture coincided with reduced *CYP1A1* mRNA expression. In addition, a ligand-independent Ah-receptor pathway may drive transcriptional activation of the *CYP1A1* gene including hypoxia and regulation via the electrophile response element (ERE) (Guigal *et al.* 2000, Nebert *et al.* 2000, Dieter *et al.* 2001).

The expression of certain *CYP* genes and enzyme activity in various intestinal cell lines and cultured Caco-2 cells were reported by Lampen *et al.* (1998). The present findings are now extended to an additional identification of gene transcripts for *CYP2D6*, *CYP2C8-19*, *CYP3A5*, *FMO1* and 2, *FMO5* and epoxide hydrolase and thus provide comprehensive information on the major human *CYP* genes in Caco-2 cells. Additionally, Lampen *et al.* (2001) reported mRNA expression of *CYP26* in cultures of Caco-2 cells and this particular CYP form plays an important role in retinoid acid metabolism and retinoid acid receptor signalling pathways.

Based on the present study and other reports (Schmiedlin-Ren *et al.* 1997), gene expression of *CYP3A4* in Caco-2 cells remains controversial, as the CYP3A expression was reported for a particular clone of Caco-2 cells, namely Caco-2 clone TC7 (Carriere *et al.* 1994) (but the isoform was not specified). Others have reported *CYP3A4* mRNA expression to be inhibited by NO (Hara *et al.* 2000) or cytokines (Bertilsson *et al.* 2001) and it is probable that polyclonal variation of various Caco-2 cell clones might account for some of the differences in the gene expression of DMEs observed by others. Notably, human colorectal adenocarcinoma tissue has been reported to exhibit a significant reduction in *CYP3A4* and epoxide hydrolase gene expression when compared with normal colon tissue of the same donor (de Waziers *et al.* 1991). In addition, functional carboxylesterase and N-acetyltransferase (NAT) activities have also been reported for cultured

Caco-2 cells (Prueksaritanont *et al.* 1996) and, thus, Caco-2 cells appear to be metabolically competent tumour cells.

Total liver RNA extracts from a pool of donors were used to compare gene expression profiles, but note that no direct comparisons are suggested between a colon adenocarcinoma cell line and liver tissue. In addition, the liver material was derived from patients of poor health and the sample size was too small to draw firm conclusions. Despite the fact that the liver is a major site for drug metabolism, it is now accepted that intestinal metabolism might be very significant as well leading to metabolic inactivation and reduced therapeutic efficacy of drugs. A good correlation of *CYP* gene expression and enzyme activity has been shown for cultured Caco-2 cells and the findings agree well with previous observations from other investigators (Lampen *et al.* 1998).

Also investigated was the mRNA expression of liver-enriched transcription factors in cultures of Caco-2 cells because of their important role in the transcriptional activation of DMEs (Schrem *et al.* 2002). It was also demonstrated that gene expression of HNF-1 α + β and HNF-4 and some of the splice variants were modulated by Aroclor 1254 treatment. Induction of nuclear transcription factors on treatment of rat hepatocyte cultures with Aroclor 1254 was recently reported by Borlak and Thum (2001). The present findings were extended to the induction of HNF-1 and HNF-4 forms and some of their splice variants. The concomitant induction of nuclear transcription factors and CYP monooxygenases fitted well with a mechanism of transcriptional activation of DMEs driven by induction of nuclear transcription factors, as shown herein. Additionally, there were several reports detailing the expression of HNF genes in relation to epithelial cellular differentiation, including the study of Suaud *et al.* (1997) who reported the mRNA expression of certain HNF forms including HNF-4 α 2 and HNF-4 α 4 to be of critical importance in epithelial cell differentiation. For example, Erickson *et al.* (2000) reported expression of HNF-1 α to be increased three- to fourfold during Caco-2 cell differentiation and HNF-1 α and HNF-4 play a critical role in the transcriptional regulation of the α 1-antitrypsin gene during cellular differentiation of Caco-2 cells (Hu and Perlmutter 1999). Guanylin gene regulation was also dependent on DNA binding of HNF-1 α , as evidenced in cotransfection experiments. HNF-1 binding to the regulatory elements of the human sucrase-isomaltase promoter was crucial for its transcriptional activation (Rodolosse *et al.* 1998) and HNF-4 regulated expression of the guanylin/heat stable toxin receptor (Swenson *et al.* 1999). Not all of the splice variants had the same transactivation potential, as reported by Drewes *et al.* (1996) who showed that HNF-4 γ was significantly less active than HNF-4 α 2, and the HNF-4 α splice variant HNF-4 α 4 has no detectable transactivation potential for HNF-4 responsive genes. There is substantial evidence for HNFs to regulate transcriptional activation of drug-metabolizing genes (Schrem *et al.* 2002) and, thus, it was not surprising to observe several HNF genes being expressed in cultures of Caco-2 cells.

The expression of STAT3 and STAT4 was also investigated because of their importance in the differentiation of Caco-2 cells. The STAT proteins are a family of cytoplasmic proteins with roles as signal messengers and transcription factors and they participate in normal cellular response to cytokines and growth factors (Horvath 2000). Certain STAT proteins were associated with a wide range of human tumours and STAT proteins are considered to be

valuable targets for drug treatment of cancer (Turkson and Jove 2000). STAT proteins are implicated in programming gene expression in embryonic development, apoptosis, innate and adaptive immunity, and cell growth regulation (Horvath 2000), but there is very limited information on the expression of STAT gene and protein expression in Caco-2 cells. Of the few studies available, Wang and Evers (1999) reported that protein expression of STAT3 and STAT5 was increased in preconfluent and confluent Caco-2 cells, but declined thereafter, whereas in Morton *et al.* (1998) a leptin-mediated induction of a number of immediate early genes and particularly activation of STAT3 and STAT5 was reported. It has been suggested that lack of leptin or resistance to leptin action might contribute to an insufficient handling of lipids in the epithelium of the gut (Morton *et al.* 1998). Expression of STAT4 in Caco-2 cells has not been reported to date and this particular STAT is involved in signal transduction of the IL-4 and IL-12 receptors (Wurster *et al.* 2000). Whether increased STAT3 and STAT4 expression in Aroclor 1254-treated cell cultures are linked to its tumour-promoting ability requires further investigation.

Finally, the expression of ATP-binding cassette (ABC) transporters and particular expression of genes coding for MDR-1 and MRP3–5 were investigated in cultures of Caco-2 cells, the latter being important transporters of endogenous and xenobiotic substrates (Kerb *et al.* 2001). These protein pumps play an important role in multidrug resistance and have been the subject of intense research for a variety of reasons, including drug transport across the intestinal barrier and in cancer therapy. On average, threefold induction of MRP1 and its isoforms were observed in addition to MDR-1, in Aroclor 1254-treated Caco-2 cell cultures. The function, gene and protein expression of MRP isoforms 1, 3 and 5 has been reported by Hirohashi *et al.* (2000), who reported continuous expression of MRP2 in cultures of Caco-2 cells, but MRP3 was not expressed in confluent monolayers, and expression of MRP1 and MRP5 was minimal. The cloning and expression of human MRP3 was reported by Kiuchi *et al.* (1998) and evidence was presented that MRP3 can act as an inducible transporter in the intestinal excretion of organic anions. Others have reported increased expression of MRP mRNA levels and increased protein expression of MRP1 and MRP6 by immunohistochemistry in various tumour tissues (Nooter *et al.* 1995). Additional factors that control MRP gene transcription include pH and osmotic shock and these findings might help to explain why colon tumours develop MDR before chemotherapy, since tumour growth can be regulated by acidic conditions (Wei and Roepe 1994). Note that MDR-1 expression is not constant in Caco-2 cell cultures and this is of importance when this cell line is used for drug transport studies (Hosoya *et al.* 1996, Anderle *et al.* 1998).

In conclusion, the expression of an array of DMEs, certain hepatic nuclear factors, STAT3 and STAT4, and genes coding for ATP binding cassette (ABC) transporters was reported. It has been shown that cultures of Caco-2 cells respond to treatment with Aroclor 1254 with increased mRNA expression of a number of genes. This cell line thus mimics biological responses also seen with cultures of primary cells and animal studies after treatment with Aroclor 1254 (Borlak and Thum 2001). Caco-2 cells are thus viewed as being valuable in predicting biological responses to digested chemicals and toxins based on gene expression and enzyme activity studies.

Acknowledgement

The authors thank T. Hansen for advice on the Caco-2 cell culture work and T. Thum for advice on the RT-PCR studies.

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Evaluation of the role of the asialoglycoprotein receptor in the clearance of UK-279,276 (recombinant neutrophil inhibitory factor)

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Received 3 April 2003

1. UK-279,276 or recombinant neutrophil inhibitory factor (rNIF) is a glycoprotein that has been investigated as an intravenous treatment for stroke. Previous data indicate that the asialoglycoprotein receptor (ASGPR) in the liver might have a role in the uptake and clearance of UK-279,276 from plasma in rats and dogs.

2. Biliary elimination of sialo UK-279,276 can be inhibited by the co-administration of asialo fetuin, a known substrate for ASGPR. These data are in keeping with sialo UK-279,276 being cleared by ASGPR.

3. *In vitro* experiments have demonstrated that asialo UK-279,276 (Sialo UK-279,276 treated to remove the sialic acid residues present on the glycans) shows a significantly greater uptake in rat, dog and human precision-cut liver slices than the sialylated protein. The addition of asialo fetuin to precision-cut liver slice incubations (rat, dog and man) containing asialo UK-279,276 reduced the uptake to levels similar to those seen for the sialylated protein. These data support the potential role of the ASGPR in the clearance of UK-279,276 in rat, dog and man.

Introduction

Native neutrophil inhibitory factor (NIF) is a 41 kD glycoprotein extracted from the canine hookworm, *Ancylostoma caninum* (Moyle *et al.* 1994). NIF protein is made up of 257 amino acids, including 10 cysteines and seven potential N-linked glycosylation sites (Moyle *et al.* 1994). Biological investigations with rNIF have shown this molecule to be effective in reducing infarct size in a rat re-perfusion model of stroke (Jiang *et al.* 1995, 1998). The molecular weight of the amino acid core of NIF is 28.9 kD. Approximately 40% of the total molecular weight of NIF consists of sugar moieties (glycans) attached at a series of N-linked glycosylation sites. The NIF used in these experiments (designated UK-279,276) was produced using recombinant technologies in Chinese hamster ovary cells and had the same amino acid sequence as the native material. This recombinant NIF (UK-279,276) has glycan chains that are complex in nature and highly sialylated (Pfizer Internal Communication, Sandwich, UK). This material is referred to as sialo UK-279,276 for present purposes.

It was proposed that UK-279,276 could be cleared from the systemic circulation by hepatic asialoglycoprotein receptors (ASGPR; Webster *et al.* 1999).

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The ASGPR is predominantly located on hepatocytes and has been shown to interact with galactose residues exposed at the termini of the glycan chains present on glycoproteins (Spiess 1990). As more galactose residues are exposed at the termini of the glycan chains, the affinity for the ASGPR increase in an exponential manner. Studies with artificial glycans have demonstrated that the affinity for the ASGPR shows stepwise increases from 10^{-3} to 10^{-9} M as the number of exposed terminal galactose residues increases from one to four (Spiess 1990). The capping of a terminal galactose residue with sialic acid ablates its affinity for this receptor. Therefore, assuming a constant core glycan structure, as the sialylation of a protein increases its affinity for the ASGPR should decrease, as should its clearance by this mechanism.

The pharmacokinetics of UK-279,276 with varying degrees of sialylation has been studied in the rat and marked differences have been observed. These differences in pharmacokinetics are dependent on the sialylation profile of the molecule (Webster *et al.* 1999). For example, sialo UK-279,276 has a low clearance ($0.08 \text{ ml min}^{-1} \text{ kg}^{-1}$) and a long half-life (11.5 h) in the rat. Asialo UK-279,276 (sialo UK-279,276 treated to remove the sialic acid residues present on the glycans), however, has significantly higher clearance and shorter half-lives ($1.11 \text{ ml min}^{-1} \text{ kg}^{-1}$ and 0.8 h, respectively). A similar sialylation-dependent pharmacokinetic profile has been observed in the dog (Webster 2000). These data indicate a potential role for the ASGPR in the clearance of UK-279,276 in these species and overall these data are in keeping with the properties of the ASGPR.

Further work has been conducted to improve confidence that the ASGPR has a role in the clearance of the sialo UK-279,276 intended for use in the clinic. To evaluate the role of this receptor in the clearance of sialo UK-279,276, experiments were carried out in rat to determine whether the clearance of sialo UK-279,276 could be inhibited by a known ASGPR substrate, asialo fetuin (Borges *et al.* 1986, Otter *et al.* 1992, Huseby *et al.* 1993).

Whilst there is evidence that the ASGPR has a role to play in the clearance of UK-279,276 in dogs and rats, the role of this receptor had not been investigated in man. Literature data suggest that these receptors are closely related between species. For example, there is 80% amino acid homology between the major human (H1) and rat (RHL1) ASGPR subunits (Spiess 1990). In addition, a review of protein clearance has shown that excellent allometric relationships can exist between animals and man (Mordenti *et al.* 1991). This may indicate a conservation of the function/affinity of this clearance mechanism across species. Therefore, the involvement of this receptor in the clearance of UK-279,276 in other species, including man, was considered likely.

As no human data are available to demonstrate the role of the ASGPR in the clearance of UK-279,276, a series of *in vitro* experiments were carried out to investigate the role of this receptor across a variety of species including man. Experiments were carried out using precision-cut liver slices to study the role of ASGPR-mediated hepatic uptake in the clearance of this protein. The data obtained in these precision-cut liver slice experiments were compared with the available *in vivo* animal data (rat, dog). In addition, the *in vitro* animal data were compared with data obtained in human precision-cut liver slice experiments, allowing comparison of the disposition in animals and man.

Materials and methods

Animal experimentation

All studies involving animals were carried out in accordance with the requirements of UK national legislation and conducted under necessary authorities. At all stages, consideration was given to experimental refinement, reduction in animal numbers and replacement with *in vitro* techniques. Rat and dog liver samples were obtained from animals being culled as part of other procedures. An inbred strain of rats, Fischer 344, was selected for these studies in an effort to reduce experimental variability.

Production of asialo UK-279,276

Sialo UK-279,276 was desialylated using neuraminidase enzymes. Sialo UK-279,276 was incubated (37°C) with neuraminidase isolated from *Clostridium perfringens* Type X (Sigma, Poole, UK; 10 Units ml⁻¹), in a solution (pH 5.6) containing 0.05 M sodium acetate, 0.165 M sodium chloride and 0.002 M calcium chloride (final concentration of UK-279,276 was 0.25 mg ml⁻¹). The sialic acid liberated by the enzymatic reaction was determined using the thiobarbituric acid method (Warren 1959) and the incubation was terminated, by cooling on ice, when no further increase in free sialic acid was observed.

Preparation of fluorescein isothiocyanate (FITC)-labelled asialo UK-279,276

Asialo UK-279,276 solution was initially concentrated, by reducing the volume of fluid it was dissolved in, to achieve a final concentration of approximately 4 mg ml⁻¹ using a molecular weight cut-off filter device (10 kD cut-off Centricon from Millipore Corporation, Bedford, MA, USA; centrifuged at 500g in a fixed angle centrifuge). The protein solution was labelled using the FITC-labelling kits obtained from Sigma (FluoroTagTM, FITC-1).

Losses during the reaction to label UK-279,276 were evaluated by subjecting a control batch of UK-279,276 to an identical regimen to that used for the labelling (however, without FITC). This UK-279,276 solution was analysed using a gradient hplc system. UK-279,276 was chromatographed using a Jupiter, 5 μ , C18, 300A, hplc column (250 $\times$ 4.6 mm, Phenomenex, Macclesfield, Cheshire, UK). The column was equilibrated at a flow rate of 1 ml min⁻¹ using a mobile phase of 90% water/10% acetonitrile containing 0.05% trifluoroacetic acid. On injection of sample (10 μ l), a 15-min linear gradient was started, terminating at a mobile phase composition of 10% water/90% acetonitrile containing 0.05% trifluoroacetic acid. UK-279,276 was detected by UV absorbance at 230 nm. Both sialo and asialo UK-279,276 had a retention time of approximately 12 min on this gradient hplc system. Data indicated that little (< 10%) UK-279,276 was lost during the labelling procedure. Also samples of the FITC-labelled UK-279,276 were evaluated using the Delfia assay (below) and concentrations were in keeping with those expected at the end of the labelling process.

Initially, the buffer used to store UK-279,276 was replaced with the reaction buffer (0.1 M carbonate/bicarbonate buffer, pH 9.0). This was achieved by placing 0.25 ml UK-279,276 solution (1 mg total) into a preweighed filtration device (10 kD cut-off) and 2 ml 0.1 M carbonate/bicarbonate buffer, pH 9.0, was then added. This filtration device was centrifuged at 500g in a fixed angle centrifuge for 15 min (this returned the total volume of the solution to approximately 0.5 ml). This procedure was repeated three times. After the final wash, the total volume of the solution was adjusted to 0.25 ml.

This sample was removed from the filtration device and placed in a small volume, amber reaction vessel (1 ml). To the UK-279,276 solution contained in the reaction vessel, FITC-labelling solution (2 μ l 2.5 mg ml⁻¹ solution) was added. This was mixed, covered in tin foil (to eliminate light) and incubated at 37°C, with gentle agitation for 2 h.

After 2 h, the incubation mixture was transferred into the previously used preweighed filtration device, together with 2 $\times$ 1 ml saline rinses from the reaction vessel. The device was centrifuged in a fixed angle centrifuge at 500g for 20 min (approximately 0.2 ml remained unfiltered). After this period, a further 2 ml saline were added to the filtration device and the centrifugation process repeated. The resultant filtrate was analysed for the presence of FITC using a fluorescence spectrophotometer. This washing process was repeated until no further FITC was detected in two consecutive filtrate samples. This required approximately 10 cycles.

At this point, the FITC-labelled UK-279,276 solution, which remained in the upper chamber of the filtration device, was adjusted to a final concentration of 1 mg ml⁻¹ using saline.

The molar ratio of FITC to UK-279,276 in the final sample was analysed using the methodology detailed in the FITC labelling kit. The absorbance of the protein conjugate was determined at 280 and 495 nm in a PBS buffer containing sodium azide. These absorbances were then used to calculate the molar ratio. These data indicated that asialo UK-279,276 was labelled with an FITC:UK-279,276 molar ratio in the region of 0.5–1:1.

Preparation of precision-cut liver slices

Rat and dog precision-cut liver slices were freshly prepared on the day of incubation. Male rats (approximately 250 g, Fischer 344, Charles River, Ramsgate, Kent, UK) and male dogs (12 kg, Beagle, Pfizer colony, Sandwich, Kent) were euthanized using anaesthetic overdose, the livers were rapidly excised and placed into ice-cold Earl's balanced salt solution (EBSS) containing 25 mM D-glucose (pre-oxygenated with 95% O₂/5% CO₂). Tissue cores (10 mm diameter) were made using a motorized tissue coring press. Slices were prepared using a Krumdieck tissue slicer at a nominal thickness of 300 µm thickness (setting on the tissue slicer) in ice-cold preoxygenated EBSS with 25 mM D-glucose.

Dog precision-cut liver slices not used immediately were cryopreserved using an in-house method for use in future experiments by immersion in increasing concentrations of dimethyl sulphoxide (1.2, 2.4 and 4.7 M) in foetal bovine serum (FBS), at 0°C for 5 min at each concentration. Slices were stored in liquid nitrogen (−196°C) until used. Before use, cryopreserved dog liver slices were rewarmed by plunging the grids directly into FBS at 40°C for 2 min and then washing in FBS at 0°C for 5 min.

Human precision-cut liver slices were purchased from the International Institute for the Advancement of Medicine (IIAM) and cryopreserved upon arrival using an in-house method, as described above. All tissues were procured with informed consent in accordance with the Uniform Anatomical Gift Act USA. The donor was a male aged 44 years. The recent drug history of the donor was known. The donor had not been treated with known P450 inhibitors or inducers and did not have a history of alcohol abuse. Slices were stored and prepared for use as described for the dog.

Precision-cut liver slice incubation procedure

Precision-cut liver slices were pre-incubated in RPMI 1640 media (Sigma) containing 5% foetal bovine serum, 0.5 mM L-methionine, 1 µM insulin, 0.1 mM hydrocortisone 21-hemisuccinate, 2.5 µg ml^{−1} fungizone and 50 µg ml^{−1} gentamicin sulphate, which had been pregassed with 95% O₂/5% CO₂, at 37°C for 30 min. After this time, rat, dog and human slices (four slices/vial) were transferred to vials containing 2 ml supplemented RPMI 1640 media. The vials containing liver slices were incubated in a orbital shaking water bath (30 rpm) at 37°C. All uptake determinations were carried out in triplicate (three vials each containing four liver slices). Separate experiments ($n=3$ vials, each containing four liver slices) were carried out in which precision-cut liver slices were incubated for 5 h at 37°C with: (1) sialo UK-279,276 (1 µg ml^{−1}), (2) asialo UK-279,276 (1 µg ml^{−1}) and (3) asialo UK-279,276 (1 µg ml^{−1}) plus asialo fetuin (200 µg ml^{−1}). A control experiment was carried out where human cryopreserved precision-cut liver slices were incubated as described above but 100% CO₂ was substituted for 95% O₂/5% CO₂.

At the end of the 5-h incubation, the slices were separated from the incubation media and rinsed in water. The slices were then weighed, added to 0.5 ml water and homogenized using an Polytron PT3000 (Kinematica AG, Littau, Switzerland).

Precision-cut liver slice experiments with FITC-labelled asialo UK-279,276

Rat precision-cut liver slice experiments were carried out as described above; however, the incubation was carried out at an FITC asialo-UK-279,276 concentration of 40 µg ml^{−1} (about 1 µM) and the experiment lasted for 1 h. At the end of this incubation period, the liver slices were removed from the incubation media, washed and stored in a neutral buffered 4% formaldehyde solution (Cellstor Pot, CellPath, Newtown, Powys, UK). A control precision-cut liver slice (not dosed with FITC asialo UK-279,276) was treated similarly. These samples were then cyrosectioned and observed using fluorescence microscopy (magnification $\times 100$).

Bile duct cannulated rat experiments

Male F344 rats (Charles River) weighing between 250 and 300 g were used. The rats were anaesthetized using halothane and placed on a heated blanket to maintain body temperature at 37°C. The bile duct was isolated and cannulated using 0.61 mm o.d./0.28 mm i.d. polythene tubing, held in place using a ligature. Following cannulation, a predose bile sample was collected. A cannula (24 g, 19 mm Jelco IV catheter, Johnson and Johnson Medical, Ascot, UK) was inserted into the tail vein and sialo UK-279,276 was administered as an intravenous bolus (nominal dose 0.5 mg), this was followed by either a saline or asialo fetuin (Sigma A1908, 10 mg ml^{−1} in saline) infusion at 1 ml kg^{−1} h^{−1} (dose of asialo fetuin of 10 mg kg^{−1} h^{−1}). Bile samples were collected every 10 min up to 2 h post-dose. Anaesthesia was monitored and maintained for the duration of the study. At the end of the study, the animals were culled with an anaesthetic overdose. Bile samples were stored frozen before analysis for drug.

Analysis of samples

The samples were analysed using a sandwich non-competitive immunoassay format. Polyclonal rabbit anti-UK-279,276 antibody was bound to plates coated with anti-rabbit antibody. UK-279,276 in samples or standards binds to the polyclonal antibodies and finally europium-labelled monoclonal antibody binds to another epitope on the bound UK-279,276. Bound europium is determined after addition of 'Enhancement' solution (Webster *et al.* 1999).

Liver slice homogenate (above) and incubation buffer (1:10 diluted with control incubation buffer) were analysed using an assay with an analytical range of $5\text{--}200\text{ ng ml}^{-1}$. Bile samples were analysed using an analytical range of $0.1\text{--}40\text{ }\mu\text{g ml}^{-1}$.

Results

Biliary excretion of sialo UK-279,276 in the rat

The biliary elimination of sialo UK-279,276 following intravenous administration, as determined using a Delfia immunoassay, is summarized in figure 1. Over the 2 h of the experiment, $0.63 \pm 0.11\%$ of the dose was eliminated by the biliary route. The co-administration of asialo fetuin ($10\text{ mg kg}^{-1}\text{ h}^{-1}$ intravenous infusion), following administration of sialo UK-279,276, markedly reduced the biliary elimination of sialo UK-279,276 to $0.10 \pm 0.01\%$. Analysis of bile flow data (figure 2) indicated that asialo fetuin infusion had no effect on bile flow.

Cellular location of FITC UK-279,276 in the precision-cut liver slices

The cellular distribution of FITC-labelled asialo UK-279,276 was also studied in precision-cut liver slices following incubation for 1 h, and these data

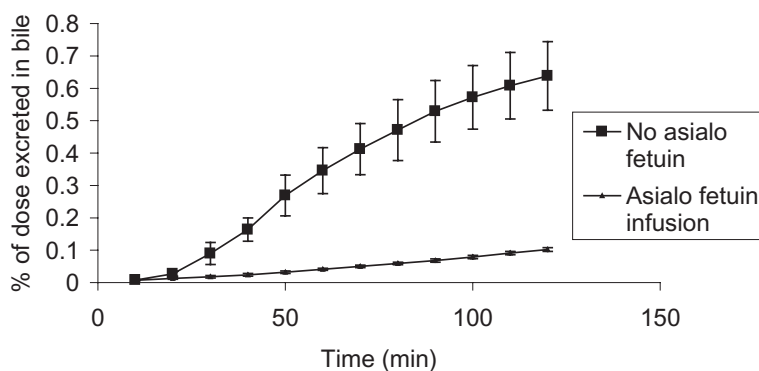


Figure 1. Biliary excretion (mean $\pm$ SD) of sialo UK-279,276 following intravenous administration to the rat in the presence and absence of asialo fetuin ($n = 3$ rats/experiment). The biliary excretion of sialo UK-279,276 was significantly reduced ($p < 0.01$) in the presence asialo fetuin at all time points greater than 40 min.

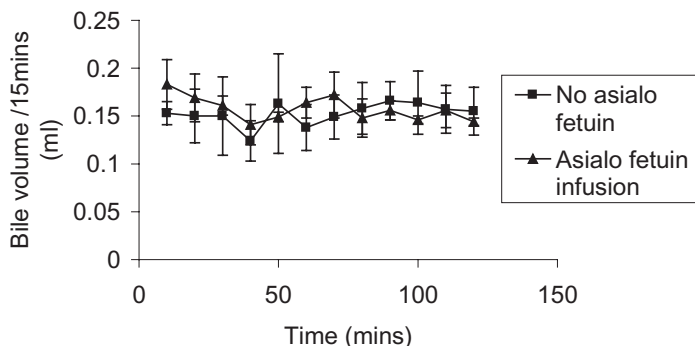


Figure 2. Volume of bile (mean $\pm$ SD) collected from rats when studying the impact of the asialo fetuin on the biliary excretion of UK-279,276. Bile flow was similar in the presence and absence of asialo fetuin at all time points.

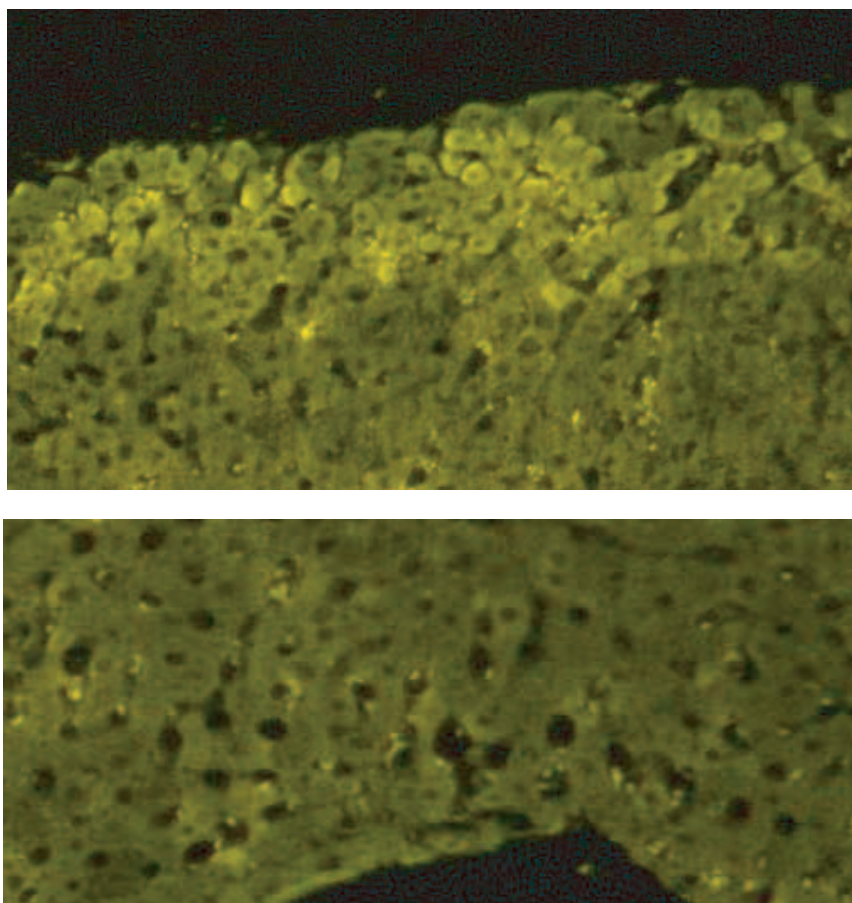


Figure 3. Distribution of FITC-labelled asialo UK-279,276 following a 1-h incubation in precision-cut rat liver slices (top) and in control precision-cut rat liver slice analysed under identical conditions (bottom). Fluorescence labelling was concentrated in the hepatocytes near the surface of the liver slice. No fluorescence was identified in the hepatocytes in the control incubation.

demonstrated that the fluorescence label was concentrated in hepatocytes (figure 3) near the surface of the precision-cut liver slice. No native fluorescence was present in hepatocytes.

Precision-cut liver slice experiments with UK-279,276 in rats, dogs and man

The uptake of sialo and asialo UK-279,276 was quantified in rat (fresh), dog (fresh) and human (cryopreserved) *in vitro* precision-cut liver slices incubations. In all cases, the uptake of the asialo protein was significantly higher than that of sialo UK-279,276 (table 1). Addition of asialo fetuin (a known substrate for the ASGPR) to precision-cut liver slice incubations containing asialo UK-279,276 reduced the uptake of asialo UK-279,276 to levels similar to those observed for the sialo protein. In experiments where liver slices were incubated in 100% CO₂, there was no significant difference between the tissue concentrations of sialo and asialo UK-279,276 (table 2).

The data from the experiments carried out with precision-cut liver slices incubated in the presence of 100% carbon dioxide indicate that there were

Table 1. UK-279,276 concentration in rat, dog and human precision-cut liver slices following incubation for 5 h.

	UK-279,276 concentration in liver slices (% of buffer concentrations)		
	Rat (fresh)	Dog (fresh)	Man (cryopreserved)
Sialo UK-279,276	22.1 ± 2.3	13.0 ± 0.5	68.5 ± 4.9
Asialo UK-279,276	42.91 ± 1.2*	33.6 ± 5.8*	88.7 ± 4.7*
Asialo UK-279,276 + asialo fetuin	18.4 ± 6.5**	12.6 ± 5.8**	62.8 ± 0.4**

Data are expressed as a percentage of the final buffer concentrations (*n* = 3, mean ± SD).
*Asialo-UK-279,276 significantly different from sialo-UK-279,276 and asialo UK-279,276/ asialo fetuin competition experiments (*p* < 0.01).
**Not significantly different from sialo UK-279,276.

Table 2. Effects of incubation in 95% O₂/5% CO₂ and 100% CO₂ on concentrations of UK-279,276 in cryopreserved precision-cut human liver slices following incubation for 5 h.

	UK-279,276 concentration in liver slices % of buffer concentrations	
	95% O ₂ /5% CO ₂	100% CO ₂
Sialo UK-279,276	33.3 ± 2.6	34.3 ± 1.7
Asialo UK-279,276	40.7 ± 2.8**	30.8 ± 3.5

Data are expressed as a percentage of the buffer concentrations (*n* = 3, mean ± SD).
***p* < 0.01.

significant levels of non-specific UK-279,276 binding to the precision-cut liver slices (binding was not mediated by uptake via the ASGPR). Data also suggest that this non-specific binding might differ between species and experiments. In the light of this observation, the data obtained in these experiments were normalized relative to the uptake of the sialo protein (likely to have the lowest ASGPR-mediated hepatic uptake) in each species (figure 4).

The effects of cryopreservation on the disposition of UK-279,276 in precision-cut dog liver slices was also investigated. The data obtained are summarized in figure 5. Again, the asialo UK-279,276 showed a greater uptake into the slice than sialo UK-279,276 and the uptake of asialo UK-279,276 could be inhibited with asialo fetuin.

Discussion

Role of the ASGPR in the clearance of sialo UK-279,276

It is proposed that both sialo and asialo UK-279,276 were cleared by the ASGPR and that the differences in clearance seen for molecules with different sialylation patterns were due to differences in exposed galactose moieties on the termini of the glycan chains of UK-279,276 (Webster *et al.* 1999, Webster. 2000). Two extreme examples are asialo UK-279,276, which is believed to have many exposed galactose moieties on its glycans giving rise to high affinity for the receptor and high clearances, and sialo UK-279,276, which has few exposed galactose residues and low affinity/clearance. Molecules in between these extremes have

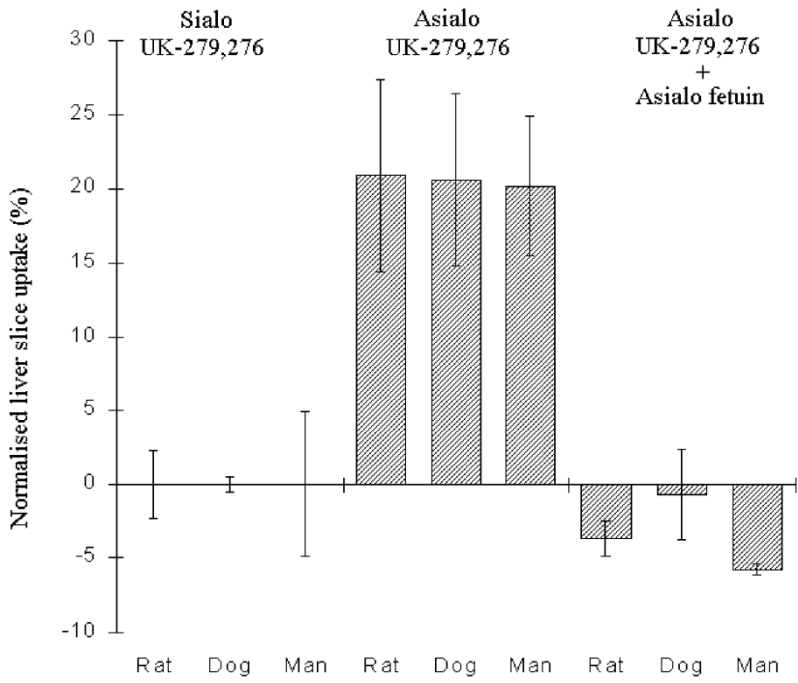


Figure 4. Distribution of sialo and asialo UK-279,276 ($n=3$, mean $\pm$ SD) in rat (fresh), dog (fresh) and human (cryopreserved) precision-cut liver slices following incubation for 5 h. Asialo UK-279,276 different significantly from sialo UK-279,276 and asialo UK-279,276/asialo fetuin competition experiments ($p < 0.01$). Data were normalized by subtracting the slice:buffer concentration ratio of sialo UK-279,276 obtained in an experiment from all other values obtained in liver slices for each species.

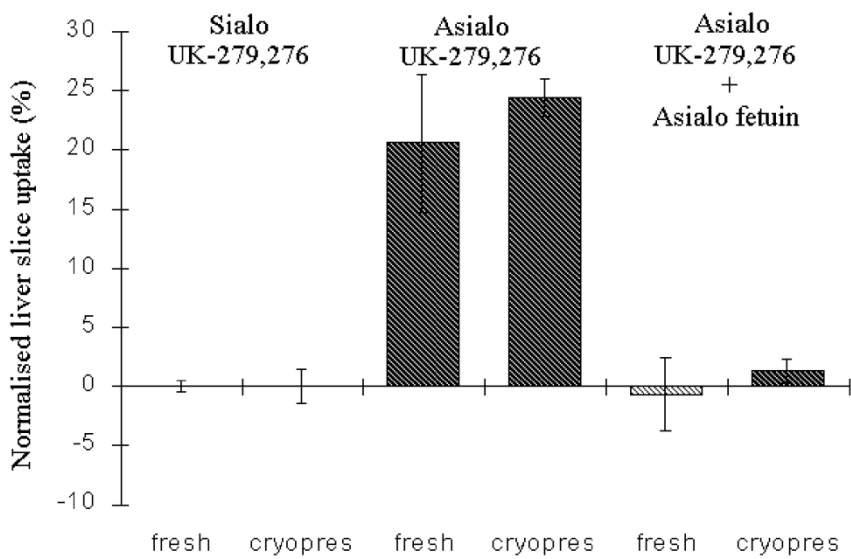


Figure 5. Effect of cryopreservation (cryopres) on the uptake of UK-279,276 by precision-cut dog liver slices following incubation for 5 h ($n=3$, mean $\pm$ SD). Data were normalized by subtracting the slice:buffer concentration ratio of sialo UK-279,276 obtained in an experiment from all other values obtained in liver slices for each species.

clearances dependent on the number of exposed galactose residues and the affinity of these glycans for the ASGPR.

The clearance of asialo UK-279,276 was investigated in the isolated perfused rat liver. These studies have shown that the co-administration of a known substrate for the ASGPR, asialo fetuin (Borges *et al.* 1986, Otter *et al.* 1992, Huseby *et al.* 1993), reduced the hepatic extraction of asialo UK-279,276 (Webster *et al.* 1999). These data support the role of the ASGPR in the clearance of asialo UK-279,276 in the rat. A similar experiment could not be carried out with sialo UK-279,276 and, therefore, the role of the ASGPR in the clearance of sialo UK-279,276 species remained speculative. For this reason, a similar study involving the co-administration of sialo UK-279,276 and asialo fetuin was carried out.

To carry out this investigation, a simple intravenous rat pharmacokinetic study was considered. However, maintaining sufficient asialo fetuin to compete with sialo UK-279,276 would be impractical owing to the marked difference in the half-lives of the two proteins: sialo UK-279,276 having a long half-life (11.5 h) and asialo fetuin having a very short half-life (< 10 min) (Morell *et al.* 1971). An infusion of asialo fetuin was considered, but this would require surgical preparation to allow for both continuous infusion and plasma sampling. In addition, the effects of long-term infusions of asialo fetuin ($0.2\text{--}0.3\text{ g kg}^{-1}\text{ day}^{-1}$) could not be evaluated from the literature data. This pharmacokinetic approach was therefore considered impractical.

The biliary excretion of several proteins known to be cleared by the ASGPR was investigated previously and included: alkaline phosphatase (Scholten *et al.* 1982), human orosomucoid (Schiff *et al.* 1984) and IgA (Schiff and Underdown 1985). These reports indicated that there was a relationship between the plasma disappearance of these proteins and their biliary elimination. In addition, the biliary excretion of proteins cleared by the ASGPR can be inhibited by co-administration of asialo fetuin (Scholten *et al.* 1982). The presence in bile of proteins cleared from the systemic circulation by the ASGPR is surprising. However, it is believed that these proteins are found in bile because a small proportion of the endocytotic vesicles containing the proteins are missorted to the bile canalicular membrane and liberate unchanged protein into the bile (Scholten *et al.* 1982, Schiff *et al.* 1984, Schiff and Underdown 1985). As this process is a random event linked to the uptake of the protein via the ASGPR, it is likely that appearance of drug in bile by misdirected endocytotic vesicles will be proportional to the formation of these vesicle and, therefore, clearance.

Sialo UK-279,276 was therefore evaluated in a similar experiment. A single dose of sialo UK-279,276 was administered to the bile duct cannulated rat and the biliary elimination of UK-279,276 was evaluated in the presence ($10\text{ mg kg}^{-1}\text{ h}^{-1}$, based on Scholten *et al.* 1982) and absence (saline) of asialo fetuin infusion.

Following intravenous administration of sialo UK-279,276 to bile duct cannulated rats, concentrations of sialo UK-279,276 could be detected in bile (figure 1). Over the period of the experiment, $0.63 \pm 0.11\%$ of the dose was eliminated in the bile. Following intravenous administration of sialo UK-279,276 to the rat, approximately 15% of the dose was eliminated from the plasma in the same period. These data indicate that approximately 4% of the dose lost from the systemic circulation was excreted in bile, in keeping with the observations detailed for alkaline phosphates ($2.6 \pm 0.4\%$, Sholtens *et al.* 1982), orosomucoid (1–4%, Schiff *et al.* 1984) and IgA (4%, Schiff and Underdown 1985). The intravenous infusion

of asialo fetuin ($10 \text{ mg kg}^{-1} \text{ h}^{-1}$) following administration of sialo UK-279,276 markedly reduced the biliary elimination of sialo UK-279,276 (figure 1). Analysis of bile flow data (figure 2) indicates that asialo fetuin infusion had no effect on bile flow and thus cannot explain the difference in sialo UK-279,276 biliary excretion. These data indicate that sialo UK-279,276 and asialo fetuin were competing for the same uptake receptor, the ASGPR.

Is UK-279,276 cleared by the ASGPR in rats, dogs and man?

The clearance of UK-279,276 has been shown to be sialylation dependent in both the rat and dog, indicating that in both these species the ASGPR has a role to play in the clearance of UK-279,276. *In vivo* studies in rats and dogs have shown that the clearance of sialo UK-279,276 is low, whilst the clearance of poorly sialylated UK-279,276 is significantly higher (Webster *et al.* 1999, Webster. 2000). A series of isolated perfused rat liver (IPRL) experiments reflected the rat *in vivo* data and also demonstrated that the hepatic extraction of asialo UK-279,276 could be inhibited by the addition of asialo fetuin to the IPRL perfusate. These data indicate that in rats and dogs the ASGPR has a potential role in clearing UK-279,276 from plasma. However, no data were available to demonstrate the clearance of UK-279,276 in man was sialylation dependent and therefore potentially mediated by the ASGPR.

A series of *in vitro* experiments have been carried out to evaluate the role of the ASGPR in the clearance of UK-279,276 in man. Hepatocytes have been used by several authors to investigate the clearance of glycoproteins. For example, isolated hepatocytes have been used to study the uptake of tPA (Otter *et al.* 1992). However, it was decided to use precision-cut liver slices to investigate the role of the ASGPR in the clearance of UK-279,276 in animals and man. The use of precision-cut liver slices offer several advantages over hepatocyte culture including the speed and ease with which precision-cut liver slices can be prepared and the maintenance of intact cell architecture (Worboys *et al.* 1996). Most importantly, precision-cut liver slices contain all cell types present in the liver, whereas the use of hepatocytes would only allow study of this particular cell type. Precision-cut liver slices provide a realistic and reliable representation of the *in vivo* situation (Ekins 1996) and have been used extensively to study the uptake and metabolism of small organic molecules. However, the uptake of large protein molecules has not been extensively evaluated in this preparation.

The uptake of sialo and asialo UK-279,276 was quantified in rat, dog and human *in vitro* precision-cut liver slices incubations. In all cases the uptake of the asialo protein was significantly higher than that of sialo UK-279,276 and the uptake of asialo UK-279,276 could be inhibited by the co-administration of asialo fetuin, a known substrate for the ASGPR (table 1 and figure 4). This observation is in keeping with the properties ascribed to the ASGPR (Spiess 1990). These data support the role of the ASGPR in the clearance of sialo UK-279,276 in all species studied, including man. To ascertain if the uptake of asialo UK-279,276 into precision-cut liver slices was an active process, a series of experiments were carried out using human precision-cut liver slices poisoned with carbon dioxide. In these experiments, there was no significant difference between the tissue concentrations of sialo and asialo UK-279,276, demonstrating that the uptake of asialo UK-279,276 is an energy-dependent process (table 2). The level of non-specific binding seen in the initial dog experiments (table 1) and in the experiments used to

determine whether uptake into slices was an active process (table 2) was markedly different. The experimental design was identical in both cases and it is unclear why there was a marked increase in the apparent non-specific binding.

At the time of these experiments, only cryopreserved human precision-cut liver slices were available to investigate the uptake of UK-279,276 in man. Therefore, before the use of cryopreserved precision-cut human liver slices, a series of experiments were carried out using dog precision liver slices to investigate the effects of cryopreservation on the disposition of UK-279,276. The data obtained are summarized in figure 5. A comparison of the uptake profile obtained for UK-279,276 in fresh and cryopreserved precision-cut dog liver slices demonstrated that this cryopreservation technique did not markedly effect the uptake of UK-279,276 into precision-cut dog liver slices or its behaviour when co-incubated with asialo fetuin. It has been assumed the same will apply for precision-cut human liver slices.

The *in vitro* precision-cut liver slice observations were in keeping with the previous *in vivo* data generated for UK-279,276 in rat and dog. The similarities in the *in vivo* and *in vitro* data generated in rat and dog suggest that precision-cut liver slices can be used successfully to mimic the *in vivo* situation in these species. In addition, the FITC asialo UK-279,276 has been shown to distribute to hepatocytes in precision-cut liver slices. Therefore, the *in vitro* data generated in precision-cut human liver slices support the potential role of the ASGPR in the clearance of UK-279,276 in man.

In conclusion, this series of experiments demonstrates that both sialo and asialo UK-279,276 interact with the ASGPR in rat and that asialo UK-279,276 can interact with this receptor in rat, dog and man. In the light of these observations, it is believed that UK-279,276 with a range of sialylation profiles ranging from highly to poorly sialylated is cleared via ASGPR in rat, dog and man. The differences in the clearances obtained for UK-279,276 molecules with different sialylation profiles are believed to be due to the differing number of exposed galactose moieties on the termini of the glycan chains and their effects on the affinity of the molecule for the ASGPR.

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Dose-response studies on the induction of liver cytochromes P4501A1 and 1B1 by polycyclic aromatic hydrocarbons in arylhydrocarbon-responsive C57BL/6J mice

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Received 30 January 2003

1. To determine the biological effects of 23 polycyclic aromatic hydrocarbons (PAHs) and 3,4,3',4'-tetrachlorobiphenyl, the dose-response studies of the induction of CYP1-dependent xenobiotic oxidation activities by these chemicals in liver microsomes of C57BL/6J mice were studied.

2. In arylhydrocarbon-responsive C57BL/6J mice, the liver microsomal xenobiotic oxidation with substrates of 7-ethoxyresorufin, 7-ethoxycoumarin, ($\pm$)-benzo[*a*]pyrene-7,8-diol, dibenzo[*a,l*]pyrene-11,12-diol and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline increased by increasing the doses of PAHs to mice, particularly when the PAHs that have been reported to be carcinogenic in experimental animals were used. In arylhydrocarbon receptor-knockout mice, there were no increases in liver microsomal 7-ethoxyresorufin *O*-deethylation activities nor in liver mRNA levels of CYP1A1, 1A2 and 1B1 by these chemicals.

3. Of the chemicals examined, benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, benzo[*j*]-fluoranthene, 3-methylcholanthrene, dibenz[*a,h*]anthracene, dibenz[*a,c*]anthracene and 3,4,3',4'-tetrachlorobiphenyl were potent inducers of the induction of liver microsomal 7-ethoxyresorufin *O*-deethylation in mice.

4. Other PAHs such as 5-methylchrysene, benzo[*a*]pyrene, dibenzo[*a,l*]pyrene, dibenz[*a,j*]acridine, benzo[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene moderately induced 7-ethoxyresorufin *O*-deethylation activities in mice. PAHs reported to be weak or less carcinogenic in experimental animals did not induce the xenobiotic oxidation activities of CYP1A1 and 1B1 in the mice.

5. The results suggest that induction of liver microsomal CYP1-dependent xenobiotic oxidation activities is a good tool in determining the potencies of carcinogenic PAHs in arylhydrocarbon-responsive C57BL/6J mice.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental carcinogens that cause several types of cancers in various organs of animals including man (Conney 1982, Pelkonen and Nebert 1982). The carcinogenic PAHs are chemically inert and require metabolic activation by several drug-metabolizing enzymes to active metabolites that interact with DNA to initiate cell transformation (Gelboin 1980, Conney 1982, Guengerich and Shimada 1991). Cytochrome

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P450 (P450 or CYP) and epoxide hydrolase are the principal enzymes that convert PAHs to highly carcinogenic metabolites, namely diol-epoxides (Gelboin 1980, Conney 1982, Guengerich and Shimada 1991, Shimada *et al.* 1999). For example, B[a]P is first oxidized by P450s to form B[a]P-7,8-oxide and then this epoxide is hydrolysed by epoxide hydrolase to form B[a]P-7,8-diol. Finally, the B[a]P-7,8-diol is again oxidized by P450s resulting formation of highly reactive 7,8-diol-9,10-epoxides (Conney 1982, Shimada *et al.* 1999). CYP1A1 and 1B1 have been shown to have major roles in these activation steps of carcinogenic PAHs (Shimada *et al.* 2001b).

The arylhydrocarbon receptor (AhR) regulates the expression and induction of several drug-metabolizing enzymes including CYP1A1, 1A2 and 1B1 (Shimada *et al.* 2002a, b). Recently, it was reported that B[a]P does not cause tumour formation in AhR-knockout mice, suggesting that P450s and other enzymes have critical roles in initiating cell transformation by B[a]P (Shimizu *et al.* 2000). Similarly, 7,12-DMBA and DB[a,l]P have been reported not to produce tumours in CYP1B1-knockout mice (Buters *et al.* 1999, 2002). Collectively, these results indicate that the levels of expression of P450 and other drug-metabolizing enzymes may correlate with susceptibilities towards carcinogenesis by these carcinogenic PAHs. Recent studies have established that both CYP1A1 and 1B1 are induced by carcinogenic PAHs in various organs of AhR(+/+), but not AhR(-/-), mice and that CYP1A2 is only induced in the livers of AhR(+/+) mice by these PAHs (Shimada *et al.* 2002a, b). There are numerous reports attempting to determine toxic equivalency factors (TEFs) for the biological effects of a variety of PAH compounds in order to assess the carcinogenic potentials of these compounds in the environment (Nisbet and LaGoy 1992, Postlind *et al.* 1993, Petry *et al.* 1996, Sjogren *et al.* 1996, Reeves *et al.* 2001).

The present studies were undertaken to determine the biological effects of 23 PAHs and to compare these effects with those of 3,4,3',4'-TCB by measuring induction of CYP1-dependent xenobiotic oxidation in liver microsomes of AhR(+/+) mice. Twenty-three PAHs were selected to determine the ED₅₀ values for the induction of CYP1-dependent xenobiotic oxidation in mice, since most of these compounds have had their carcinogenic potencies determined in mice *in vivo* (Gelboin 1980, Conney 1982, Pelkonen and Nebert 1982). Different doses of PAHs and 3,4,3',4'-TCB (figure 1) were injected intraperitoneally (i.p.) to mice and the levels of total P450 and CYP1-related xenobiotic oxidation activities were determined in liver microsomes. The substrates used were 7-ethoxyresorufin, 7-ethoxycoumarin, (±)B[a]P-7,8-diol, DB[a,l]P-11,12-diol and MeIQ, and the latter three chemicals were examined for the determination of genotoxic activation to DNA-damaging products in *S. typhimurium* NM2009 tester strain (Shimada and Okuda 1988, Shimada *et al.* 1994). ED₅₀ values for the induction of CYP1-related xenobiotic oxidation activities were determined with these PAHs and 3,4,3',4'-TCB.

Materials and methods

Chemicals

Acenaphthene, acenaphthylene, 6-aminochrysene, anthracene, B[a]A, B[b]FA, B[j]FA, B[k]FA, B[a]P, benzo[e]pyrene, chrysene, DB[a,l]P, DB[a,e]P, DB[a,c]A, DB[a,h]A, DB[a,j]Ac, 7,12-DMBA, fluoranthene, 3-MC, 5-methylchrysene, 1-nitropyrene, phenanthrene and pyrene were purchased from

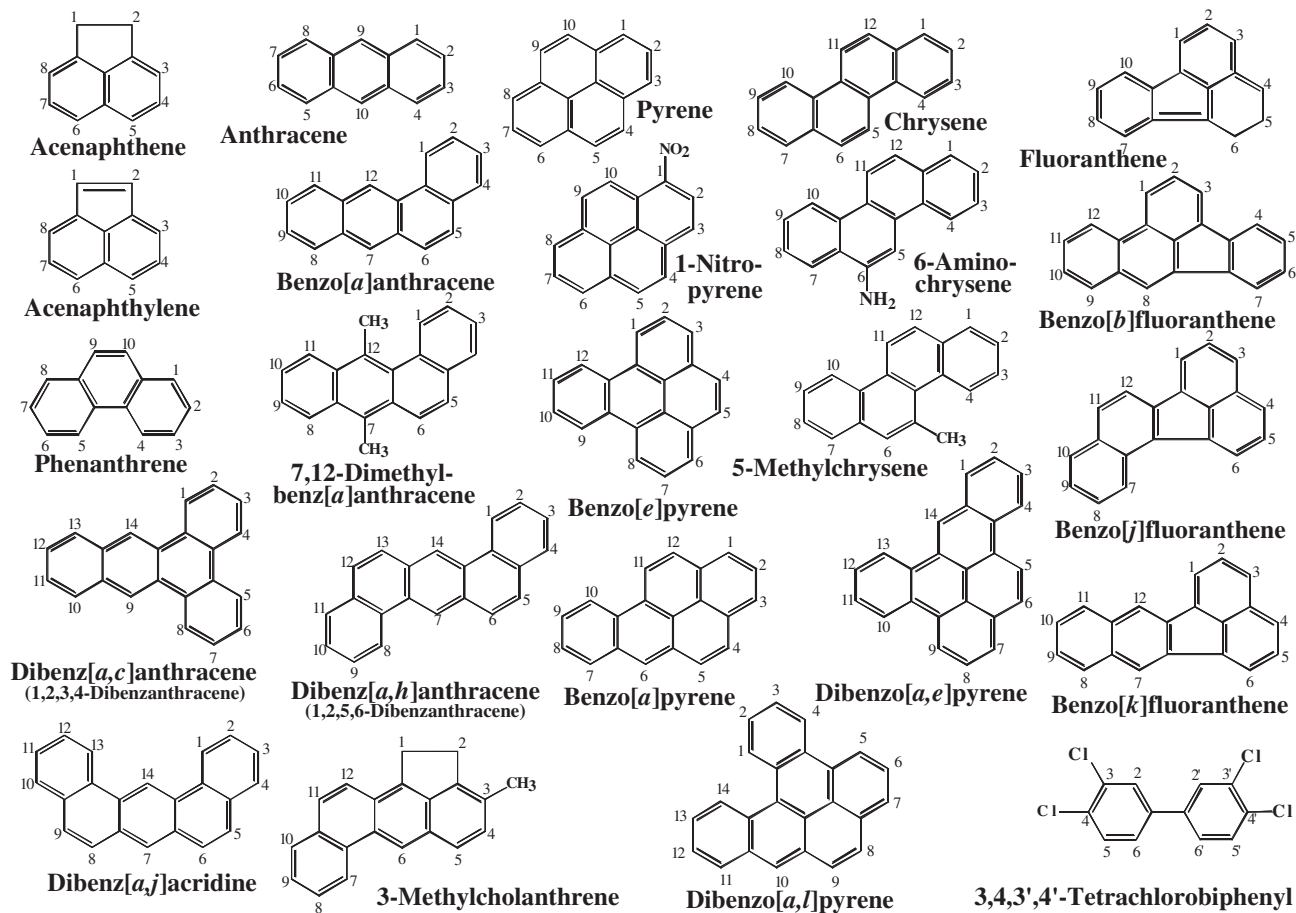


Figure 1. Chemical structures of the PAHs and 3,4,3',4'-TCB used.

Sigma Chemical Co. (St Louis, MO, USA) or Kanto Kagaku Co. (Tokyo, Japan). 3,4,3',4'-TCB was purchased from Kanto Kagaku. These chemicals were used without further purification; their purities have been described in the manufacturers' papers. Other chemicals and reagents used here were obtained from sources described previously or were of the highest qualities commercially available (Shimada *et al.* 2001a, b, 2002a, b).

AhR(+/+) and *AhR*(-/-) mice

AhR(-/-) mice generated by using a homologous recombination method were kindly donated by Dr Yoshiaki Fujii-Kuriyama, Tohoku University (Mimura *et al.* 1997, Shimizu *et al.* 2000). Male *AhR*(-/-) mice were back-crossed to C57BL/6J *AhR*(+/+) female mice to give rise to heterozygotes. The genotype of each pup was determined by analysing the presence of the mutant *AhR* allele by PCR with genomic DNA (Shimizu *et al.* 2000, 2002a). The PCR conditions used for the determination of the *AhR* genotype were described by Shimizu *et al.* (2000).

Treatment of mice with PAHs and PCBs, isolation of liver and lung RNA, and preparation of liver microsomes

Mice received a single i.p. injection of PAHs or 3,4,3',4'-TCB (both dissolved in olive oil) at dose levels of 5, 10, 20, 40 and 100 mg kg⁻¹ body weight; these dose levels were chosen on the basis of previous studies in which the mice received several PAHs at dose levels of 0.01, 0.1, 1, 10 and 100 mg kg⁻¹ (Shimada *et al.* 2002a). The number of animals used for each dose levels was one except that eight animals were used for control group in which these mice received olive oil alone (0.25 ml/25 g of mice). For the experiments to determine levels of mRNAs in livers of mice treated with PAHs, two mice in each group were used. The mice were killed at 72 h after dosing and the livers removed.

Total RNA was extracted from livers using Isogen (Nippon Gene Co., Toyama, Japan) as described by Shimada *et al.* (2002a). Briefly, 50–100 mg tissue samples were added to 1 ml Isogen (phenol and guanidine thiocyanate) and homogenized to lyse the cells. After standing the homogenates at room temperature for 5 min, 0.2 ml chloroform were added and the solutions mixed vigorously and centrifuged at 10 000g for 20 min. The RNA fraction was recovered from the upper layer and further precipitated with 0.5 ml isopropanol (Shimada *et al.* 2002a).

Liver microsomes were prepared by the methods described by Guengerich (1989) and were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v).

RT-PCR procedure for the assay of P450 mRNAs

Levels of mRNAs of CYP1A1, CYP1A2, CYP1B1 and β -actin in several tissues were determined using a mRNA Selective PCR Kit (Takara Shuzo Co., Tokyo, Japan) as described by Shimada *et al.* (2002a). Briefly, about 1 μ g total RNA were reverse-transcribed to cDNAs and the resultant cDNAs were subjected to PCR using the specific primers for the genes of CYP1A1, 1A2, and 1B1 and β -actin. The PCR reactions were performed with AMV Optimized Taq DNA polymerase (Takara PCR Kit, Tokyo, Japan) as described by Shimada *et al.* (2002a). The levels of mRNA expression of CYP1A1, 1A2 and 1B1 were calculated by obtaining ratios of staining intensities of each P450 mRNAs to β -actin mRNA on the same agarose gels.

Assay methods

7-Ethoxyresorufin and 7-ethoxycoumarin *O*-deethylation activities of liver microsomes were determined by the methods, as described (Shimada *et al.* 1998a, Yamazaki *et al.* 1999). P450-dependent activation of procarcinogens to reactive products that cause induction of *umu* gene expression in the tester strain *S. typhimurium* NM2009 was determined as described (Shimada *et al.* 1989, 1996). Standard incubation mixtures contained microsomal protein (5 μ g protein in the case of MeIQ and 10 μ g protein for ($\pm$)B[a]P-7,8-diol and DB[a,l]P-11,12-diol) and procarcinogens (0.1 μ M for MeIQ, 2.5 μ M ($\pm$)B[a]P-7,8-diol and DB[a,l]P-11,12-diol) in a final volume of 1.0 ml 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 0.5 unit ml⁻¹ glucose 6-phosphate dehydrogenase) and 0.75 ml bacterial suspension. The induction of *umu* gene expression is presented as units of β -galactosidase activity min⁻¹ mg⁻¹ protein (Shimada *et al.* 1994).

Other assays

P450 and protein contents were estimated by published methods described by Omura and Sato (1964) and Lowry *et al.* (1951). ED₅₀ values were calculated by using linear regression analysis (Litchfield and Wilcoxon 1949).

Results

The levels of induction of hepatic mRNAs of CYP1A1, 1A2 and 1B1 and activities of 7-ethoxyresorufin *O*-deethylation were first determined in liver microsomes of AhR(+/+) mice treated i.p. with the 23 PAHs and 3,4,3',4'-TCB (figure 1) at a dose level of 100 mg kg⁻¹ (figure 2). Both CYP1A1 and 1B1 were highly induced by PAHs that have been reported to be carcinogens in experimental animals (Conney 1982, Pelkonen and Nebert 1982, Shimada *et al.* 2002a, b). These PAHs include B[k]FA, B[b]FA, B[j]FA, B[a]P, 3-MC, DB[a,h]A, B[a]A and DB[a,l]P. Other PAHs such as DB[a,j]Ac, DB[a,c]A, 7,12-DMBA, 5-methylchrysene, 6-aminochrysene, chrysene and DB[a,e]P also induced mRNAs of liver CYP1A1 and 1B1, although the levels of induction of P450s were lower than for the animal carcinogens. CYP1A2 was constitutively expressed in the liver of mice and induced to a much lesser extent than for CYP1A1 and CYP1B1 (Shimada *et al.* 2002a, b).

Induction of hepatic 7-ethoxyresorufin *O*-deethylation activities were closely related to the levels of expression of liver CYP1A1 ($r=0.91$) and CYP1B1 ($r=0.94$) in mice treated with PAHs and 3,4,3',4'-TCB (figure 2).

Note that the 23 PAHs and 3,4,3',4'-TCB did not induce liver mRNAs of CYPs1A1, 1A2 and 1B1 or 7-ethoxyresorufin *O*-deethylation activity in liver microsomes of AhR(-/-) mice (data not shown).

Dose-response curves for the induction of P450-dependent xenobiotic oxidation activities were determined with the PAHs and 3,4,3',4'-TCB at dose levels of 5, 10, 20, 40 and 100 mg kg⁻¹ in AhR(+/+) mice. We measured total P450 contents, 7-ethoxyresorufin and 7-ethoxycoumarin *O*-deethylation activities as well as the activities of metabolic activation of (±)B[a]P-7,8-diol, DB[a,l]P-11,12-diol and MeIQ to genotoxic metabolites in liver microsomes of these mice. Dose-response curves for the induction of 7-ethoxyresorufin *O*-deethylation in mouse liver microsomes suggested that 3-MC, B[b]FA, DB[a,h]A, B[k]FA, B[j]FA, DB[a,c]A and 3,4,3',4'-TCB were potent inducers and 5-methylchrysene, B[a]P, DB[a,l]P, DB[a,j]Ac, B[a]A and 7,12-DMBA were moderate inducers (figures 3 and 4). 6-Aminochrysene, chrysene, DB[a,e]P, 1-nitropyrene and B[e]P were very weak inducers of liver microsomal 7-ethoxyresorufin *O*-deethylation activities, if at all. Other PAHs such as acenaphthene, acenaphthylene, anthracene, pyrene and phenanthrene were not inducers in mice. Similar tendencies in the dose-dependent induction were also observed for the metabolic activation of (±)B[a]P-7,8-diol and DB[a,l]P-11,12-diol to DNA-damaging products in *S. typhimurium* NM2009 (figures 4 and 5).

P450 contents, 7-ethoxyresorufin and 7-ethoxycoumarin *O*-deethylation activities, and metabolic activation of the three potential carcinogens correlated well with each other (figure 6). Among the parameters examined, there was a good correlation between activation of (±)B[a]P-7,8-diol and DB[a,l]P-11,12-diol to genotoxic metabolites with the correlation line passing through the origin (figure 6).

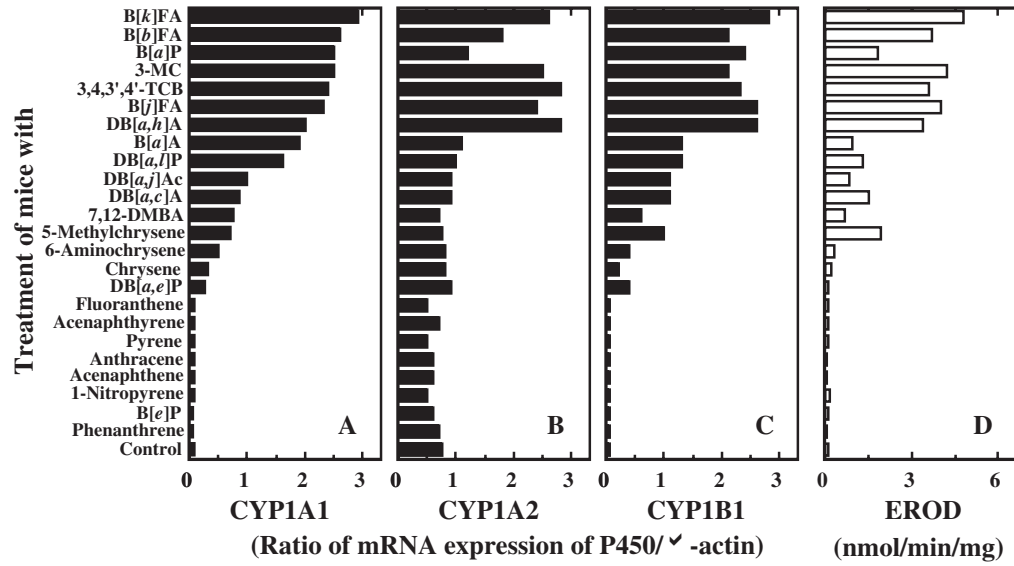


Figure 2. Levels of expression of liver mRNA of CYP1A1 (A), CYP1A2 (B) and CYP1B1 (C), and activities of liver microsomal 7-ethoxyresorufin *O*-deethylation (D) in mice treated i.p. with 100 mg kg⁻¹ of xenobiotics. P450 mRNA expression was determined relative to that of β -actin mRNA. Data are means from two mice.

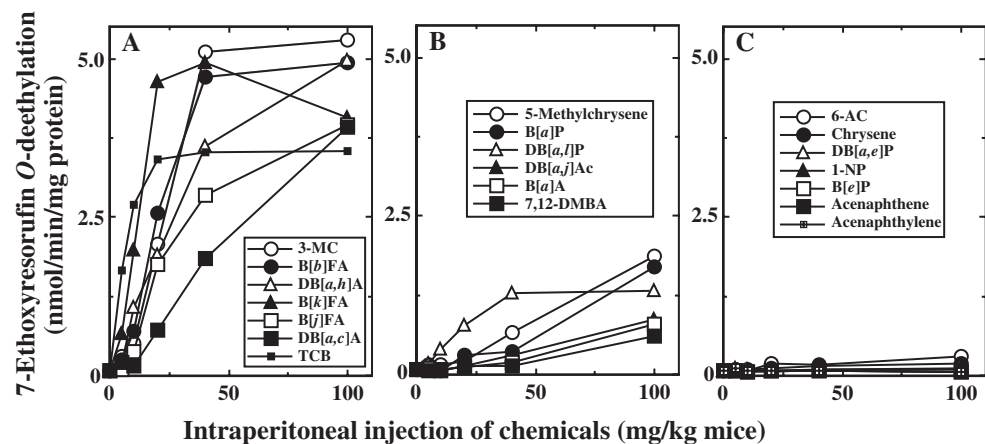


Figure 3. Dose-response curves for the induction of 7-ethoxyresorufin *O*-deethylation in liver microsomes of mice treated i.p. with 0, 5, 10, 20, 40 and 100 mg kg⁻¹ mice (*n* = 8 for control, *n* = 1 for dose groups).

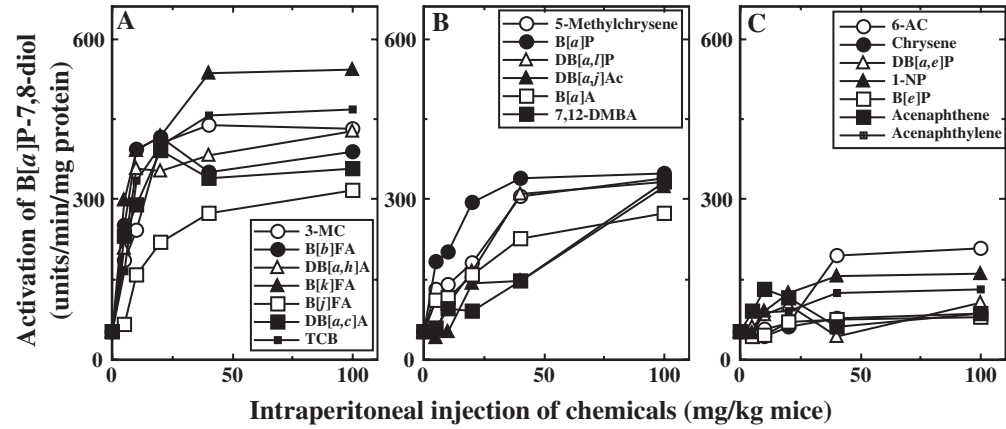


Figure 4. Dose-response curves for the induction of activities of (±)B[a]P-7,8-diol to DNA-damaging products in liver microsomes of mice treated i.p. with 0, 5, 10, 20, 40 and 100 mg kg⁻¹ mice (*n* = 8 for control, *n* = 1 for dose groups).

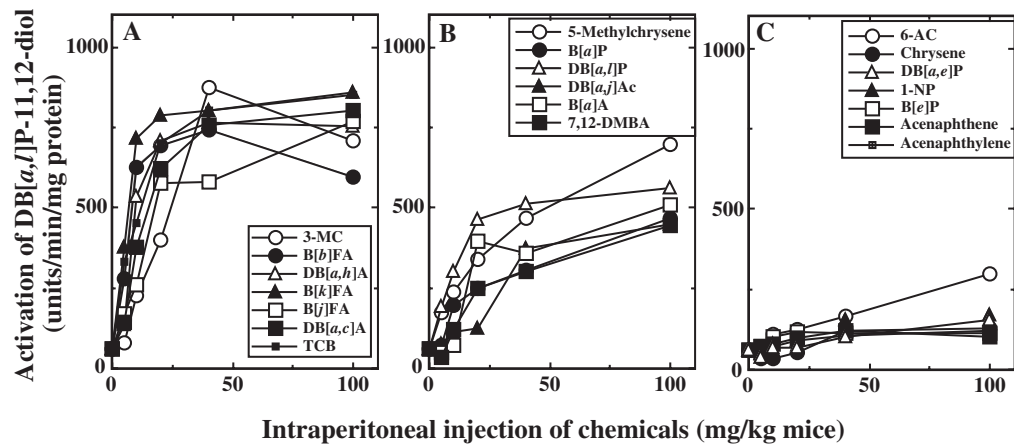


Figure 5. Dose-response curves for the induction of activities of DB[a,l]P-11,12-diol to DNA-damaging products in liver microsomes of mice treated i.p. with 0, 5, 10, 20, 40 and 100 mg kg⁻¹ mice (*n* = 8 for control, *n* = 1 for dose groups).

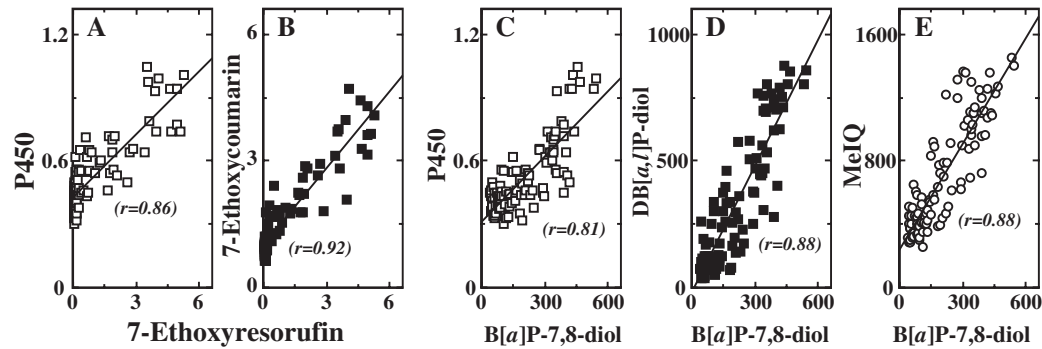


Figure 6. Correlation of P450 contents and 7-ethoxyresorufin *O*-deethylation activity and of activation of ($\pm$)B[a]P-7,8-diol, DB[a,I]/P-11,12-diol and MeIQ in liver microsomes of mice treated with various chemicals at dose levels of 0, 5, 10, 20, 40 and 100 mg kg⁻¹ of mice. Control mice ($n=8$) and mice treated with 23 PAHs and 3,4,3',4'-TCB (total=120) were used for calculation.

Table 1. ED₅₀ values for induction of P450 content and P450-dependent xenobiotic oxidation activities by PAHs in liver microsomes of AhR(+/+) mice.

	ED ₅₀ (mg of dose/kg ⁻¹ mice)					
	P450 ^a	7-Ethoxy- resorufin ^b	7-Ethoxy- coumarin ^b	(±)B[a]P- 7,8-diol ^c	DB[a,l]P- 11,12-diol ^c	MeIQ ^c
3-MC	56	30	25	18	36	13
B[b]FA	45	31	36	9	5	20
DB[a,h]A	46	36	60	10	4	13
B[k]FA	33	18	8	40	5	33
B[j]FA	75	55	66	65	38	5
DB[a,c]A	65	72	52	20	18	14
3,4,3',4'-TCB	32	22	18	12	15	18
5-Methylchrysene	95	130	160	60	46	39
B[a]P	90	200	140	40	100	52
DB[a,l]P	300	105	200	50	40	30
DB[a,j]Ac	160	230	380	100	80	65
B[a]A	107	240	330	101	60	50
7,12-DMBA	105	410	410	85	104	100
6-Aminochrysene	> 1000	750	250	260	250	170
Chrysene	> 1000	> 1000	> 1000	800	580	> 1000
DB[a,e]P	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
1-Nitropyrene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
B[e]P	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Acenaphthene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Acenaphthylene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Phenanthrene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Anthracene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Fluoranthene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Pyrene	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000

Units of activity are as described in the text.

^aP450 content; ^bO-deethylation activity; ^ccarcinogen activation.

Finally, the ED₅₀ values were calculated for the induction of the above parameters in liver microsomes of these mice treated with different dose levels of the 23 PAHs and 3,4,3',4'-TCB (table 1). To determine accurately the ED₅₀ values, the base values (of control animals) were subtracted from the experimental values of animals dosed with PAHs and 3,4,3',4'-TCB. The base values used were 0.41 nmol mg⁻¹ protein for P450, 0.08 nmol min⁻¹ mg⁻¹ protein for 7-ethoxyresorufin O-deethylation, 0.85 nmol min⁻¹ mg⁻¹ protein for 7-ethoxycoumarin O-deethylation, 51 units min⁻¹ mg⁻¹ protein for the activation of (±)B[a]P-7,8-diol, 61 units min⁻¹ mg⁻¹ protein for the activation of DB[a,l]P-11,12-diol, and 285 units min⁻¹ mg⁻¹ protein for the activation of MeIQ. The maximal rates (100% values) for the induction of P450, 7-ethoxyresorufin O-deethylation and 7-ethoxycoumarin O-deethylation and the activation of (±)B[a]P-7,8-diol, DB[a,l]P-11,12-diol and MeIQ by PAHs and 3,4,3',4'-TCB were tentatively determined as 1.11, 5.48, 4.95, 550, 900 and 1500 nmol min⁻¹ mg⁻¹, respectively. These rates were taken from the values that were the highest in mice treated either with potent inducers of PAHs and 3,4,3',4'-TCB.

3-MC, B[b]FA, DB[a,h]A, B[k]FA, B[j]FA, DB[a,c]A and 3,4,3',4'-TCB had relatively low ED₅₀ values for total P450 contents and 7-ethoxyresorufin and 7-ethoxycoumarin O-deethylation activities and for the metabolic activation of (±)B[a]P-7,8-diol, DB[a,l]P-11,12-diol and MeIQ to genotoxic metabolites (table 1). ED₅₀ values for the induction of liver microsomal P450-dependent

activities by different doses of 5-methylchrysene, B[a]P, DB[a,l]P, DB[a,j]Ac, B[a]A and 7,12-DMBA were determined to be higher than the former PAHs and 3,4,3',4'-TCB. Other compounds examined had high ED₅₀ values for the induction of microsomal CYP1-dependent activities ($> 1000 \text{ mg kg}^{-1}$) (table 1).

Discussion

The present results have demonstrated that potent carcinogenic PAHs are strong inducers of CYP1-dependent xenobiotic oxidation activities in liver microsomes of AhR(+/+) mice. Since these PAHs do not induce the liver microsomal activities at all in AhR(-/-) mice, it is suggested that the increases in total P450 contents and CYP1-dependent xenobiotic oxidation activities are due to the induction of CYP1A1, 1A2 and 1B1 that are regulated through AhR (Hankinson 1995, Whitlock 1999, Shimada *et al.* 2002a, b). Potent carcinogenic PAHs and 3,4,3',4'-TCB highly induced several CYP1-dependent xenobiotic oxidation activities including 7-ethoxyresorufin *O*-deethylation, 7-ethoxycoumarin *O*-deethylation, activation of ($\pm$)B[a]P-7,8-diol, DB[a,l]P-11,12-diol and MeIQ to DNA-damaging products in *S. typhimurium* NM2009.

There were good correlations between liver mRNA levels of CYP1A1 and 1B1 and liver microsomal 7-ethoxyresorufin *O*-deethylation activities in liver microsomes of AhR(+/+) mice treated with 23 PAHs and 3,4,3',4'-TCB (figure 2). Constitutive levels of liver CYP1A2 mRNA were usually significant in control animals as compared with the low levels of CYP1A1 and 1B1 mRNAs in the liver; the extent of induction of CYP1A2 mRNA was smaller than that for CYP1A1 and 1B1 in AhR(+/+) mice treated with PAHs and 3,4,3',4'-TCB (figure 2). These results indicate that liver microsomal 7-ethoxyresorufin *O*-deethylation is a good marker for induction of CYP1A1 and 1B1 in mouse liver. Our previous results suggested that human CYP1A1 is more active (about several fold) than human CYP1B1 in catalysing 7-ethoxyresorufin *O*-deethylation activities in recombinant enzymes systems with *E. coli* and *Trichoplusia ni* cells (Shimada *et al.* 1998b, 2001a). However, it is not known at present whether mouse CYP1A1 is more active than mouse CYP1B1 in the *O*-deethylation of 7-ethoxyresorufin. Savas *et al.* (1997) reported that there are differences in substrate specificities in mouse and human CYP1B1.

The dose-response effects of several PAHs were determined for the induction of CYP1A1, 1A2 and 1B1 at dose levels of 0.01, 0.1, 1, 10 and 100 mg kg⁻¹ body weight (Shimada *et al.* 2002a). However, the ED₅₀ values in the paper by Shimada *et al.* have not been determined precisely. The present study also determined the ED₅₀ values for the induction of CYP-related activities with PAHs and 3,4,3',4'-TCB at dose levels of 5, 10, 20, 40 and 100 mg kg⁻¹. ED₅₀ values for PAHs and 3,4,3',4'-TCB for the induction of 7-ethoxyresorufin *O*-deethylation indicated that 3-MC, B[b]FA, DB[a,h]A, B[k]FA, B[j]FA, DB[a,c]A and 3,4,3',4'-TCB are potent inducers of CYP1A1 and CYP1B1, and that B[a]P, DB[a,l]P, DB[a,j]Ac, B[a]A and 7,12-DMBA induced this activity only moderately. The former group of inducers have ED₅₀ values below 100 mg kg⁻¹ and the latter group of 100–400 mg kg⁻¹. Other PAHs including 6-aminochrysene, chrysene, DB[a,e]P, 1-nitropyrene and B[e]P were only very weak inducers of liver microsomal 7-ethoxyresorufin *O*-deethylation activity, if at all. Acenaphthene, acenaphthylene, phenanthrene, anthracene, fluoranthene and pyrene did not induce microsomal

7-ethoxyresorufin *O*-deethylation activities and these chemicals have been reported to be weak carcinogens *in vivo* in experimental animals (Conney 1982, Pelkonen and Nebert 1982, Reeves *et al.* 2001).

Metabolic activation of ($\pm$)B[a]P-7,8-diol and DB[a,l]P-11,12-diol to DNA-damaging metabolites in *S. typhimurium* NM2009 were well correlated with each other and also with 7-ethoxyresorufin *O*-deethylation activity in liver microsomes of mice treated with different doses of 23 PAHs and 3,4,3',4'-TCB (figure 6). Both ($\pm$)B[a]P and DB[a,l]P have been previously reported to be potent carcinogens in mice (Conney 1982, Higginbotham *et al.* 1993, Nesnow *et al.* 1998, Shimizu *et al.* 2000) and recently it has been reported that DB[a,l]P is non-carcinogenic in mice when the *CYP1B1* gene is disrupted (Buters *et al.* 2002). The PAH chemicals are activated to reactive metabolites by CYP1A1 and CYP1B1 and are induced by the PAH themselves. Although 3,4,3',4'-TCB is a potent inducers of liver microsomal CYP1-dependent xenobiotic oxidation activities, this chemical, like other poly-halogenated biphenys and TCDD, is not converted to active metabolites that cause DNA-damage, and thus does not initiate cell transformations in mammals (Hankinson 1995, Hu and Bunce 1999).

Most of the 23 PAH chemicals have been previously examined for their carcinogenic potential in experimental animal models. However, some of the PAHs have not been examined for *in vivo* carcinogenicity in detail (Freudenthal and Jones 1976). For example, although B[b]FA has been reported to be carcinogenic in experimental animals, two other isomers, B[j]FA and B[k]FA, have not been examined for their carcinogenicity *in vivo* (Freudenthal and Jones 1976, Hecht *et al.* 1995, Hecht 1999). The present results have demonstrated that B[b]FA, B[j]FA and B[k]FA are equally potent at inducing liver microsomal CYP1-dependent xenobiotic oxidation activities in AhR(+/+) mice. The present study has also demonstrated that the two isomeric B[a,c]A and B[a,h]A compounds are potent inducers of CYP1-dependent xenobiotic oxidation activity, although only B[a,h]A has been identified as a strong carcinogen in experimental animals (Freudenthal and Jones 1976, Higginbotham *et al.* 1993, Shou *et al.* 1996). DB[a,e]P, as with DB[a,l]P, has been reported to be carcinogenic in rodents. However, the former chemical induces CYP1-dependent xenobiotic oxidation activities to a lesser extent than DB[a,l]P in AhR(+/+) mice (Freudenthal and Jones 1976, Higginbotham *et al.* 1993, Amin *et al.* 1995, Hecht 1999).

In conclusion, the present results have demonstrated that CYP1-related xenobiotic oxidation activities in liver microsomes of AhR(+/+) mice are good tools to determine the biological effects of carcinogenic PAHs. 7-Ethoxyresorufin *O*-deethylation activity was a good indicator for the detection of induction of CYP1A1 and CYP1B1 in mouse liver microsomes and it was also found that the activities of activation of ($\pm$)B[a]P-7,8-diol and DB[a,l]P-11,12-diol to DNA-damaging products in *S. typhimurium* NM2009 were also good indicators for the detection of CYP1A1 and CYP1B1 activities in the mouse.

Acknowledgements

The work was supported in part by Grants from the Ministry of Education, Science, and Culture of Japan, the Ministry of Health and Welfare of Japan, the Ministry of Economy, Trade, and Industry of Japan, and the Petroleum Energy

Research Center, Japan. The authors thank Professor Yoshiaki Fujii-Kuriyama for supplying engineered C57BL/6J mice.

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Mechanism-based inhibition of CYP activities in rat liver by fluoxetine and structurally similar alkylamines

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Received 29 January 2003

1. The inhibition of cytochrome P450 (CYP)-mediated substrate oxidations by alkylamine-based drugs was investigated in rat hepatic microsomes. The effects of pre-incubation of the drugs with NADPH-fortified microsomes on inhibition potency was evaluated in relation to the formation of metabolite intermediate (MI) complexes with CYP *in vitro*.

2. The selective serotonin-reuptake inhibitor fluoxetine (FLU) emerged as a potent and preferential inhibitor of CYP2C11 in rat liver microsomes. After FLU biotransformation in NADPH-supplemented microsomes, IC_{50} values of 2 and $1\mu M$ were determined against CYP2C11-dependent testosterone 2α - and 16α -hydroxylation; in the absence of pre-incubation, the corresponding IC_{50} values were 47 and $39\mu M$. MI complexation of CYP appeared to contribute significantly to inhibition by FLU, as evidenced by the 21% decrease in apparent microsomal CYP content produced by $50\mu M$ FLU in the presence of NADPH.

3. The secondary amines nisoxetine (NIS), and especially, desipramine (DES) and nortriptyline (NOR), also inhibited CYP2C11 and generated MI complexes with microsomal CYP. In contrast, with the exception of SKF-525-A, tertiary alkylamines (10 compounds) inhibited specific CYP activities but did not form MI complexes. Pre-incubation of these agents with NADPH-supplemented microsomes did not enhance inhibition of CYP activities, thus suggesting that formation of inhibitory metabolites was minimal for these compounds.

4. These findings implicate drug-mediated MI complexation of CYPs in the inhibition of hepatic biotransformation processes by secondary alkylamines. In contrast, tertiary amines did not generate significant quantities of CYP–MI complexes under the test conditions. Despite their diffusion from the CYP active site, inhibition produced by tertiary amines and stable metabolites of other drugs may be significant. However, such inhibition would be of shorter duration than that from MI complexation, which involves quasi-covalent binding to the haem and prevention of oxygen activation.

Introduction

The alkylamine structural feature is present in many drugs from different therapeutic classes, including selective serotonin reuptake inhibitors (SSRIs) like fluoxetine (FLU) and nisoxetine (NIS), tricyclic antidepressants such as desipramine (DES), and macrolide antibiotics and antihistamines such as dicyclomine and pheniramine. Therapy with SSRIs and tricyclic antidepressants is likely to be ongoing and involve combinations with tranquillisers, sedative-hypnotics and other drugs. Pharmacokinetic interactions have been reported during therapy with FLU and other SSRIs (Baumann 1996, Harvey and Preskorn 2001) and

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in vitro studies have established that the drugs inhibit hepatic CYPs that catalyse oxidative drug metabolism (Kobayashi *et al.* 1995, Mayhew *et al.* 2000, Daniel *et al.* 2002). The precise mechanism of the inhibitory action of FLU on CYP enzymes remains unclear.

Studies undertaken in several mammalian species have shown that some alkylamines undergo CYP-dependent oxidation to reactive metabolites that remain within the active site of the enzyme and generate metabolite-intermediate (MI) complexes (Delaforge *et al.* 1983, Murray and Field 1992). Most studies have been concerned with MI complexation of the quantitatively important CYP3A enzymes in rat, rabbit and human liver by the macrolide antibiotics (Pessayre *et al.* 1982, Pershing and Franklin 1982, Delaforge *et al.* 1983). Such mechanism-based inhibition of constitutive and inducible CYPs from the 3A subfamily by a number of other alkylamine drugs has also been reported (Bensoussan *et al.* 1995, Franklin 1995, Jones *et al.* 1999, Trieu and Murray 2000). In contrast, drug-mediated complexation of CYPs from other families has not been extensively studied.

Family 2 CYPs are also important catalysts of drug oxidation pathways in mammalian species and are subject to MI complexation by certain alkylamines, which could impact on drug biotransformation rates. Tricyclic antidepressants, including DES and nortriptyline (NOR) (Murray 1992, Murray and Field 1992) and the model CYP inhibitor SKF 525-A (Murray 1988), generate MI complexes with CYPs 2C, and orphenadrine produces MI complexes with CYPs 2B (Bast and Noordhoek 1982, Reidy *et al.* 1989) and possibly also CYP2C11 and CYP3A (Roos and Mahnke 1996).

The present study evaluated the role of MI complexation in the inhibition of the major hepatic monooxygenases in rat liver by the SSRIs FLU and NIS in comparison with a series of structurally similar alkylamines, including tricyclic antidepressants and SKF 525-A (figure 1). The 2 α -, 6 β -, 7 α - and 16 α -hydroxylations of testosterone were used as catalytic indicators of CYP 2C11, 3A, 2A1 and 2C11, respectively (Waxman *et al.* 1983). The major finding to emerge was that FLU was a preferential and extremely potent inhibitor of CYP2C11, whereas the other agents were less effective inhibitors of the enzyme. FLU and other secondary amines were mechanism-based inhibitors that generated MI complexes with CYP2C11, but, with the exception of SKF 525-A, tertiary amines appeared to be reversible CYP inhibitors. These findings indicate that MI complexation of CYP2 enzymes may contribute significantly to inhibitory effects of certain secondary alkylamine agents on drug metabolism.

Materials and Methods

Chemicals

Hyperfilm, ACS II, Hybond-N⁺ filters, [4-¹⁴C]-testosterone (specific activity about 55 mCi mmol⁻¹; 98.2% radiochemical purity), [4-¹⁴C]-androst-4-ene-3,17-dione (AD; specific activity about 55 mCi mmol⁻¹; 98.5% radiochemical purity) and reagents for enhanced chemiluminescence were from Amersham Pharmacia Biotech Australia (Castle Hill, Australia). The hydrochloride salts of desipramine, nortriptyline, dicyclomine, drofenine, amitriptyline, imipramine, phentoloxamine and *N,N*-dimethyldiphenylacetamide, doxylamine succinate, nafronyl oxalate, pheniramine maleate, oxeladin citrate (all drug purities $\geq$ 98%), unlabelled steroids and biochemicals were from Sigma Chemical Co. (St Louis, MO, USA). FLU and NIS hydrochlorides were gifts from Eli Lilly (Indianapolis, IN, USA), and SKF 525-A hydrochloride was provided by Smith, Kline and French (Ermington, Australia). High-performance liquid chromatography grade solvents were from Rhone-Poulenc Chemicals (Baulkham Hills, Australia) and analytical grade reagents were from Ajax Chemicals (Sydney, Australia).

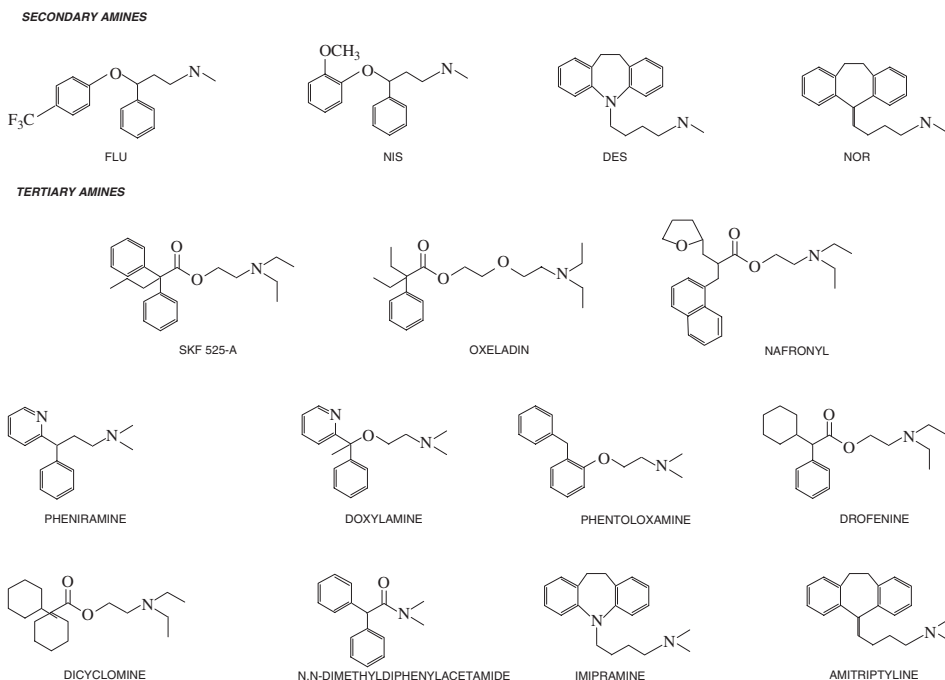


Figure 1. Structures of secondary and tertiary alkylamines. FLU, fluoxetine; NIS, nisoxetine; DES, desipramine; NOR, nortriptyline.

Animal treatments

Studies were performed in accordance with the guidelines of the Australian National Health and Medical Research Council, as approved by the Institutional Animal Care and Ethics Committee. Male Wistar rats (about 200 g) were held at constant temperature and lighting (12 h light–dark cycle) and had access to rat chow (Young Stock Feeds, Young, Australia) and water.

Animals were killed by exsanguination under enflurane anaesthesia, and livers were removed and perfused with ice-cold saline and used in the preparation of hepatic microsomes by differential ultracentrifugation. Briefly, tissue was homogenized in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 100 mM sucrose using a Potter–Elvehjem homogenizer and centrifuged at 10 000 g for 10 min. The supernatant was ultracentrifuged at 105 000 g for 60 min, followed by resuspension in buffer and resedimentation at 105 000 g for 30 min. The final microsomal pellets obtained were resuspended in 50 mM potassium phosphate buffer, pH 7.4, that contained 20% glycerol and 1 mM EDTA, frozen in liquid nitrogen and stored at -70°C until required (Murray *et al.* 1999). Microsomal protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Microsomal steroid hydroxylation

Microsomal testosterone and AD hydroxylation activities were determined by methods described by Martini *et al.* (1995). [^{14}C]-labelled substrates (50 μM , 0.18 μCi) were incubated with microsomal protein (0.15 mg) and NADPH (1 mM) at 37°C (in 0.1 M phosphate buffer, pH 7.4; 0.4 ml). Chloroform (5 ml) was added after 2.5 min and the tubes were transferred to ice to terminate the reaction. The organic phase was evaporated under N_2 and applied in a small volume of chloroform to thin-layer chromatography (TLC) plates (Merck silica gel 60 F₂₅₄ type; Darmstadt, Germany). For the determination of testosterone hydroxylation, plates were run in dichloromethane:acetone (4:1) and then chloroform:ethyl acetate:ethanol (4:1:0.7) with air drying between runs and for AD hydroxylation plates were run twice in chloroform:ethyl acetate (1:2) (Waxman *et al.* 1983). Radioactive metabolites were located on TLC plates by autoradiography (Hyperfilm-MP; Amersham) over 48–60 h and were quantified by scintillation counting (ACS II; Amersham). The identity of steroid metabolites was confirmed. Radioactive regions were scraped from the TLC plates, eluted from silica gel, rechromatographed on TLC plates along with the authentic metabolite and autoradiographed. Thus, metabolites comigrated in two solvent systems and radioactivity was localised to a single region. Intra-assay variation was 3% (analysis of three samples in triplicate) and the interassay variation was 8% ($n=3$).

microsomal fractions were analysed on 3 consecutive days). Metabolite formation with protein concentration and time was linear in each case; substrate conversion was $15 \pm 2\%$ in microsomes from control rat liver.

IC₅₀ values were determined in duplicate with four to six concentrations of drug prepared in distilled water; these concentrations of drugs did not affect the pH of the incubations. IC₅₀ values were calculated from plots of percentage activity remaining versus log₁₀ (inhibitor concentration). Control incubations (no drug) were included in which solvent (water) alone was added.

The impact of NADPH-dependent drug biotransformation on the extent of CYP inhibition was assessed. Drugs were pre-incubated with NADPH (1 mM) and microsomes, aliquots were withdrawn and IC₅₀ values determined, as described above. Similarly, in experiments to define the time dependence of inhibition, aliquots were withdrawn at various times and transferred to tubes containing [¹⁴C]-labelled substrate. Incubations were continued for a further 2.5 min for the determination of steroid hydroxylation activities.

Binding interactions between alkylamine drugs and CYP in NADPH-supplemented rat hepatic microsomes

The interaction of drugs with CYP in rat hepatic microsomes was monitored by difference spectroscopy essentially as described by Murray (1992). Binding to ferric CYP was done in microsomal suspensions (2 mg protein ml⁻¹ in 0.1 M potassium phosphate buffer, pH 7.4) divided between two cuvettes and after a baseline was established in a Varian Cary 2300 spectrophotometer at 37°C. Drugs were added to the sample cuvette in microlitre volumes of distilled water and the difference spectrum was scanned between 380 and 500 nm. The absorbance difference (ΔA) was calculated between the apparent absorbance maximum and minimum of the binding spectrum. Double-reciprocal plots of drug concentration versus ΔA were constructed, and the *x*- and *y*-axis intercepts were used to calculate K_s and $\Delta A_{\max}$, respectively (Jefcoate 1978).

The interaction of drugs with CYP in NADPH-fortified rat liver microsomes was also monitored by difference spectroscopy (Murray 1992). Drugs were added in microlitre volumes of distilled water to the sample cuvette (2 mg microsomal protein ml⁻¹) and NADPH (1 mM final) was added to both cuvettes. Formation of a CYP-MI complex was indicated by the intensification of a 452-nm absorbance maximum in the optical difference spectrum; the reaction was conducted for at least 10 min. After the reaction was complete, CYP content was determined by the ferrous-carbonyl method of Omura and Sato (1964). In some experiments, potassium ferricyanide (FCN; 50 μ M final) was added to restore CYP that had been sequestered as an MI complex (Murray 1988).

Statistics

Differences between means from multiple treatment groups were detected using one-way analysis of variance in conjunction with Fisher PLSD test.

Results

Inhibition of microsomal CYP steroid hydroxylation activities by FLU and other secondary alkylamines

FLU was found to inhibit CYP2C11-dependent steroid hydroxylation pathways in rat hepatic microsomes. Initial studies with a number of alkylamine agents showed that FLU (100 μ M) decreased 2 α - and 16 α -hydroxylation activities to 27 and 19% of control (figure 2). IC₅₀ values against these pathways were 47 and 39 μ M, respectively, and against CYP3A- and CYP2A1-dependent 6 β - and 7 α -hydroxylation, were 81 and 57 μ M, respectively. Pre-incubation of FLU (100 μ M) with NADPH-supplemented microsomes for 10 min before substrate biotransformation effected almost complete inhibition of CYP2C11-catalysed pathways (residual activity about 1% of control; figure 2). Thus, IC₅₀ values of 2 and 1 μ M were measured for the inhibition of 2 α - and 16 α -hydroxylation activities after the pre-incubation step. More extensive inhibition of CYP2A1-mediated steroid 7 α -hydroxylation (IC₅₀ 4 μ M after pre-incubation) and, to a lesser extent, testosterone 6 β -hydroxylation (IC₅₀ 46 μ M) was also noted. Thus, pre-incubation enhanced the potency of FLU 24- and 39-fold against 2 α - and 16 α -hydroxylation,

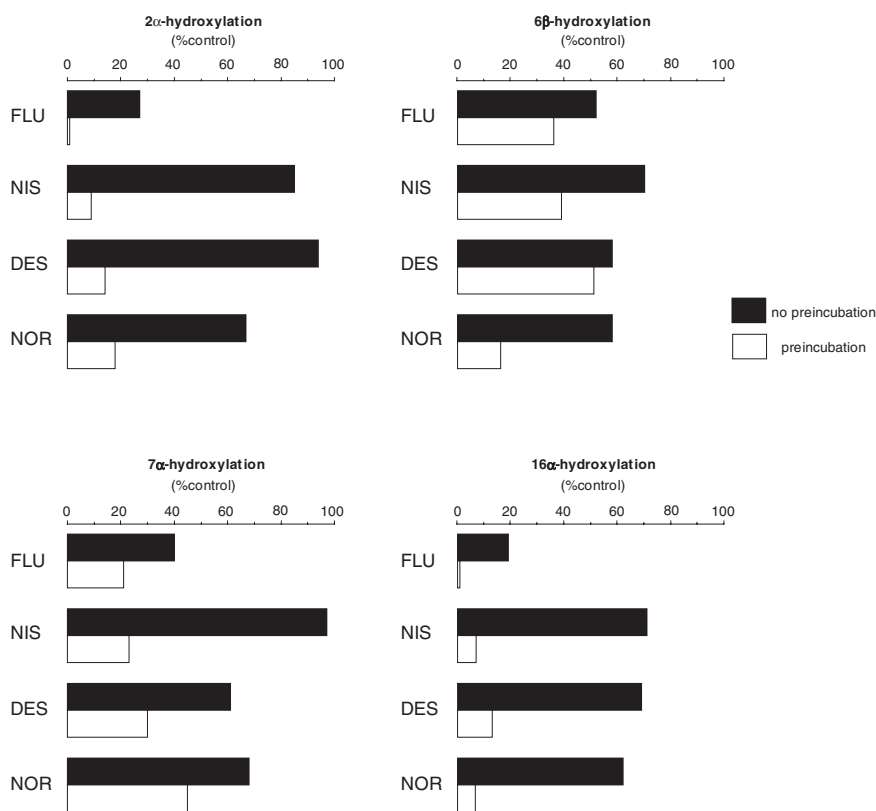


Figure 2. Inhibitory effects of the secondary amines FLU (fluoxetine), NIS (nisoxetine), DES (desipramine) and NOR (nortriptyline) on regioselective testosterone hydroxylation in rat hepatic microsomes *in vitro*. Drugs were tested at 100 μ M and included directly into microsomal incubations (no pre-incubation; solid bars) or were pre-incubated (open bars) with NADPH-supplemented microsomal fractions for 10 min (FLU, NIS) or 20 min (DES, NOR) before transfer to the substrate. After a further 2.5-min incubation, hydroxysteroid metabolite formation was quantified. Formation of 2 α -, 6 β -, 7 α - and 16 α -hydroxytestosterone products, reflecting the activities of CYPs 2C11, 3A, 2A1 and 2C11, respectively, were: 1.86 ± 0.29 , 3.32 ± 0.18 , 0.64 ± 0.05 and 2.47 ± 0.08 nmol mg protein⁻¹ min⁻¹, respectively. Data were obtained from duplicate incubations and product formation varied by < 8% from the stated means.

respectively, and 14-fold against 7 α -hydroxylation, but only 1.8-fold against 6 β -hydroxylation.

In contrast, NIS was much less effective than FLU against all pathways of testosterone hydroxylation (< 50% inhibition of each pathway was observed at 200 μ M NIS); IC₅₀ values were not determined. However, pre-incubation of drug with microsomes and NADPH (1 mM) intensified inhibition considerably. IC₅₀ values of 20 and 18 μ M were determined for NIS against the CYP2C11-dependent 2 α - and 16 α -hydroxylations of testosterone and 50 μ M versus CYP2A1-dependent 7 α -hydroxylation, but CYP3A-mediated testosterone 6 β -hydroxylation remained relatively refractory to inhibition.

Comparative studies were also undertaken with the tricyclic antidepressants DES and NOR. Like NIS, these drugs were relatively ineffective inhibitors of steroid hydroxylation pathways when added directly to microsomal incubations along with substrate but inhibitory potencies were markedly enhanced by the

pre-incubation step (figure 2). Thus, after pre-incubation, IC_{50} values for DES against CYP2C11-dependent 2α - and 16α -hydroxylation activities were 3 and $2\mu M$, respectively, and for NOR were 2 and $2\mu M$, respectively. It is noteworthy, however, that a longer pre-incubation step (20 min) was used in these experiments because shorter pre-incubations were less effective in intensifying inhibition. These experiments emphasize that potent inhibitory metabolites are formed during the oxidative biotransformation of FLU and the other three secondary amine drugs.

The time-dependent intensification of CYP2C11 inhibition by FLU ($10\mu M$) was evaluated further. Pseudo first-order kinetic analysis revealed potent inhibition of CYP2C11 activities after pre-incubation of the drug with NADPH-fortified microsomes (figure 3), with half-times of 5.6–7.7 min for the loss of enzyme activity (table 1). In contrast, intensification of the inhibition of CYP2A1- and CYP3A-mediated testosterone hydroxylation pathways was somewhat less pronounced under the experimental conditions (figure 3). Analogous experiments were done with NIS but, consistent with the earlier IC_{50} experiments, the potency of this agent was much less pronounced than was the case with FLU (table 1). DES and NOR were also tested for their capacity to undergo biotransformation in microsomes to inhibitory metabolites. Half-times for DES ($50\mu M$) and NOR (10 and $50\mu M$) against the CYP2C11-mediated steroid hydroxylation pathways were similar to those measured for FLU (table 1). Taken together, these findings establish that CYP2C11 is sensitive to inhibition by metabolites of secondary amines.

Inhibition of microsomal CYP steroid hydroxylation activities by tertiary alkylamine drugs

The preceding studies indicated that four secondary alkylamine drugs were effective inhibitors of CYP2C11 activity, with FLU and NOR in particular exhibiting considerable potency. Several tertiary (*N,N*-dialkyl-substituted) alkylamines (figure 1) also produced significant inhibition of CYP2C11 activity (figure 4). Thus, the 2α - and 16α -hydroxylations of testosterone were inhibited by

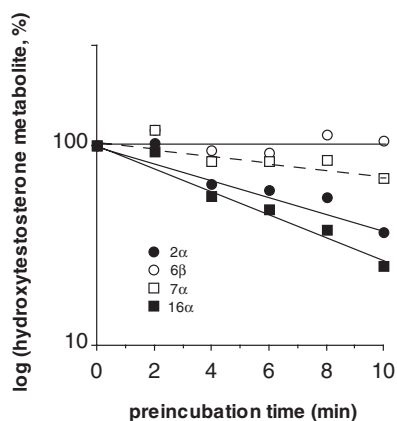


Figure 3. Time-dependent intensification of the inhibition of CYP-mediated testosterone hydroxylation activities during NADPH-dependent FLU biotransformation. Note that the scale on the y-axis is logarithmic (pseudo first-order kinetic analysis). 2α -, 6β -, 7α - and 16α - indicate formation of the 2α -, 6β -, 7α - and 16α -hydroxy metabolites of testosterone. Data are averages of experiments conducted in duplicate and varied by $< 8\%$ from the values shown.

Table 1. Intensification of CYP2C11 inhibition by alkylamine metabolites.

Drug	Concentration (μM)	CYP2C11 reaction	Half-time (loss of 50% activity; min)
FLU	10	testosterone 2 α -hydroxylation	7.7
		testosterone 16 α -hydroxylation	5.6
NIS	50	testosterone 2 α -hydroxylation	> 15
		testosterone 16 α -hydroxylation	> 15
DES	50	testosterone 2 α -hydroxylation	8.5
		testosterone 16 α -hydroxylation	7.0
NOR	10	testosterone 2 α -hydroxylation	4.8
		testosterone 16 α -hydroxylation	3.8
NOR	50	testosterone 2 α -hydroxylation	4.0
		testosterone 16 α -hydroxylation	4.0
SKF 525-A	10	AD 16 α -hydroxylation	10.7
SKF 525-A	100	AD 16 α -hydroxylation	11.0

Half-times were determined as described in the Materials and methods: alkylamines were incubated with NADPH-supplemented microsomes for varying periods, aliquots were withdrawn and transferred to tubes containing steroid substrate. Reactions continued for 2.5 min and metabolite formation was measured. Data are averages of duplicate determinations with three to six pre-incubation times; variation from stated values was within 12%.

dicyclomine, drofenine, amitriptyline and imipramine, as well as SKF 525-A (100 μM). Inhibition of CYP3A-dependent 6 β -hydroxylation of the steroid was observed with 100 μM dicyclomine, nafronyl, amitriptyline and SKF 525-A, but CYP2A1-mediated testosterone 7 α -hydroxylation activity was only inhibited to a significant extent ($\geq 50\%$) by drofenine and SKF 525-A.

Pre-incubation of the tertiary alkylamines with NADPH-supplemented microsomal fractions before transfer to tubes containing steroid substrate led to more intense inhibition of the four testosterone hydroxylation pathways in the case of SKF 525-A (figure 4). Apart from SKF 525-A, however, more potent inhibition was only produced by oxeladin, nafronyl and amitriptyline, and, interestingly, only against 7 α -hydroxylation (figure 4).

The characteristics of inhibition by SKF 525-A were evaluated further. IC_{50} values of 84 and 65 μM were measured for SKF 525-A against CYP2C11-dependent AD 16 α -hydroxylation and CYP3A-dependent AD 6 β -hydroxylation, respectively. In contrast, CYP2A-dependent 7 α -hydroxylation of this steroid was refractory to inhibition ($\text{IC}_{50} > 300 \mu\text{M}$). After a 10-min pre-incubation with NADPH-supplemented microsomal fractions, IC_{50} values against the 16 α - and 6 β -hydroxylation pathways were 7.8 and 3.9 μM , respectively, thus indicating approximate 11- and 17-fold intensification of inhibition of CYP 2C11 and 3A by SKF 525-A metabolites; CYP2A remained essentially uninhibited.

Time-dependent inhibition of the CYP reactions was also evaluated for SKF 525-A. As shown in table 1, 10 and 100 μM were similarly effective in terms of the propensity for formation of inhibitory metabolites: half-times for the loss of CYP2C11-mediated 16 α -hydroxylation activity were 10.7 and 11.0 min, respectively.

Spectroscopic studies of binding interactions of alkylamine drugs with microsomal CYPs

The binding of alkylamines to CYP in oxidized microsomes was measured. All the test compounds elicited characteristic type I interactions ($\lambda_{\text{max}} \sim 386 \text{ nm}$,

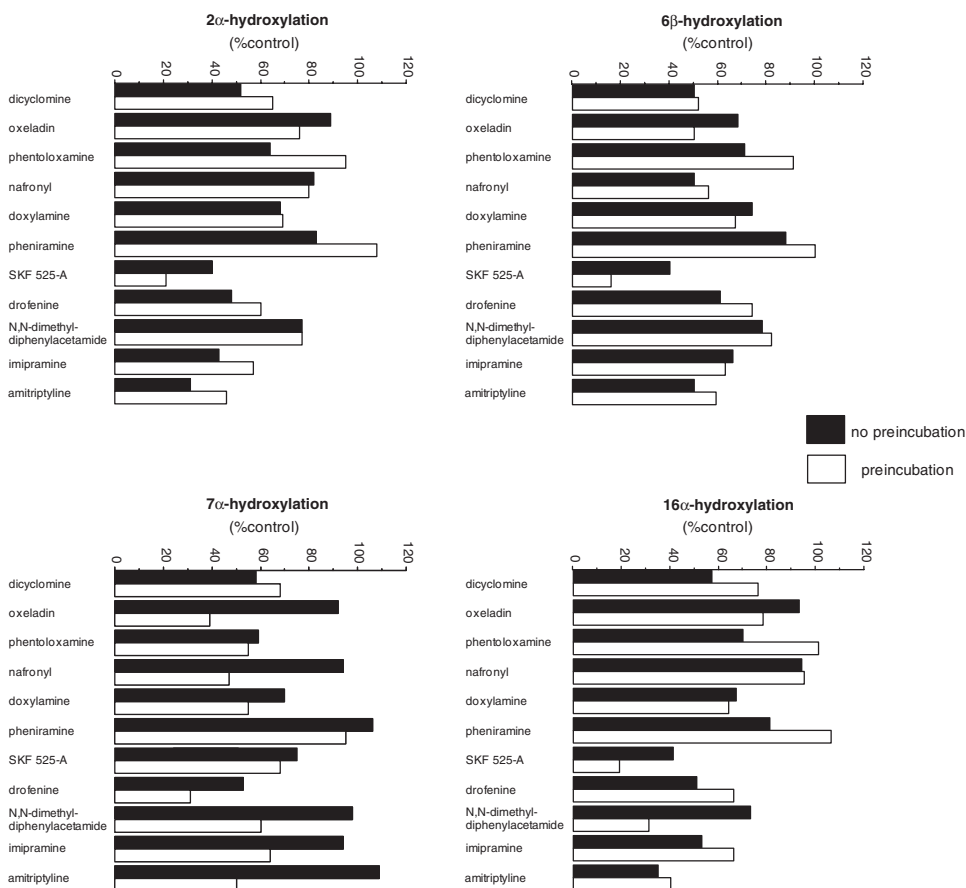


Figure 4. Inhibitory effects of tertiary amines on regioselective testosterone hydroxylation in rat hepatic microsomes *in vitro*. Drugs were tested at 100 μ M and included directly into microsomal incubations (no pre-incubation; solid bars) or were pre-incubated (open bars) with NADPH-supplemented microsomal fractions for 10 min before transfer to substrate. Incubations were conducted at least in triplicate and product formation was measured as described in the legend to figure 2.

$\lambda_{\min} \sim 420$ nm in the optical difference spectrum; as exemplified by the interactions of FLU and dicyclomine with microsomal fractions; figure 5A, C), which indicate a shift in the spin equilibrium from the low- to high-spin component (Rein *et al.* 1979). From these analyses, spectral dissociation constants (K_s) were calculated for FLU and dicyclomine to be 3.2 ± 0.3 and 16 ± 2 μ M, respectively (table 2 and figure 5B, D); K_s values for other agents were in the range 0.9–3.6 μ M (table 2). The extent of binding followed a bimodal distribution, with several drugs demonstrating extensive type I binding ($\Delta A_{\max} = 0.026$ –0.048 absorbance units nmol^{-1} CYP; dicyclomine, oxeladin, nafronyl, drofenine, FLU and DES) (table 2) and a number of drugs exhibiting much less pronounced spectral interactions ($\Delta A_{\max} = 0.009$ –0.018 absorbance units nmol^{-1} CYP; phentoloxamine, doxylamine, pheniramine, SKF 525-A, *N,N*-dimethyldiphenylacetamide and NIS) (table 2). From these findings, it emerged that several of the type I binding interactions were of high efficiency ($\Delta A_{\max}/K_s = 9000$ –33000 absorbance units nmol^{-1} CYP/M).

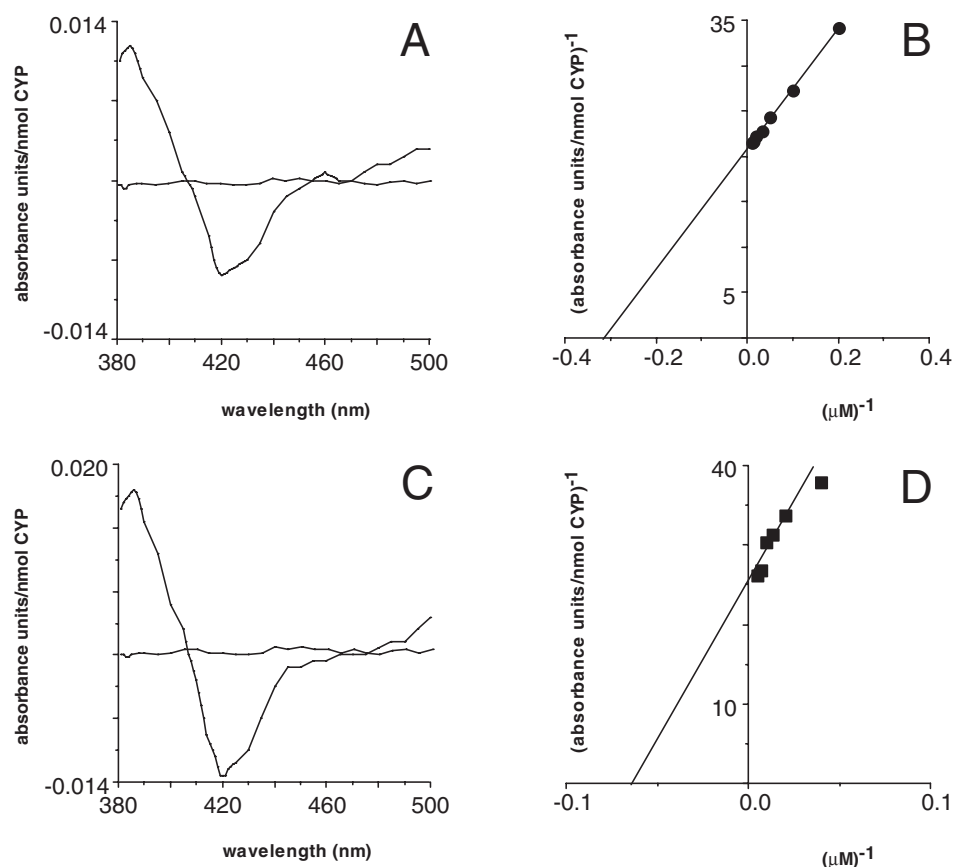


Figure 5. Type I binding spectra produced by (A) FLU (10 μM) and (C) dicyclomine (25 μM) in oxidized rat hepatic microsomes (2 mg protein ml⁻¹). Double reciprocal replot of the binding data obtained with FLU (B) and dicyclomine (D); data are representative plots of spectral titrations performed in triplicate.

Table 2. Binding interactions of alkylamine agents to microsomal CYP in rat liver.

Compound	K_s (μM)	$\Delta A_{\max}$ (absorbance units/nmol P450)	$\Delta A_{\max}/K_s$ (absorbance units/nmol P450/M)	MI complex
FLU	3.2 ± 0.3	0.048 ± 0.012	15 000	yes
NIS	0.9	0.026	28 900	yes
DES	1.4	0.009	6430	yes
SKF 525-A	1.3 ± 0.1	0.018 ± 0.001	13 800	yes
Dicyclomine	16 ± 2	0.040 ± 0.005	2500	no
Oxeladine	3.2	0.031	9690	no
Phentoloxamine	1.0	0.012	12 000	no
Nafronyl	3.0	0.027	9000	no
Doxylamine	3.6	0.009	2500	no
Pheniramine	2.5	0.015	6000	no
Drofenine	1.0 ± 0.1	0.033 ± 0.005	33 000	no
N,N-dimethyldiphenylacetamide	3.6	0.008	2200	no

Data are means ± SEM of $n=3$ individual spectral titrations. Where SEM are not stated, the difference spectra were obtained at least in duplicate and the means varied by < 8%.

Interactions of alkylamines with CYP in NADPH-fortified microsomes were also evaluated. Time-dependent formation of an absorbance maximum near 452 nm was observed in the optical difference spectrum with FLU (figure 6A). A 21% decrease in CYP holo-enzyme was produced by the incubation of FLU (50 μ M) with microsomes; at 10 μ M FLU, less MI complexation was observed. Sequestration of CYP in the form of an MI complex was confirmed by addition of FCN to oxidize the complex and restore CYP (figure 6B). MI complexation with NIS was minimal when tested at a concentration of 50 μ M, but was measurable at 100 μ M (13% of microsomal CYP complexed; data not shown). By comparison, NOR (50 and 200 μ M) and DES (200 μ M) trapped 15 and 14%, respectively, of total CYP as an MI complex (15-min incubation). Thus, all of the secondary amines underwent oxidative biotransformation to generate MI complexes with microsomal CYP *in vitro*.

With the exception of SKF 525-A, addition of NADPH (1 mM) to microsomal fractions containing the other alkylamines (figure 1) did not give rise to a typical MI complex absorbance maximum around 455 nm. Like the secondary alkylamines, SKF 525-A (100 μ M) trapped 15% of total CYP as an MI complex; neither dicyclomine nor drofenine elicited a decrease in measured CYP data (not shown).

Discussion

Oxidative biotransformation of a range of *N*-alkylamine substituted drugs can result in extensive CYP inhibition as a result of MI complexation. The present study establishes that the SSRI drug FLU is a potent and preferential inhibitor of the major drug-metabolizing CYP2C11 and that MI complexation contributes to the inhibitory mechanism. Although most studies of MI complexation have concentrated on agents that inhibit CYPs 3A, the present finding that FLU generates an inhibitory complex with CYP2C11 is of considerable interest.

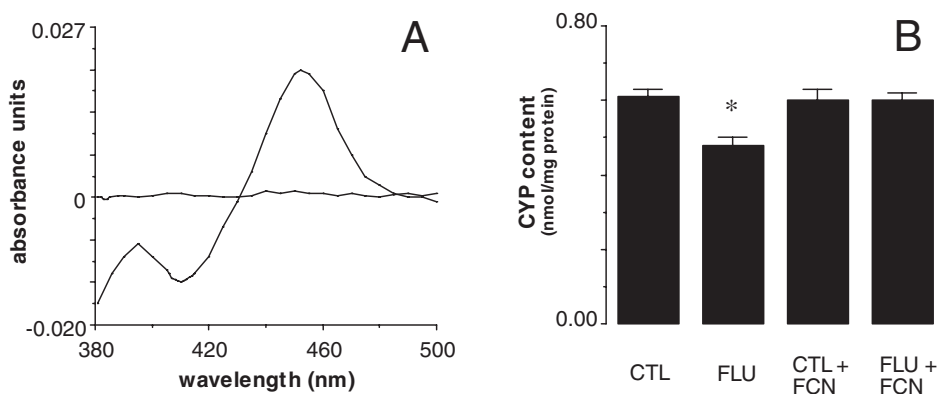


Figure 6. Formation of an MI complex between a FLU metabolite and CYP in NADPH-supplemented rat hepatic microsomes. (A) FLU (50 μ M) was incubated for 10 min with NADPH and microsomes, and the optical difference spectrum between 380 and 500 nm was scanned. The absorbance near 455 nm indicates formation of the MI complex. (B) Microsomes were incubated with FLU (50 μ M) or vehicle (water) and the NADPH (1 mM) for 10 min and total CYP content was measured ($n=3$ independent experiments). In some incubations, potassium ferricyanide (FCN; 50 μ M) was added before CYP was estimated. *Different from control (CTL): $p < 0.01$.

Previous studies have shown that tricyclic antidepressants like DES and NOR also form an MI complex with this enzyme (Murray 1992, Murray and Field 1992), but FLU has emerged from the present study as a particularly potent inhibitor of CYP2C11.

SSRIs, especially FLU (Prozac), are widely used in clinical practice. Other SSRIs include NIS, fluvoxamine, sertraline and paroxetine. Most of these drugs have been associated with significant inhibitory interactions during therapy, the characteristics of which are consistent with the formation of inhibitory metabolites and possibly also MI complexation. CYP-MI complexes are formed when a reactive metabolite, probably the nitroso product following oxidation of the parent alkylamine, binds tightly to the CYP haem iron (Mansuy *et al.* 1978, Lindeke *et al.* 1979). MI complexation leads to highly specific CYP inhibition because the reactive metabolites do not readily dissociate from the active site of the CYP involved in their formation (the corresponding reaction for FLU is shown in figure 7A). The polymorphically distributed CYP2D6 and CYP2C19 appear to be major human CYPs that are susceptible to inhibition by FLU, which may be significant because of the role of these enzymes in the oxidation of numerous drugs (Avenoso *et al.* 1997, Fjordside *et al.* 1999, Hemeryk *et al.* 1999). Interestingly, Caccia (1998) reported that some metabolites, including the major *N*-demethylated metabolite of FLU, norfluoxetine, have longer half-lives than the parent drug. This report is consistent with the notion that FLU as well as its reactive and

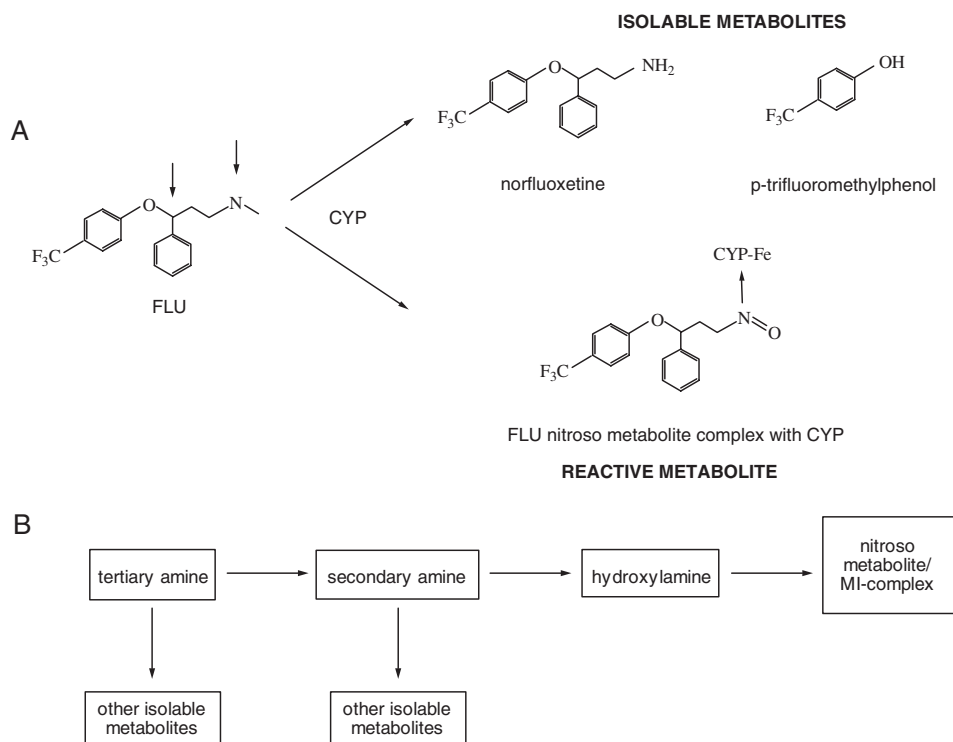


Figure 7. Scheme showing (A) formation of isolable (stable) and reactive metabolites during the CYP-dependent biotransformation of FLU and (B) general pathway of oxidative conversion of tertiary and secondary amines to stable and MI complex-forming metabolites. The arrows in (A) indicate the sites of metabolic oxidation of FLU.

isolable metabolites may contribute to significant inhibitory effects on specific CYP enzymes. Thus, it is noteworthy that Harvey and Preskorn (2001) reported that the human CYP2C19 was extensively inhibited during long-term FLU therapy.

Although CYP inhibition by FLU has been shown previously, the possibility that FLU may generate an MI complex has not been fully explored. In the present study, about one-fifth of the available microsomal CYP in rat liver was sequestered as an *in vitro* MI complex by FLU (50 μ M), but NIS was less effective. These findings are consistent with the observed relative inhibitory potencies of the two agents against CYP2C11. By comparison, the tricyclic antidepressants DES and NOR effected MI complexation of a similar proportion of CYP in rat liver microsomes to that seen with FLU, which is consistent with earlier reports (Murray and Field 1992). Apart from MI complexation, CYP inhibition may also occur when certain drugs or their stable metabolites bind reversibly to the enzyme (Rein *et al.* 1979, McNeil and Murray 1996, Sutton *et al.* 1997). Inhibitory chemicals can compete with substrates and prevent the reduction of CYP by an electron from NADPH, which is a prerequisite step for oxygen activation by the cytochrome. However, because the inhibitory agent is not bound quasi-covalently to the CYP active centre, these processes are reversible and of shorter duration than MI complexation. Reversible inhibition by parent alkylamines in the present study seems likely because pre-incubation of drugs with NADPH-supplemented microsomes did not accentuate the observed inhibition; thus, formation of inhibitory metabolites seems unlikely.

An ongoing interest in several laboratories concerns the structural features of drugs that promote MI complexation. In accord with earlier findings, the present study confirms that secondary alkylamines are more effective MI complexation agents than most tertiary amines (Bast and Noordhoek 1982, Murray and Field 1992, Roos and Mahnke 1996, Roos 1999). The hydrophobic and chiral properties of certain secondary alkylamines also influences MI complex formation (Jonsson and Lindeke 1992). Oxidation of tertiary alkylamines proceeds in part by *N*-dealkylation to the secondary amine, but this seems to be generally inefficient in forming sufficient amounts of the precursor for further biotransformation to the MI complexing metabolite. This may relate to the greater basicity of the tertiary amino nitrogen, which might also promote *N*-oxidation, or may be a consequence of alternate metabolic pathways that direct substrate away from the pathway to MI complexation (figure 7B).

Although most tertiary amines appear to follow alternate biotransformation pathways apart from MI complexation, an induction regimen may promote complex formation. Franklin (1995) and Bensoussan *et al.* (1995) demonstrated that induction of CYP3A by dexamethasone increased MI complexation by a number of tertiary and secondary amines, including FLU. This implicates CYPs 3A as targets for FLU metabolites under certain conditions. Mayhew *et al.* (2000) modelled the inhibition of human CYP3A by FLU and suggested that *in vivo* treatment with the drug would decrease the available CYP3A4 by about 30%, an effect that was attributed to MI complexation. However, clinical studies by Bergstrom *et al.* (1997) suggested that FLU had only minimal effect on terfenadine metabolism — a substrate for CYP3A. Indeed, Ring *et al.* (1995) reported that the K_i for the inhibition of midazolam 1'-hydroxylation by FLU was relatively high, suggesting that inhibition of CYP3A activity is of minimal clinical importance.

Alkylamine hydrophobicity has been shown to influence the magnitude of MI complex formation (Jonsson and Lindeke 1992). Parameters that describe the hydrophobic and steric properties of several of the present alkylamine agents (FLU, NIS, DES, NOR, SKF 525-A, dicyclomine and amitriptyline) were calculated according to established literature principles (Hansch *et al.* 1973, 1977). Thus, octanol/water partition coefficients ($\log P$) were used to reflect hydrophobicity and ranged from 3.72 for DES to 6.81 for SKF 525-A. Steric properties were reflected by molar refractivities and ranged from 82.45 for FLU to 111.22 for SKF 525-A. Thus, all seven alkylamines were quite hydrophobic and large, but simple relationships between these parameters and the propensity for MI complexation were not found. The interatomic distance between the alkylamine nitrogen and the lipophilic cyclic systems in the present group of tertiary alkylamines is a possible factor that could influence the extent of CYP inhibition. Analogues with fewer than four sp^3 hybridized atoms between the alkylamine nitrogen and the carbocyclic system appeared to bind to CYP with low efficiency and effected minimal inhibition of CYP-dependent testosterone hydroxylation. Four or more sp^3 hybridized atoms between the ring and alkylamine nitrogen promoted binding and inhibition. The exception to this trend, however, was dicyclomine. This drug possesses four sp^3 hybridized atoms between the cyclohexyl ring and alkylamine nitrogen and was a relatively effective inhibitor of monooxygenation but exhibited a relatively high K_s for binding to ferric CYP. Structure-complexation relationships have been reported previously for methylenedioxyphenyl derivatives, although the nature of the subsequent CYP-MI complex differs from that which is operative in the case of alkylamines. Within series of 4-alkyl and 4-alkoxy-substituted methylenedioxyphenyls, increasing chain length increased the extent of MI complexation to an apparent optimum; beyond this, the magnitude of the complex declined, but its stability increased (Murray *et al.* 1983, Marcus *et al.* 1990). Electron withdrawing substituents on the aromatic system in methylenedioxyphenyl agents detracted from MI complexation and promoted oxidation of the methylene bridge to carbon monoxide. Electron donating substituents favoured MI complexation (Yu *et al.* 1980).

In summary, the present study established the SSRI agent FLU as a potent and preferential inhibitor of CYP2C11 in rat liver microsomes; FLU metabolites are implicated in the inhibitory process. Compared with other secondary alkylamines, FLU was extremely potent and MI complexation of CYP appeared to contribute significantly, but not exclusively, to inhibition. A range of tertiary alkylamines that were structurally similar to FLU were also tested, and some were moderately potent CYP inhibitors. However, neither stable nor reactive MI complex-forming metabolites of these agents appeared to contribute to inhibition. The oxidizability of the aliphatic nitrogen atom and the proximity of the alkylamine drug to the eventual complex-forming metabolite are major determinants of the extent of MI complexation. Further studies are now required to test this possibility further.

Acknowledgments

This study was supported by a grant from the Australian National Health and Medical Research Council. The technical assistance of Rachel Sefton and Louise

Nadin in aspects of the study is also gratefully acknowledged. FLU and NIS were generous gifts from Eli Lilly, Indianapolis, USA.

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Identification of cytochrome P450 enzymes involved in the metabolism of 4'-methoxy- α -pyrrolidinopropiophenone (MOPPP), a designer drug, in human liver microsomes

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Received 10 March 2003

1. The metabolism of 4'-methoxy- α -pyrrolidinopropiophenone (MOPPP), a novel designer drug, to its demethylated major metabolite 4'-hydroxy-pyrrolidinopropiophenone (HO-PPP) was studied in pooled human liver microsomes (HLM) and in cDNA-expressed human hepatic cytochrome P450 (CYP) enzymes.

2. CYP2C19 catalysed the demethylation with apparent K_m and V_{max} values of $373.4 \pm 45.1 \mu\text{M}$ and $6.0 \pm 0.3 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ CYP, respectively (mean $\pm$ SD). Both CYP2D6 and HLM exhibited clear biphasic profiles with apparent $K_{m,1}$ values of 1.3 ± 0.4 and $22.0 \pm 6.5 \mu\text{M}$, respectively, and $V_{max,1}$ values of $1.1 \pm 0.1 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ CYP and $169.1 \pm 20.5 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein, respectively.

3. Percentages of intrinsic clearances of MOPPP by particular CYPs were calculated using the relative activity factor (RAF) approach with (*S*)-mephenytoin-4'-hydroxylation or bufuralol-1'-hydroxylation as index reactions for CYP2C19 or CYP2D6, respectively.

4. MOPPP, HO-PPP and the standard 3',4'-methylenedioxy-pyrrolidinopropiophenone (MDPPP) were separated and analysed by liquid chromatography–mass spectrometry in the selected-ion monitoring (SIM) mode.

5. The CYP2D6 specific chemical inhibitor quinidine ($3 \mu\text{M}$) significantly ($p < 0.0001$) inhibited HO-PPP formation by $91.8 \pm 0.5\%$ (mean $\pm$ SEM) in incubation mixtures with HLM and $2 \mu\text{M}$ MOPPP.

6. It can be concluded from the data obtained from kinetic and inhibition studies that polymorphically expressed CYP2D6 is the enzyme mainly responsible for MOPPP demethylation.

Introduction

The cytochrome P450 (CYP) family accounts for more than 90% of oxidative metabolic reactions of xenobiotics (Guengerich *et al.* 2000). The involvement of particular CYP enzymes in the biotransformation of a new drug is usually thoroughly investigated before it can be marketed. Such investigations allow the prediction of possible drug–drug interactions, if a drug is co-administered that influences (i.e. induces or inhibits) the corresponding enzyme. Furthermore, some CYPs (e.g. CYP2D6) are expressed polymorphically in humans and can therefore lead to interindividual variations in pharmacokinetic profiles and an increased appearance of side-effects and serious poisonings (Evans and McLeod 2003).

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However, such risk assessment is typically performed for substances intended for therapeutic use, but not for drugs of the illicit market.

4'-Methoxy- α -pyrrolidinopropiophenone (MOPPP, IUPAC: 2-(pyrrolidine-1-yl)-1-(*p*-methoxy)propane-1-one) is a new designer drug which has appeared on the illicit drug market (Roesner *et al.* 1999). Large amounts of tablets were distributed for recreational use and have been seized by the police. Screening for drugs of abuse do not detect MOPPP in biosamples (Springer *et al.* 2003). So far, little information about the pharmacological and toxicological properties of MOPPP is available. The chemical structure of MOPPP is closely related to anorectics like amfepramone and metamfepramone, drugs of abuse like cathinone and methcathinone, or antidepressants like bupropion. Having dopaminergic and noradrenergic properties, all these substances show amphetamine-like effects (Bryant *et al.* 1983, Kalix and Glennon 1986, Glennon *et al.* 1987, Martinez *et al.* 1998). Therefore, it can be assumed that MOPPP shows similar effects, particularly since it is misused as a substitute for amphetamines. Possible clinical effects of MOPPP should include tachycardia, hypertension, mydriasis and tremor.

Previous *in vivo* studies in the rat have shown that MOPPP is mainly metabolized by demethylation of the 4'-methoxy group to 4'-hydroxy-pyrrolidinopropiophenone (HO-PPP) (Springer *et al.* 2003). Unfortunately, no authentic human samples were available, but incubation of MOPPP with pooled human liver microsomes (HLM) showed that HO-PPP formation was the main CYP-catalysed reaction. The aim of the current study was to identify the human hepatic CYPs involved in demethylation, to determine the kinetic constants for this metabolic reaction and to draw conclusions concerning the intrinsic clearance.

Materials and methods

Materials

MOPPP-HCl and MDPPP-HCl (3',4'-methylenedioxy- α -pyrrolidino-propiophenone, INN: 1-(1,3-benzodioxol-5-yl)-2-(pyrrolidine-1-yl)propane-1-one) were of analytical grade and provided by the Hessian State Criminal Office (Wiesbaden, Germany). NADP⁺ was obtained from Biomol (Hamburg, Germany), isocitrate and isocitrate dehydrogenase from Sigma (Taufkirchen, Germany), all other chemicals and reagents from Merck (Darmstadt, Germany). The following microsomes were from Gentest and delivered by NatuTec (Frankfurt/Main, Germany): baculovirus-infected insect cell microsomes containing 1 nmol ml⁻¹ human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 (Supersomes[®]), wild-type baculovirus-infected insect cell microsomes (control Supersomes[®]) and HLM (20 mg microsomal protein ml⁻¹, 400 pmol total CYP mg⁻¹ protein). After delivery, the microsomes were thawed at 37°C, aliquoted, shock-frozen in liquid nitrogen and stored at -80°C until use.

Microsomal incubations

Incubation mixtures (final volume 50 μ l) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM Mg²⁺, 5 mM isocitrate, 1.2 mM NADP⁺, 0.5 U ml⁻¹ isocitrate dehydrogenase, 200 U ml⁻¹ superoxide dismutase and substrate at 37°C. The substrate was added after dilution of a 250 mM methanolic stock solution in buffer. The methanol concentration did not exceed 0.4%. Reactions were started by addition of the ice-cold microsomes and terminated with 5 μ l 60% (w/w) perchloric acid. After addition of 1 μ l 0.1 mg ml⁻¹ MDPPP as standard, the samples were centrifuged and the supernatants transferred to autosampler vials.

Initial screening studies

To investigate the involvement of particular CYP enzymes in MOPPP demethylation, incubations were performed with 50 μ M MOPPP and 50 pmol ml⁻¹ CYP1A2, CYP2A6, CYP2B6, CYP2C8,

CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 or 90 mM Tris-buffer, respectively, according to the Gentest manual.

Kinetic studies

Duration of and protein content for all incubations were in the linear range of metabolite formation (data not shown). Kinetic constants were derived from incubations with the following MOPPP concentrations, incubation times and protein concentrations: 10, 20, 30, 40, 50, 75, 100, 150, 200, 300, 400, 700, 1000 and 1500 μM MOPPP with 50 pmol CYP2C19 ml^{-1} for 30 min; 1, 2, 3, 5, 7.5, 10, 15, 20, 30, 50, 75, 100, 200, 300 and 500 μM MOPPP with 50 pmol CYP2D6 ml^{-1} for 20 min; 1, 3, 5, 10, 20, 40, 70, 100, 150, 200, 300, 500, 700, 1000 and 1500 μM MOPPP with 1.0 mg HLM protein ml^{-1} for 45 min. Less than 20% of substrate were metabolized in all incubations. Apparent K_m and $V_{\max}$ values for a single enzyme system were estimated by non-linear regression according to the Michaelis–Menten equation:

$$V = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (1)$$

If visual inspection of the data points suggested that a two-site binding model was the more appropriate model, equation (2) was applied for fitting the curve (Korzekwa *et al.* 1998):

$$V = \frac{V_{\max,1} \times [S]}{K_{m,1} + [S]} + \frac{V_{\max,2} \times [S]}{K_{m,2} + [S]} \quad (2)$$

Eadie–Hofstee plots were used to confirm biphasic kinetics (Clarke 1998). The kinetic data were determined using GraphPad Prism 3.02 software (San Diego, CA, USA).

Calculation of relative activity factors

Taking into account differences in functional levels of redox partners between the two enzyme sources, the relative activity factor (RAF) approach was used (Crespi and Miller 1999, Venkatakrishnan *et al.* 2000). The activities of CYP2C19 (specific substrate (S)-mephenytoin) and CYP2D6 (specific substrate bufuralol) in insect cell microsomes and HLM were taken from the supplier's data sheets. The formation rate of 4'-hydroxymephenytoin was given to be 2857 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein in cDNA-expressed microsomes and 82 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein in HLM. The formation rate of 1'-hydroxybufuralol was given as 8293 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein in cDNA-expressed microsomes and 84 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein in HLM. The RAFs were calculated according to equation (3) yielding $\text{RAF} = 0.029$ for CYP2C19 and 0.01 for CYP2D6.

$$\text{RAF}_{\text{enzyme}} = \frac{\text{turnover rate for probe substrate in HLM (pmol min}^{-1} \text{mg}^{-1} \text{protein)}}{\text{turnover rate for probe substrate in cDNA – expressed CYP (pmol min}^{-1} \text{mg}^{-1} \text{protein)}} \quad (3)$$

$V_{\max}$ values for MOPPP demethylation obtained from incubations with cDNA-expressed CYPs were then multiplied with the corresponding RAF leading to a value, which is defined as 'contribution':

$$\text{Contribution}_{\text{enzyme}} = (V_{\max} \text{ for MOPPP demethylation with cDNA – expressed CYP}) \times \text{RAF}_{\text{enzyme}} \quad (4)$$

From these corrected activities (contributions), the percentage of intrinsic clearance by a particular CYP can be calculated according to equation (5), where intrinsic clearance equals contribution/ K_m :

$$\text{Percentage of clearance by enzyme} = \frac{\text{clearance}_{\text{enzyme}}}{\sum \text{clearances}_{\text{enzyme}}} \times 100\% \quad (5)$$

Chemical inhibition studies

The effect of 3 μM quinidine on HO-PPP formation was assessed in incubations containing 1.0 mg HLM protein ml^{-1} and 2 μM MOPPP. Controls contained no quinidine, but the same amount of methanol ($\leq 0.1\%$) to control for any solvent effects ($n = 6$ each). Significance of inhibition was tested by a one-tailed unpaired *t*-test with Welch's-correction using GraphPad Prism 3.02 software.

Liquid chromatography-mass spectrometry (LC-MS) conditions and metabolite quantification

MOPPP, HO-PPP and MDPPP were separated and quantified using an Agilent Technologies (AT, Waldbronn, Germany) AT 1100 series atmospheric pressure chemical ionization (APCI) electrospray LC-MSD, SL version and an LC-MSD ChemStation using the A.08.03 software.

LC conditions. Gradient elution was achieved on a Merck LiChroCART[®] column (125 × 2 mm i.d.) with Superspher[®]60 RP Select B as stationary phase and a LiChroCART[®]10-2 Superspher[®]60 RP Select B guard column. The mobile phase consisted of ammonium formate (5 mM, adjusted to pH 3 with formic acid) (eluent A) and acetonitrile (eluent B) according to Maurer *et al.* (2002). The gradient and the flow rate were programmed as follows: 0–3 min 15% B (flow: 0.4 ml min⁻¹), 3–5 min 40% B (0.4 ml min⁻¹), 5–8 min 90% B (0.6 ml min⁻¹), 8–8.75 min 90% B (0.9 ml min⁻¹), and 8.75–10 min 15% B (0.4 ml min⁻¹). The injection volume was 2 µl.

Electrospray conditions. The following APCI inlet conditions were applied: drying gas (7000 ml min⁻¹, 300°C) and nebulizer pressure (25 psi) (both nitrogen); capillary voltage, 4000 V; drying gas temperature set at 300°C, vaporizer temperature set at 400°C; corona current was 5.0 µA; positive selected-ion monitoring (SIM) mode; fragmentor voltage 100 V.

MS conditions. For quantification, the following target ions (*m/z*) were used in SIM mode: time window 2–4.5 min: 220 for HO-PPP; time window 4.5–6 min: 248 for the standard MDPPP; time window 6–8 min: 234 for MOPPP.

Metabolite quantification. The concentration of HO-PPP in the incubation mixture was estimated from the known concentration of the standard MDPPP, because reference substance of HO-PPP for an accurate quantification was not available.

Results

LC-MS procedure

The mass fragmentograms in figure 1 show that the applied LC-MS conditions provided baseline separation of HO-PPP, the standard MDPPP and MOPPP. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and standard) and zero samples (control microsomes without substrate, but with standard).

Initial screening studies

Figure 2 illustrates that among the nine CYPs tested, only CYP2C19 and CYP2D6 supported demethylation of the 4'-methoxy moiety of MOPPP. HO-PPP was not detectable in incubations with other cDNA-expressed CYPs.

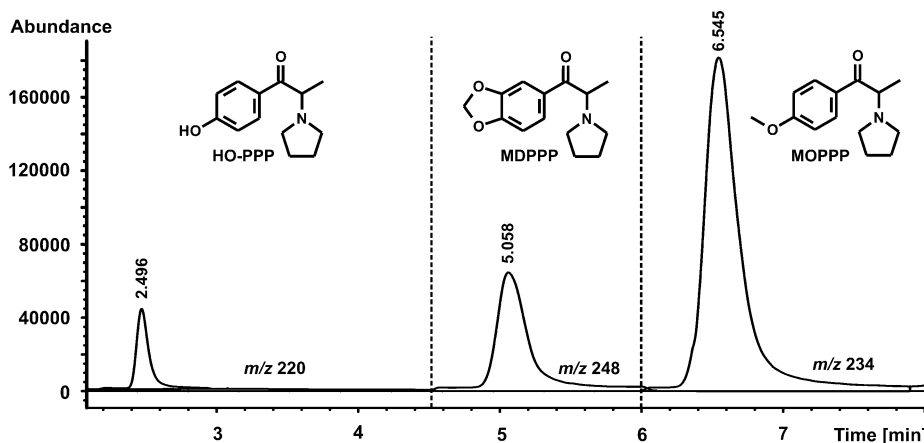


Figure 1. Typical mass fragmentograms of a supernatant of an incubation mixture of 10 µM MOPPP with cDNA-expressed CYP2D6. Dotted lines indicate the time of switch between the monitored ions (*m/z* 220, 248 or 234 for HO-PPP, standard MDPPP or MOPPP, respectively) during the LC-MS run.

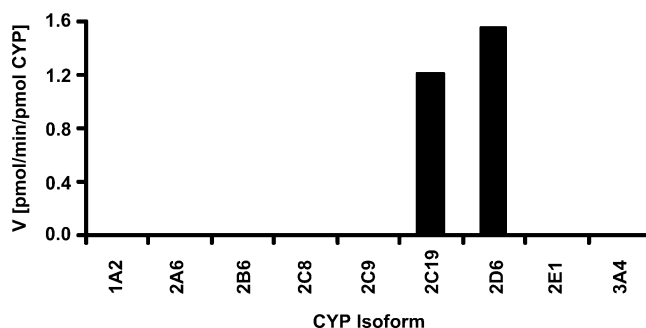


Figure 2. Estimated turnover rates (V) of 50 μM MOPPP with 50 pmoles ml^{-1} of the given individual CYPs. Data are the average of two determinations.

Kinetic studies

CYP2C19 showed a typical hyperbolic metabolite formation profile (figure 3A). Kinetic parameters for CYP2C19 were estimated to be $373.4 \pm 45.1 \mu\text{M}$ for apparent K_m and $6.0 \pm 0.3 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ CYP}$ for $V_{\max}$ (mean $\pm$ SD). In a first approach using equation (1), the kinetic parameters for CYP2D6 were estimated to be $9.9 \pm 2.5 \mu\text{M}$ for apparent K_m and $2.1 \pm 0.1 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ CYP}$ for $V_{\max}$ (solid line in figure 3B). Visual inspection of the CYP2D6 Michaelis–Menten plot suggested that the enzyme was not saturated even at the highest substrate concentration. A linear increase in the turnover rate from 100 to 500 μM was observed, and the concave course of the corresponding Eadie–Hofstee plot (figure 3C) clearly demonstrated atypical, biphasic, kinetics (Clarke 1998, Korzekwa *et al.* 1998). Therefore, CYP2D6 kinetic parameters were estimated by fitting the data into equation (2) for a two-site binding model (figure 3B, dotted line), which gave significantly better results (F -test, $p < 0.001$) than the one-site binding model. $K_{m,1}$ was calculated as $1.3 \pm 0.4 \mu\text{M}$ and $V_{\max,1}$ was estimated as $1.1 \pm 0.1 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ CYP}$. As more than one enzyme was involved in MOPPP demethylation, equation (2) was used to fit into the data points of the HLM experiments (figure 3D). Apparent $K_{m,1}$ and $V_{\max,1}$ values were $22.0 \pm 6.5 \mu\text{M}$ and $169.1 \pm 20.5 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$, respectively. The corresponding Eadie–Hofstee plot confirmed the biphasic kinetic profile (figure 3E).

The K_m , $V_{\max}$, RAF, contributions and intrinsic clearances are presented in table 1. With roughly 80–95% of the net clearance (depending on the data applied for the calculation), CYP2D6 should be the predominant enzyme responsible for MOPPP demethylation.

Chemical inhibition studies

To underline the importance of CYP2D6 in MOPPP metabolism, the CYP2D6 specific inhibitor quinidine (3 μM) was added to incubation mixtures and the metabolite formation rate compared with incubations without inhibitor. In the presence of inhibitor and 2 μM MOPPP, the metabolite formation was inhibited by about 92% (figure 4).

Discussion

HO-PPP was not available for its validated determination. However, the peak areas of HO-PPP and MDPPP were about equal, if their concentrations

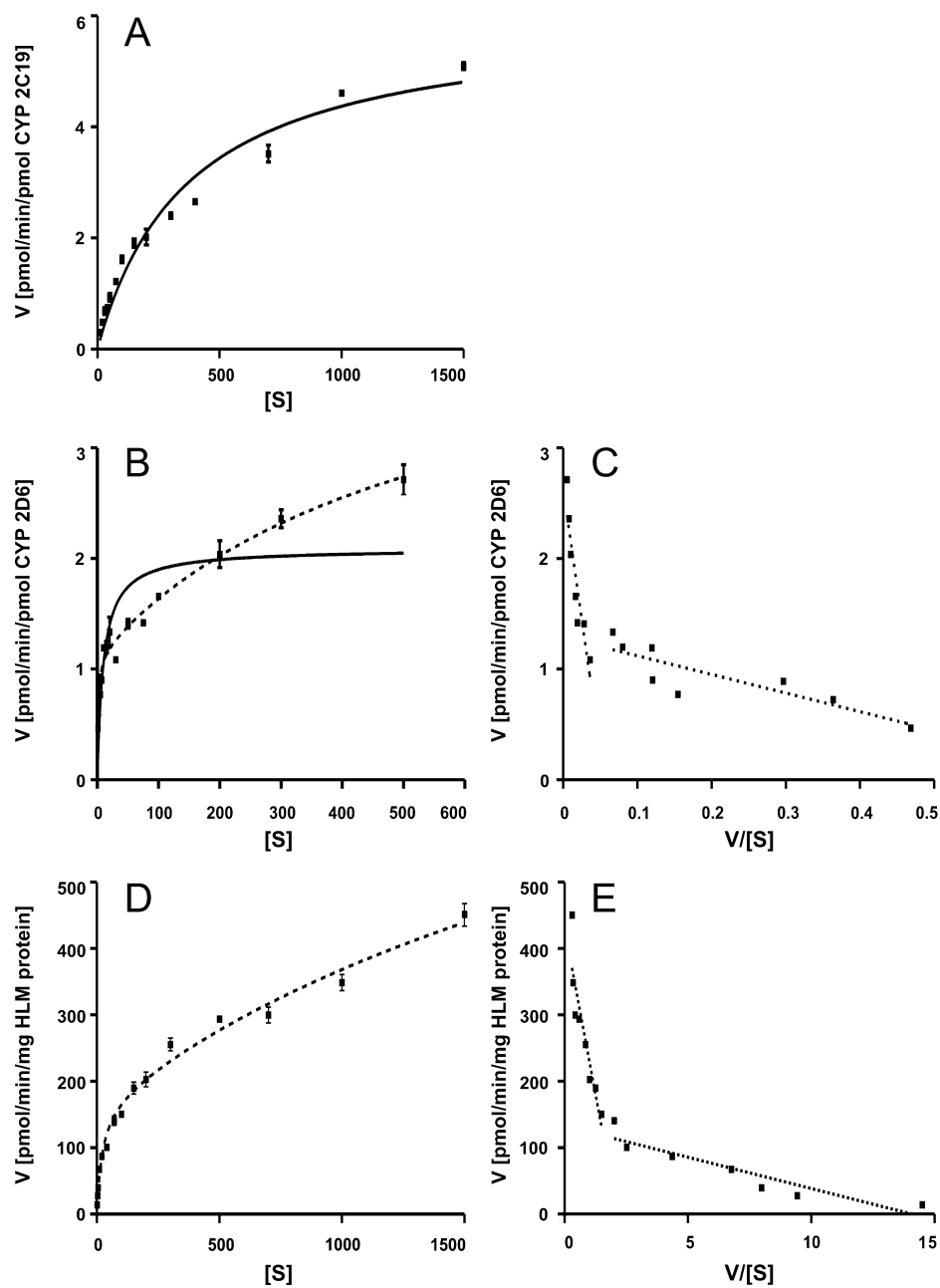


Figure 3. Michaelis-Menten (A, B, D) and Eadie-Hofstee plots (C, E) for MOPPP demethylation catalysed by CYP2C19 (A), CYP2D6 (B, C) and HLM (D, E). Data points in A, B and D are means (solid squares) and ranges (error bars) of two individual measurements. Solid curves in A and B were calculated by non-linear regression according to equation (1) (one site binding model), dotted curves in B and D according to equation (2) (two site binding model).

were the same. This could be concluded from the following observations. It could be demonstrated in many LC-MS runs that the peak areas of the standard MDPPP and MOPPP were similar, if injected in equimolar amounts. Furthermore, the peak areas of MOPPP and HO-PPP were essentially equal in

Table 1. Kinetic data of MOPPP demethylation by CYP2D6, CYP2C19 and HLM.

	CYP2D6	CYP2D6	CYP2C19	HLM
K_m (best fit $\pm$ SE)	9.9 ± 2.5^a	$K_{m,1} 1.3 \pm 0.4^b$	373.4 ± 45.1^a	$K_{m,1} 22.0 \pm 6.5^b$
V_{max} (best fit $\pm$ SE)	2.1 ± 0.1^a	$V_{max,1} 1.1 \pm 0.1^b$	6.0 ± 0.3^a	$V_{max,1} 169.1 \pm 20.5^b$
RAF	0.01	0.01	0.029	
Contribution	0.02	0.11	0.18	
Intrinsic clearance ^c	0.0021	0.0087	0.00047	
Percentage of net clearance	81.6	94.9	5.1	

K_m (μ M), V_{max} and contribution in $\text{pmol min}^{-1} \text{pmol}^{-1}$ CYP (CYP2D6 and CYP2C19) or $\text{pmol min}^{-1} \text{mg}^{-1}$ protein (HLM), intrinsic clearance in $\text{nl min}^{-1} \text{pmol}^{-1}$ CYP.

^aKinetic data estimated according to equation (1).

^bKinetic data estimated according to equation (2).

^cContribution/ K_m .

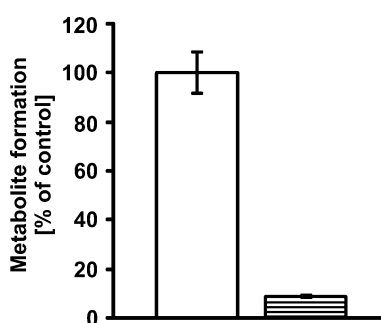


Figure 4. Effect of 3 μ M quinidine (striped bar) on metabolite formation in incubation mixtures containing 2 μ M MOPPP. Controls (open bar) were set to 100%. Each bar represents the mean of six incubations $\pm$ SEM.

incubations, in which half of the substrate (half of the initial peak area) had been demethylated to HO-PPP. No further metabolites were detected in microsomal incubations. The levels of HO-PPP were estimated in MDPPP equivalents, and as a first approximation, MDPPP equivalents and HO-PPP levels can be about the same. In addition, the apparent K_m values and conclusions concerning the percentage of clearance by a specific CYP enzyme are not affected by this quantification.

The initial screening studies with the nine most abundant human hepatic CYPs were performed to identify their possible role in MOPPP demethylation. According to the supplier's advice, the incubation conditions chosen were adequate enough to make a statement on a general involvement of a particular CYP enzyme. The data revealed that only CYP2C19 and CYP2D6, two CYPs expressed polymorphically in humans, were capable of demethylating MOPPP. For this reason, the kinetic profiles of only CYP2C19 and CYP2D6 as single CYP enzyme sources and that of HLM were further investigated.

CYP2C19 was saturated within the substrate concentration range tested and the data showed, as expected, a classical hyperbolic Michaelis–Menten plot (figure 3A). The apparent K_m and V_{max} values of CYP2C19 and in a first approach of CYP2D6 were calculated by nonlinear regression fit according to equation (1). However, closer inspection of the CYP2D6 Michaelis–Menten plot (figure 3B) revealed that CYP2D6 was not saturated at the highest substrate concentration and that the profile did not become asymptotic. Instead, a linear increase in turnover

rate from 100 to 500 μM was observed. This resulted in the inability to predict apparent K_m and $V_{\max}$ according to equation (1). Moreover, the corresponding Eadie–Hofstee plot (figure 3C) clearly demonstrated atypical biphasic kinetics (Clarke 1998). Therefore, CYP2D6 kinetic parameters were estimated by fitting equation (2) for a two-site binding model (figure 3B, dotted line), which fitted significantly better (F -test $p < 0.001$) than the one-site binding model. The linear increase in turnover rate at higher substrate concentrations therefore did not allow the determination of $K_{m,2}$ and $V_{\max,2}$. Such profiles, despite using single enzyme sources, have already been observed for CYP1A1 with aminopyrine (Inouye *et al.* 2000), CYP1A2 with 1-methoxy-4-nitrobenzene (Miller and Guengerich 2001), CYP2C9 with naproxen (Korzekwa *et al.* 1998, Hutzler and Tracy 2002) and CYP3A4 with levo- α -acetylmethadol or naphthalene (Korzekwa *et al.* 1998, Oda and Kharasch 2001), but not for CYP2D6. For these biphasic plots, Korzekwa *et al.* proposed that two substrate molecules at one time have access to the reactive oxygen. This is more likely if the binding pocket is large and the substrate molecules are small, as is the case with MOPPP.

Like CYP2D6, HLM could not be saturated with MOPPP at the highest substrate concentration (figure 3D), and the corresponding Eadie–Hofstee plot revealed a biphasic kinetic profile (figure 3E). As more than one CYP was involved in MOPPP demethylation, the data obtained from the multiple enzyme source HLM were fitted into the two-site binding model equation. The apparent K_m of the high-affinity enzyme differed somewhat from that estimated from experiments with cDNA-expressed CYP2D6 (table 1). The shifts of the apparent K_m values could be explained by the fact that the lowest HO-PPP concentrations were difficult to quantify and slight changes in the lower substrate range of the Michaelis–Menten plot can dramatically change the K_m values for the high-affinity enzyme. If the low substrate concentration data points of the Eadie–Hofstee plot were taken for the apparent K_m calculation of the high-affinity enzyme in HLM (9.5 μM), this value would be in the same order of magnitude as that of CYP2D6. Furthermore, equation (2) only yields accurate results if exactly two enzymes (one high affinity/low capacity and one low affinity/high capacity) participate in a reaction. With CYP2C19 and virtually ‘two’ CYP2D6 enzymes, the equation yields a mixture of the apparent K_m values of the high affinity/low capacity enzymes.

The RAF approach (Crespi 1995, Crespi and Penman 1997, Venkatakrishnan *et al.* 2000) and the use of immunologically determined CYP liver content (Shimada *et al.* 1994, Rodrigues 1999) have been the two main strategies to correct recombinant CYP formation rates for native human liver enzyme activity. In contrast to immunoquantified CYP levels, a RAF not only depends on the liver abundance of the CYP, but also reflects the specific activity of the cDNA-expressed preparation used. RAFs provided by the manufacturer of the enzyme preparations yielded more reliable results than using the immunoquantification approach, as demonstrated with mirtazapine as substrate (Stormer *et al.* 2000).

To confirm the role of CYP2D6 in MOPPP demethylation, inhibition studies with the CYP2D6-specific chemical inhibitor quinidine were performed. The concentration of the inhibitor (3 μM) was based on average literature data (Newton *et al.* 1995, Bourrie *et al.* 1996, Clarke 1998). The substrate concentration examined was 2 μM . This concentration was chosen because it was near the apparent K_m for the high-affinity CYP2D6 enzyme and plasma levels of MOPPP are

expected to be in this range. The results showed that at this substrate concentration, the overall turnover was inhibited markedly (about 92%). This notable inhibition was consistent with the observation that CYP2D6 accounted for about 80–95% (depending on the data applied for the calculation) of the net intrinsic clearance of MOPPP. Usually, the accuracy of prediction of a CYP contribution to a particular reaction can be readily assessed by comparison with chemical inhibition data in HLM. At a given substrate concentration, a CYP-specific inhibitor should reduce the formation rate of the metabolite in HLM by approximately the same fraction that the particular CYP is estimated to account for. RAF-corrected kinetic studies, as well as inhibition experiments at low MOPPP concentrations, demonstrated that CYP2D6 contributed predominantly to the clearance of the designer drug. CYP2D6 poor metabolizers, which account for about 7% of the Caucasian population (Bertilsson 1995, Smith *et al.* 1998), might exhibit a lower clearance than extensive metabolizers. This should not be true for CYP2C19 poor metabolizers (frequency of occurrence is 3% in Caucasians). Simultaneous intake of potent CYP2D6 inhibitory drugs, such as fluoxetine or paroxetine (Crewe *et al.* 1992), might also lead to a decreased clearance of MOPPP and, consequently, lead to elevated plasma concentrations. Whether these genetic polymorphisms and drug interactions are of clinical relevance for MOPPP toxicokinetics cannot be concluded unequivocally. The impact of MOPPP on the metabolism of other (designer) drugs, i.e. whether MOPPP might be an inhibitor of certain CYPs, is currently under investigation.

Acknowledgements

The authors thank Julian E. A. Leakey (National Center for Toxicological Research, Jefferson, AR, USA), Giseller Fritschi (Hessian State Criminal Office, Wiesbaden, Germany), and Carsten Kratzsch, Frank T. Peters and Armin A. Weber for assistance and helpful discussions.

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Characterization of bropirimine *O*-glucuronidation in human liver microsomes

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Received 3 April 2003

1. The antitumour agent bropirimine undergoes significant Phase II conjugation *in vivo*. Incubation of [^{14}C]bropirimine with human liver microsomes resulted in the formation of a single product peak (M1) using high-performance liquid chromatography with radiochemical detection and was tentatively assigned as bropirimine glucuronide based on sensitivity to β -glucuronidase and by obtaining the expected mass of 442/444 amu with liquid chromatography/mass spectrometry. Following metabolite isolation, the structure of M1 was established as bropirimine *O*-glucuronide by ^1H -nuclear magnetic spectroscopy.

2. Studies aimed at identifying the human liver UDP-glucuronosyltransferase (UGT) enzyme(s) involved in the glucuronidation of bropirimine were carried out using recombinant human UGTs and it was determined that glucuronidation of bropirimine was catalysed by UGT1A1, UGT1A3 and UGT1A9. Bropirimine *O*-glucuronidation followed Michaelis–Menten kinetics and the K_m and $V_{\max}$ (mean $\pm$ SD; $n=3$) were $1217 \pm 205 \mu\text{M}$ and $667 \pm 188 \text{ pmol min}^{-1} \text{ mg}^{-1}$, respectively.

3. The activity of bropirimine *O*-glucuronidation by human liver microsomes was inhibited by bilirubin (40%) and with mefenamic acid (80%). Although buprenorphine extensively inhibited the activity of bropirimine *O*-glucuronidation by UGT1A3, the inhibition profile did not parallel that observed in HLMs.

4. The results demonstrate that UGT1A9 and to a lesser extent UGT1A1 are responsible for the majority of bropirimine *O*-glucuronidation in man.

Introduction

Cancer of the urinary bladder represents one of the more common malignant tumours and is characterized by multiple reoccurrence and typically a poor response to conventional anticancer chemotherapeutic agents (Thrasher and Crawford 1993). Bropirimine (2-amino-5-bromo-6-phenyl-4-pyrimidinone), a member of a class of antiviral agents known as aryl pyrimidinones, has demonstrated the potential to ameliorate tumour growth in select cancer populations (Sarosdy *et al.* 1992). Consequently, bropirimine is considered as an antineoplastic agent for the treatment of bladder cancer.

It is well recognized that variability in drug metabolism between patients, including glucuronidation pathways, may be an important determinant in the clinical outcomes for many therapeutic regimens (Zhou *et al.* 1993, Iyer *et al.* 2000). Consequently, strategies of phenotyping and/or genotyping are being adopted to identify patients genetically predisposed to untoward effects associated with the administration of chemotherapeutic agents (Seppen *et al.* 1994, Lévesque

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et al. 1999). Therefore, the determination of the identity of the enzyme(s) involved in drug metabolism is important to understand further variation in drug effect and metabolism. Moreover, since many drug-metabolizing enzymes (e.g. cytochrome P450 and UDP-glucuronosyltransferase, UGT) are involved in the metabolism of several xenobiotics, the determination of the enzyme involved in the metabolism of a specific drug could provide essential information about potential drug interactions (Bertz and Granneman 1997). In man, bropirimine disposition appears to be primarily mediated via Phase II conjugation and a minor contribution from Phase I oxidation with subsequent excretion of metabolites in urine (Hauer *et al.* 1997). Previously, we described that human CYP1A2 was responsible for the Phase I metabolic pathway for bropirimine in humans (Wynalda *et al.* 1998). The present paper describes the results of *in vitro* mechanistic studies aimed at the identification and characterization of the Phase II enzyme(s) responsible for bropirimine glucuronidation.

Materials and methods

Chemicals and reagents

Bropirimine (figure 2), specifically labelled with carbon-14 (specific activity $102.2 \text{ mCi mg}^{-1}$), was supplied by Dr R. S. P. Hsi and J. A. Easter (Pharmacia, Kalamazoo, MI, USA). The radiochemical purity of [^{14}C]bropirimine was greater than 99% by HPLC analysis. Imipramine, bilirubin, 1-naphthol, mefenamic acid, buprenorphine and UDP-glucuronic acid (UDPGA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Human livers were acquired from the Arizona Organ Bank and the International Institute for the Advancement of Medicine (IIAM) and human kidney microsomes were purchased from Tissue Transformation Technology (Edison, NJ, USA). Recombinant human UGT enzymes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15) were purchased from BD Gentest (Woburn, MA, USA). Human hepatic microsome isolation and protein content of individual liver microsomal preparations were determined as described by Wienkers *et al.* (1996).

Incubation conditions for bropirimine biotransformation

Incubations containing bropirimine were performed using pooled ($n=15$) human liver microsomes (Xeno Tech, Kansas City, KS, USA) and a panel of various known substrates/inhibitors of specific UGT enzymes. Initial incubations to optimize metabolite formation with protein concentration (range 0.1 – 0.7 mg ml^{-1}) and time (30, 45, 60 and 90 min) were conducted to ensure product linearity. From these studies, each incubation contained 0.1 mg microsomal protein, UDPGA 10 mM , and [^{14}C]bropirimine $200 \mu\text{M}$ in a final volume of 0.1 ml 50 mM Tris buffer with 10 mM MgCl_2 , pH 7.4. A pre-incubation equilibrium of 4 min was carried out at 37°C , and metabolic reactions were initiated by the addition of UDPGA. Incubation reactions were allowed to proceed for 90 min and then terminated by addition of $50 \mu\text{l}$ ACN. After centrifugation at $21\,000g$ for 20 min at 4°C , the 96-well plates were transferred to an HPLC system where quantitation of bropirimine and bropirimine O-glucuronide (M1) was accomplished by radiochemical detection.

β -glucuronidase assay

Glucuronidase treatment was used to identify tentatively the metabolite peak as a glucuronide. A reaction mixture ($100 \mu\text{l}$) containing the glucuronide metabolite was generated by incubating $50 \mu\text{M}$ bropirimine and 0.5 mg ml^{-1} human liver microsomes for 1 h at 37°C . After adding $100 \mu\text{l}$ 1 M acetate buffer (pH 4.5) containing (10 000 units) of *Helix pomatia* β -glucuronidase (Sigma), it was incubated for a further 1 h at 37°C and stopped by addition of $50 \mu\text{l}$ ACN. Control incubations were identical except that β -glucuronidase was added after the stop solution. The reaction products were analysed by HPLC, as described below.

HPLC analysis of bropirimine metabolite (M1)

Quantitation of M1 was achieved using a HPLC system equipped with a Perkin Elmer Series 200 pump and autosampler (Perkin Elmer, Norwalk, CT, USA) equipped with a chilled sample tray maintained at 4°C and a FLO-ONE\Beta Series A500 flow-through radioactivity detector (Packard/Radiomatic, Meriden, CT, USA). Peak areas were integrated with Windows-based Radio-HPLC Workstation software (FLO-ONE\Data for Windows). UltimaFlo M liquid scintillant was introduced

post-column at 2.5 ml min^{-1} . Analytical separations of bropirime and metabolites were performed on a Zorbax SB-C8 column ($250 \times 4.6 \text{ mm}$; Mac-Mod Analytical, Chadds Ford, PA, USA). The mobile phase conditions were: water:acetonitrile:acetic acid (v/v/v) linear gradient from 90:10:0.5 to 80:20:0.5 over 40 min at 1.0 ml min^{-1} . UV detection was at 255 nm. Radioactivity was detected by mixing the HPLC effluent with UltimaFlo M (Packard Instruments) at a 3:1 (v/v) ratio using the A-515 detector.

Characterization of M1 by LC/ESI-MS

Incubations conducted for mass spectral analysis contained microsomal protein (0.1 mg), UDPGA 10 mM and bropirime 200 μM , in a final volume of 0.1 ml 50 mM Tris buffer with 10 mM MgCl_2 , pH 7.4 at 37°C . Incubations were carried out for 90-min periods as described above before analysis by LC/ESI-MS. The identity of the primary *in vitro* bropirime glucuronide was accomplished using a Finnigan LCQ iontrap (San Jose, CA, USA) operated in electrospray ionization (ESI) mode. Nitrogen (99.9% pure, AGA, Maumee, OH, USA) was employed as a drying gas at a sheath pressure of 80 psi and auxiliary flow rate of 20 ml min^{-1} , and the heated capillary was set at 250°C . Analytical separation of bropirime and M1 was accomplished using the HPLC conditions described above.

Identification of M1 by LC-NMR

Incubations for NMR analysis required much larger amounts of material; five 1-ml incubation mixtures, each containing 1.5 mM bropirime (one containing [^{14}C]bropirime, the other four using non-radioactive bropirime) were pooled. Acetonitrile was added to quench the reaction, and the mixture was centrifuged for 20 min at 21 000g. The supernatant was concentrated to approximately 250 μl by evaporation under vacuum and subsequently directly injected for LC-NMR analysis.

LC-NMR analysis was performed in the stopped-flow mode on a Bruker DRX-500 spectrometer (Bruker Biospin, Billerica, MA, USA) equipped with a Bruker 4 mm flow probe (120 μl active volume). The chromatography system was an HP-1100 (Agilent Technologies, Palo Alto, CA, USA) which was comprised of a binary pump, a manual injector with 200- μl injection loop and a variable wavelength detector, as well as a customized stop-flow unit built in-house for NMR studies. The chromatography parameters were controlled by Agilent ChemStation Version 4.1 software and custom software written in MATLAB Version 6 (The MathWorks, Natick, MA, USA) controlled the stopped flow equipment. The column used for separation was a Zorbax 4.7 mm $\times$ 25 cm SB-C18, and an isocratic mobile phase of 85% (A) and 15% (B) was used. Mobile phase (A) was 20 mM ammonium acetate- d_7 in 99.9% deuterated water with the pH adjusted to 7.5 with NaOD and mobile phase (B) was 99.9% deuterated acetonitrile (all deuterated solvents were from Isotec, Inc., Miamisburg, OH, USA). The flow rate was 1 ml min^{-1} , and UV detection was at 254 nm.

Determination of bropirime O-glucuronosyltransferase activity across a panel of human liver and kidney preparations

The relative rates of bropirime (50 μM for liver and 200 μM for kidney, respectively) conjugation to form M1 were determined across 10 different human liver and five different human kidney microsomal preparations. Microsomal incubations were performed as described for the kinetic studies (below) and M1 formation was quantitated by radiochemical HPLC.

Bropirime biotransformation: kinetic studies

Experiments to characterize the kinetics of bropirime O-glucuronidation were performed using pooled human liver microsomes and two individual human hepatic microsomal preparations. Incubations contained either liver microsomal protein (0.1 mg) UDPGA 10 mM, and [^{14}C]bropirime 50, 150, 550, 1050, 2050 and 3050 μM in a final volume of 0.1 ml 50 mM Tris buffer with 10 mM MgCl_2 , pH 7.4. Samples were pre-incubated for 4 min at 37°C , and reactions were initiated by the addition of UDPGA. Incubation reactions were allowed to proceed for 90 min and then terminated by addition of 50 μl ACN. Sample work-up and chromatographic analysis was as described above.

Kinetic analysis

Data from human liver microsomal incubations for the rates of formation of M1 formation across varying substrate concentrations were fitted to a single enzyme Michaelis-Menten equation to determine estimates for K_m and V_{max} using non-linear regression method of sum-of-squares with the statistical analysis program Prism (GraphPad Software, Inc., San Diego, CA, USA).

Incubations with recombinant human UGTs

Potential glucuronidation of bropirime was examined in microsomes prepared from a Baculovirus-insect cell line expressing UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15. The incubations were conducted in a manner essentially as described above, with 200 μM [^{14}C]bropirime and equivalent protein concentrations (0.3 mg ml^{-1}) of each UGT enzyme.

Bropirime inhibition study

Incubations containing bropirime were performed using pooled human liver microsomes and a panel of known substrates/inhibitors of different UGT enzymes. Each incubation contained 0.1 mg microsomal protein, UDPGA 10 mM, [^{14} C]bropirime 200 μ M and a single concentration of one of the following enzyme inhibitors: bilirubin (25 μ M)/UGT1A1 (Kadacol *et al.* 2000), buprenorphine (200 μ M)/UGT1A3 (Green *et al.* 1998), imipramine (200 μ M)/UGT1A4 (Green *et al.* 1995, Nakajima *et al.* 2002), 1-naphthol (200 μ M)/UGT1A6 (Ciotti *et al.* 1996) and mefenamic acid (25 μ M)/UGT1A9 (McGurk *et al.* 1996), in a final volume of 0.1 ml Tris buffer 50 mM, with 10 mM MgCl₂, pH 7.4. Incubation condition and the following analytical procedure were as described in kinetic studies.

Selectivity of UGT inhibitors towards UGT1A1, UGT1A9 and UGT1A3

Incubations containing bropirime were performed in microsomes prepared from Baculovirus-insect cell line expressing UGT1A1, UGT1A9 or UGT1A3. Each incubation contained 0.3 mg microsomal protein, UDPGA 10 mM, [^{14} C]bropirime 200 μ M and a single concentration of either mefenamic acid (25 μ M), bilirubin (25 μ M) or buprenorphine (200 μ M) in a final volume of 0.1 ml Tris buffer 50 mM, with 10 mM MgCl₂, pH 7.4. Incubation condition and the following analytical procedure were as described in kinetic studies.

Results

In vitro glucuronidation of bropirime

Figure 1 shows representative chromatograms of bropirime O-glucuronide formation in recombinant UGT1A9 microsomes. Figure 1A, B represents

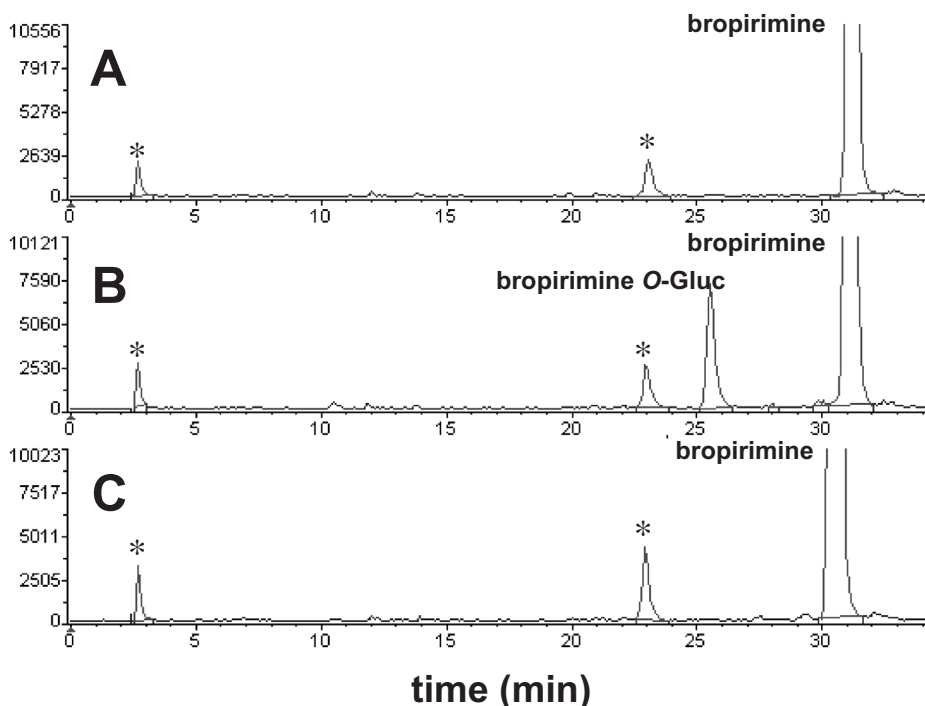


Figure 1. Representative radiochemical HPLC profiles showing glucuronidation of [^{14} C]bropirime by human liver microsomes. *Impurities associated with [^{14} C]bropirime stock solution. (A) Blank incubation of (50 μ M) bropirime with UGT1A9 microsomes without UDPGA; (B) incubation of (50 μ M) bropirime and 10 mM UDP-glucuronic acid with human liver microsomes (0.5 mg protein, 60 min at 37°C); (C) same as sample (B) but incubated with β -glucuronidase (10 000 units). Chromatographic conditions as described in the Materials and methods.

chromatograms of the incubation mixture for the bropirimine incubation without and with UDP-glucuronic acid, respectively. The retention times of bropirimine *O*-glucuronide (M1) and bropirimine were about 26 and 31 min, respectively. M1 was tentatively assigned as bropirimine glucuronide based on sensitivity to β -glucuronidase (figure 1, C). Positive-ion mass spectra of the bropirimine biotransformation product revealed stable protonated molecular ions for M1 at m/z 442/444 amu, with a corresponding fragment (m/z 266/268) formed from cleavage of the glycosidic bond (-176) (figure 2). Under LC-NMR chromatographic conditions the bropirimine *O*-glucuronide conjugate eluted at 29.6 min; these conditions were optimized to stabilize the product during the NMR experiment. A straightforward ^1H -NMR observation gave a spectrum that was easily interpretable from the aromatic region extending to 5.3 ppm. The expected phenyl resonance pattern at 7.6 ppm (5H) was observed, as well as the characteristic anomeric doublet (1H, 6.7 Hz) at 6.02 ppm. The remaining glucuronide resonances were obscured by the effects of suppressing the residual water resonance with which they coincided. The obtained anomeric shift was identical with results measured previously (Hauer *et al.* 1997). Coupled with mass spectrometric data, these results confirm M1 as the bropirimine *O*-glucuronide structure.

Bropirimine O-glucuronidation in human liver and kidney microsomes

Bropirimine *O*-glucuronidation rates were examined in 10 human liver and five human kidney microsomal samples using substrate concentration of 50 and

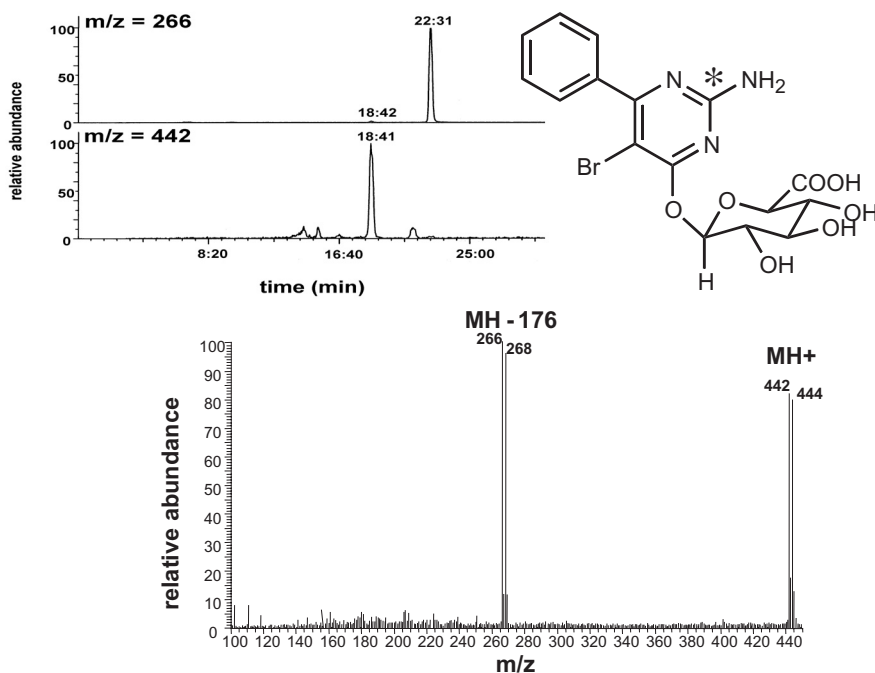


Figure 2. Positive-ion electrospray mass spectrum of bropirimine *O*-glucuronide obtained from human liver microsomal incubations. Ions at m/z 442/444 and 266/268 amu are in agreement with the molecular mass of bropirimine *O*-glucuronide and the fragment formed from cleavage of the glycosidic bond (-176 amu), respectively. LC/MS conditions are described in the Materials and methods. *Position of the carbon-14 label in the bropirimine molecule.

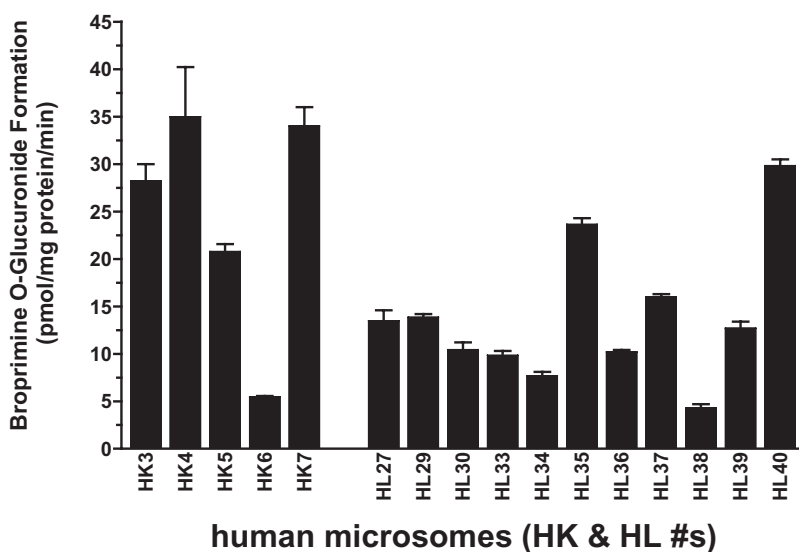


Figure 3. Intersample individual variability in the rate of broprimine *O*-glucuronidation in 10 human liver (HL) and five human kidney (HK) microsomal preparations. Incubation conditions were carried out as described in the Materials and methods. Each datum point represents the mean ($\pm$ SD) of triplicate determinations.

200 μ M (for liver and kidney, respectively). Both tissues actively metabolized broprimine with a large degree of intersample variability (figure 3); approximately eightfold for liver, (4–30 $\text{pmol mg}^{-1} \text{ protein min}^{-1}$) and sevenfold for kidney (5–35 $\text{pmol mg}^{-1} \text{ protein min}^{-1}$).

Kinetic analysis of broprimine O-glucuronide

Experiments to characterize M1 kinetic parameters were carried out in three different human liver microsomal preparations. Subsequent *in vitro* incubations revealed that product formation was linearly dependent with both time and microsomal protein (data not shown). Moreover, analysis of the kinetic data using Eadie–Hofstee graphical analysis (figure 4), revealed a linear relationship between V (the rate of M1) and $V/[S]$ (the velocity divided by the substrate concentration), which suggested that a single enzyme or two enzymes with similar K_m values were responsible for broprimine *O*-glucuronidation. The Michaelis–Menten parameters for broprimine *O*-glucuronidation determined from the single enzyme rate equation for three different human liver samples ranged are listed in table 1.

Glucuronidation of broprimine by recombinant UGTs

Of the recombinant UGT enzymes tested for the ability to metabolize broprimine to M1 (figure 5), it appears that, on an activity/mg protein basis, UGT1A9 possesses the greatest catalytic activity. As shown in figure 5, in addition to UGT1A9, minor M1 formation was also detected with recombinant UGT1A3 and UGT1A1.

Chemical inhibition of broprimine O-glucuronidation (figure 6)

To investigate further the potential UGT enzymes responsible for the formation of M1, incubations of human liver microsomes with broprimine in the

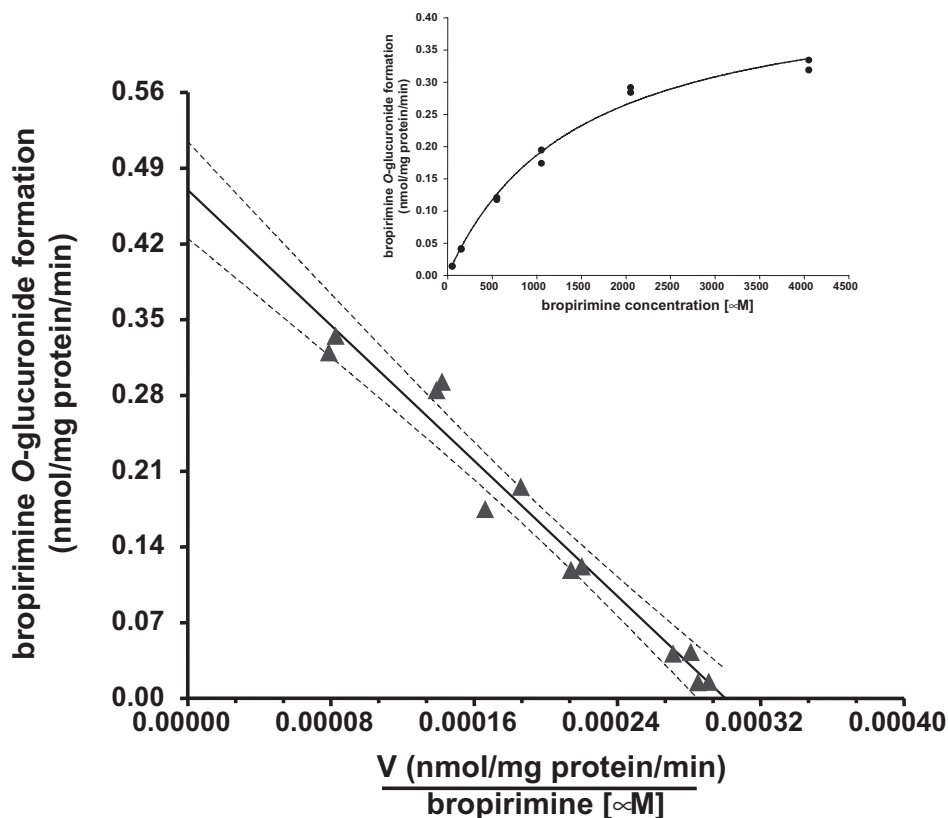


Figure 4. Representative Eadie-Hofstee plot for bropirimine *O*-glucuronidation by pooled human liver microsomes. Each value is the average of duplicate determinations, whereas the solid line is the computer-generated curve of best fit, with 95% confidence limits. Inset is the Michaelis-Menten plot showing the experimental data and the computer-generated curve.

Table 1. Estimated enzyme kinetic parameters for bropirimine *O*-glucuronidation in various human liver microsomal preparations.

Bropirimine/microsomal treatment	K_m (μM) ($\pm$ SD)	V_{max} ($pmol\ mg\ protein^{-1}\ min^{-1}$) ($\pm$ SD)
Pooled HLM	1454.20 ± 174.18	457.37 ± 23.12
HLM#36	1112.22 ± 298.17	726.07 ± 114.08
HLM#37	1086.25 ± 141.42	820.02 ± 62.05

Incubation conditions were carried out as described in the Materials and methods. Each datum point represents the mean ($\pm$ SD) of triplicate determinations.

presence of selective UGT substrates/inhibitors were performed. The results revealed that bropirimine incubations carried out in the presence of the known UGT1A9 inhibitor, mefenamic acid (McGurk *et al.* 1996, Ritter 2000), markedly reduced M1 formation. In addition, co-incubation with UGT1A1 inhibitor, bilirubin, resulted in a reduction (40%) in M1 formation, whereas all other inhibitors tested resulted in negligible M1 inhibition. As a follow-up experiment to the findings from the recombinant UGT screen, the selectivity of UGT1A1,

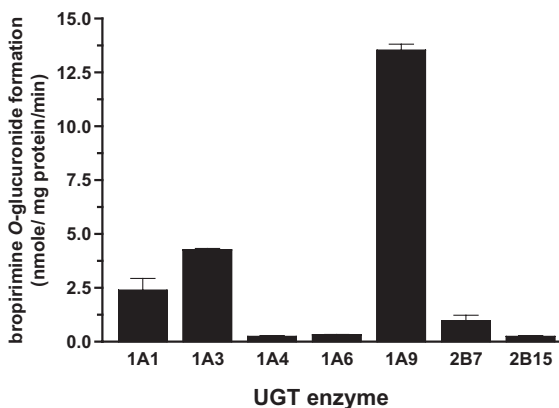


Figure 5. Comparison of the rates of bropirimine *O*-glucuronide formation across a panel of recombinant human UGT enzymes. Incubation conditions were carried out as described in the Materials and methods. Each datum point represents the mean ($\pm$ SD) of triplicate determinations.

UGT1A3 and UGT1A9 inhibitors; bilirubin, buprenorphine and mefenamic acid, on M1 formation were examined using single enzyme (recombinant UGT) microsomal preparations. The results of these limited studies demonstrated that under conditions equivalent to those carried out using human liver microsomes, buprenorphine was an inhibitor of UGT1A3 and not of UGT1A9, whereas mefenamic acid was a potent inhibitor of UGT1A9 but not of UGT1A3. Interestingly, whereas there was selectivity between the inhibitors of UGT1A3 and 1A9, there was some cross-reactivity between UGT1A1 and UGT1A9. Specifically, bilirubin elicited a slight (20%) reduction in UGT1A9-mediated M1 formation and mefenamic acid possessed inhibitory activity towards UGT1A1.

Discussion

In addition to cytochrome P450 oxidations, glucuronidation is one of the most common biotransformation reactions for therapeutic drugs. UDP-glucuronosyltransferases are endoplasmic reticulum membrane-bound enzymes that have been divided into two families, UGT1 and UGT2, based on sequence homology (Burchell *et al.* 1991, Mackenzie *et al.* 1997). The chemical mechanism of conjugation consists of an enzyme-mediated transfer of glucuronic acid from uridinediphospho-glucuronic acid (UDPGA) to non-polar compounds (Tephly and Burchell 1990). As a consequence, UGTs are involved in both the secondary metabolism of products derived from cytochrome P450 oxidative reactions (Halpin *et al.* 2002, Zheng *et al.* 2002) as well as the direct conjugation of many xenobiotics (e.g. raloxifene) and endogenous substrates (e.g. bilirubin) (King *et al.* 1996, Kemp *et al.* 2002).

In man, clearance of bropirimine is mediated via two hepatic metabolic pathways: direct Phase II conjugation yielding primarily bropirimine *O*-glucuronide and Phase I oxidation, which results in the formation of three oxidative metabolites: bropirimine dihydrodiol and *p*- and *m*-hydroxybropirimine. Previous *in vitro* mechanistic studies using human liver microsomes and recombinant cytochrome

P450 enzymes in parallel with microsomal epoxide hydrolase revealed that all three bropiramine Phase I metabolites were formed via a common CYP1A2-dependent transient arene oxide intermediate (Wynalda *et al.* 1998). The intent of the current *in vitro* study was to understand the molecular basis of bropiramine Phase II hepatic metabolism and thus provide insight into factors such as interindividual and/or ethnic variability associated with the pharmacokinetics of the drug in humans.

The liver is generally accepted as the main drug metabolic organ in human, but the kidney has also been shown capable of conjugating a wide range of drugs (McGurk *et al.* 1998). The activities detected in the present study are consistent with these observations since both liver and kidney microsomes possessed catalytic activity towards bropiramine *O*-glucuronidation (figure 3). Initial kinetic analysis of bropiramine *O*-glucuronidation revealed that M1 formation was mediated via a single enzyme or multiple enzymes that share an apparent affinity constant of approximately 1200 μ M. When bropiramine was screened against recombinant human UGT enzymes, enzymes belonging to the UGT1A subfamily possessed the greatest bropiramine *O*-glucuronosyltransferase activity (figure 5). To date, five human UGT1A enzymes have been identified and characterized from liver tissue: UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 (Tukey and Strassburg 2000). The UGT1A enzymes, like other drug-metabolizing enzymes, share many transcriptional (Owens and Ritter 1995) and structural features (e.g. carboxyl-terminal region). In some cases, however, each enzyme has its own substrate specificity (Ebner and Burchell 1993, Fisher *et al.* 2000). For example, UGT1A1 in addition to having a physiological role in the glucuronidation of bilirubin, is also capable of conjugating a variety of phenolic substrates such as flavonoids (Boersma *et al.* 2002, Sorich *et al.* 2002). In contrast, the substrate specificity for UGT1A3 and UGT1A4 usually involve the glucuronidation of tertiary amines to quaternary ammonium-linked glucuronides as well as secondary and primary amines (Green *et al.* 1995, 1998). In addition, while UGT1A6 and UGT1A9 preferentially conjugate phenols (Ethell *et al.* 2002), they may be differentiated by the chemical class of phenolic substrate. UGT1A9 typically catalyses the glucuronidation of bulky phenols, whereas UGT1A6 has been reported to catalyse the glucuronidation of phenolic substrates with the steric requirements of small size and planarity (Court *et al.* 2001). Moreover, it appears that a UGT1A6 is also sensitive to the presence of substitution at position alpha to the phenol (Ebner and Burchell 1993). Thus, in light of the complimentary structural (e.g. phenolic) and spatial (e.g. less than 14.5 Å intramolecular distance perpendicular to the phenolic *O*-position) features of bropiramine to many known UGT1A enzyme substrates (Takenaga *et al.* 2002), it was not surprising that the bropiramine *O*-glucuronosyltransferase would be a member of the UGT1A family.

Of the five possible UGT1A enzymes expressed in the liver, only three, UGT1A1, UGT1A3 and UGT1A9, possess any measurable M1 activity (figure 5). When bropiramine was co-incubated with known substrates/inhibitors of the various UGT enzymes, the proposed UGT1A9 inhibitor, mefenamic acid, exhibited the greatest inhibition (about 80%) of M1 formation compared with control (i.e. without inhibitor) incubations (figure 6). In addition, there was a marked decrease observed with co-incubation with the UGT1A1 inhibitor, bilirubin (about 40%), which suggests a role for UGT1A1 in bropiramine *O*-glucuronidation.

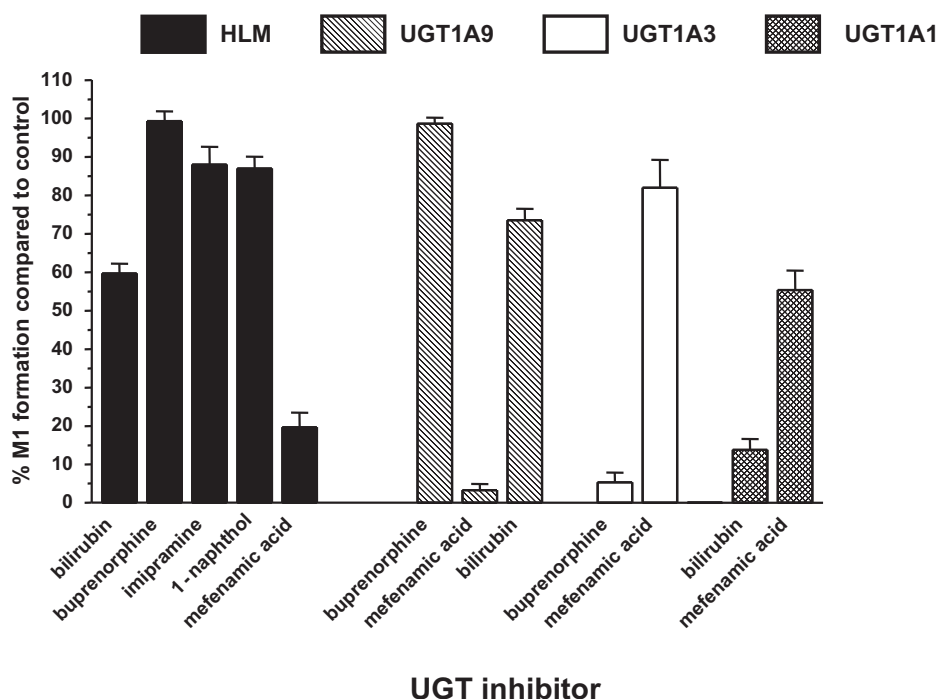


Figure 6. Percentage of control activities for bupiramine *O*-glucuronidation in the presence of various substrates/inhibitors selective for individual UGT enzymes conducted in pooled human liver microsomes (solid bars); recombinant UGT1A9 (hatched bars), recombinant UGT1A3 (open bars) and recombinant UGT1A1 (cross-hatched bars). Incubation conditions were carried out as described in the Materials and methods. Each datum point represents the mean ($\pm$ SD) of triplicate determinations.

Moreover, all other inhibitors tested were devoid of any significant inhibition of M1 formation.

To further examine the inhibition screen conducted in human liver microsomes, the UGT selectivity of mefenamic acid and bilirubin inhibition was examined against UGT1A9 and UGT1A1, respectively. The results of this investigation revealed that bilirubin was relatively selective for UGT1A1, whereas mefenamic acid has some inhibitory activity (about 40%) towards UGT1A1 (figure 6). However, as UGTs often have overlapping substrate specificities (Luukkanen *et al.* 2001) and UGT1A9 is known to recognize and conjugate many molecules also conjugated by UGT1A1 (Hagenauer *et al.* 2001, Gagne *et al.* 2002), a modest overlap between UGT1A1 and mefenamic acid in addition to the anticipated UGT1A9 inhibition was not surprising. Therefore, taking into account the inhibition by bilirubin (40%) with the inhibition observed with mefenamic acid (80%), this suggests that UGT1A1 and in particular UGT1A9 constitute the majority of bupiramine *O*-glucuronosyltransferase activity in human liver microsomes.

An anomaly to the current study was the lack of concordance between the information obtained from the inhibition experiments performed in pooled human microsomes to the results obtained with the recombinant UGT screen. In an attempt to relate these two studies, bupiramine was incubated in recombinant UGT enzymes, UGT1A3 and UGT1A9, in the presence of the potent UGT

inhibitors, buprenorphine (UGT1A3) and mefenamic acid (UGT1A9). The intent of this experiment was to examine the selectivity of the inhibitors as a means to exclude UGT1A3 as a major bropiramine *O*-glucuronosyltransferase. The results of this experiment clearly demonstrate that whereas recombinant UGT1A3 possessed a small amount of bropiramine *O*-glucuronidation activity, co-incubation of the UGT1A3 inhibitor did not result in a significant reduction in the rate of conjugation in pooled human liver or recombinant UGT1A9 microsomes. On the other hand, co-incubation of mefenamic acid with human liver microsomes resulted in a marked decrease in M1 formation, but with relatively no cross-reactivity with UGT1A3 (figure 6).

In conclusion, the identity of the hepatic enzyme(s) responsible for bropiramine *O*-glucuronidation was accomplished through several *in vitro* mechanistic studies: (1) kinetic analysis of M1 formation in human liver microsomes; (2) assessment of M1 formation across a panel of recombinant UGT enzymes; and (3) use of known substrates/inhibitors of different UGT1A enzymes against bropiramine *O*-glucuronosyltransferase activity in human liver microsomes. The results of these reaction phenotyping experiments suggest a role for UGT1A9, and to a lesser extent UGT1A1, as the glucuronosyltransferases responsible for bropiramine conjugation in human.

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Identification of some *N*-hydroxylated metabolites of (±)-3,4-methylenedioxymethamphetamine in horse urine by gas chromatography-mass spectrometry

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Received 11 March 2003

1. The *in vivo* biotransformation of (±)-3,4-methylenedioxymethamphetamine [(±)-MDMA] in the thoroughbred horse was determined after oral administration.

2. Unconjugated compounds and aglycones were isolated from enzyme-hydrolysed urine by solid-phase extraction using mixed-mode cartridges. The basic isolates were derivatized (trimethylsilyl ether, TMS) and analysed by positive-ion electron ionization/gas chromatography-mass spectrometry (EI+/GC-MS). MDMA and 10 Phase I metabolites containing the arylisopropylamine substructure were detected.

3. *N*-Hydroxy amphetamine and *N*-hydroxymethamphetamine were synthesized. The EI+ mass spectra of their *O*-TMS derivatives showed characteristic α -cleavage ions at m/z 132 and 146, respectively, as base peaks. Based upon these data, five putative *N*-hydroxylated metabolites of MDMA were detected.

4. In the horse, (±)-MDMA is metabolized by oxidative *N*-demethylation to form the primary amine methylenedioxyamphetamine (MDA). Both MDMA and MDA are further metabolized by oxidative demethylenation (cleavage and *O*-demethylation of the benzodioxole moiety) to form the corresponding catechols, 3-*O*-methylation to form the guaiacols and *N*-oxidation of the secondary and primary amine metabolites to form the hydroxylamines.

5. Both phenolic and *N*-hydroxy metabolites of (±)-MDMA undergo Phase II conjugation before excretion in urine.

Introduction

(±)-3,4-Methylenedioxymethamphetamine, (*N*, α -dimethyl-1,3-benzodioxole-5-ethanamine) (MDMA) and commonly known as 'ecstasy (XTC)', 'adam' or 'E', is a psychoactive designer drug of abuse (Pentney 2001). In humans, its use recreationally has been associated with problems of neurotoxic action on dopamine neurons, impairment in memory, renal and hepatic failure, brain damage and fatalities (Parrott 2002, Bogen *et al.* 2003). Detailed chemical profiling of trace impurities present in illegally manufactured MDMA tablets has been reported (Gimeno *et al.* 2002, Palhol *et al.* 2002).

The *in vivo* and *in vitro* metabolism of (±)-MDMA in rat, mice and man has been reported (Lim and Foltz 1988, Yousif *et al.* 1990, Maurer 1996, Maurer *et al.* 2000). The known basic metabolites are (±)-MDA (α -methyl-1,3-benzodioxole-5-ethanamine), (±)-3,4-dihydroxyamphetamine, (±)-3,4-dihydroxymethamphetamine, (±)-4-hydroxy-3-methoxyamphetamine

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(HMA) and ($\pm$)-4-hydroxy-3-methoxymethamphetamine (HMMA). Formation of 2-, 5- and 6-hydroxy phenols and the neutral and phenolic deaminooxo (phenyl-2-propanone) metabolites of ($\pm$)-MDMA in both rat and man have also been reported (Lim and Foltz 1991, Maurer 1996). Formation of glutathione adducts of the *cis*-dihydroxy (catechol) metabolites was reported by Hiramatsu *et al.* (1990). P450 enzymes involved in the oxidative metabolism of ($\pm$)-MDMA in man have been identified (Kumagai *et al.* 1994, Tucker *et al.* 1994, Kreth *et al.* 2000).

Beckett's group was the first to report on both the *N*-hydroxylation of arylisopropylamines (Beckett and Al-Sarraj 1972, Beckett and Belanger 1976) and the formation of other *N*-oxygenated compounds such as oximes, nitro, nitroso and nitrones, some possibly as metabonates (Beckett and Belanger 1974, Beckett *et al.* 1974, Beckett 1977). *N*-Hydroxy-MDA has been identified as a minor metabolite of MDMA in human urine (de Boer *et al.* 1997). *N*-Hydroxy metabolites of amphetamine and ephedrine and of *N*-desmethylelegiline and methamphetamine both formed by *N*-dealkylations from selegiline have been identified by GC-MS in horse urine (Dumasia 2001).

Blood and urine are the most common biofluids analysed for detecting misuse of MDMA and related arylisopropylamines in man. However, alternative biological matrices such as hair, saliva, sweat and vitreous humor (Kintz and Samyn 1999, Clauwaert *et al.* 2000, Navarro *et al.* 2001) have also been used. Following extraction of MDMA and its metabolites from biological matrices by a variety of methods, the identification and/or quantification of the drug and metabolites by high-performance liquid chromatography (HPLC) with fluorescence (Tagliaro *et al.* 1999), photodiode-array (Helmlin *et al.* 1996), electrochemical (Segura *et al.* 2002) and MS detection (Mortier *et al.* 2002), GC with nitrogen-selective (Ortuno *et al.* 1999) and MS detection (Jurado *et al.* 2000), electrophoresis (Ramseier *et al.* 2000, Pizarro *et al.* 2002), immunoassays (Poklis *et al.* 1993) or mass spectrometry (McCooney *et al.* 2002) have been reported. Pizarro *et al.* (2003) also determined the stereochemical disposition of MDMA and its metabolites in human plasma and urine by GC-MS.

The present study was conducted to identify the major metabolites of MDMA in horse urine for the development of forensic screening and confirmatory methods. After administration of a single oral dose to two thoroughbred horses, the drug and its basic metabolites were isolated from urine as reported (Dumasia and Houghton 1991). A combination of enzymatic hydrolysis, solid-phase extraction (SPE), TMS derivatization and capillary column GC-MS was used. This permitted the detailed multicomponent analysis and identification of MDMA and its basic metabolites, as well as reducing the risk of possible formation of chemical artefacts (metabonates) by non-enzymatic changes in the labile metabolites at any stage of the analytical procedure. The *in vivo* metabolism of ($\pm$)-MDMA and the methods used for the isolation, detection and characterization of basic metabolites in the horse are described.

Materials and methods

Chemicals and reagents

Glass distilled-grade organic solvents were purchased from Rathburn Chemicals Ltd (Peebleshire, UK). β -Glucuronidase from *Helix pomatia* (type HP2; a mixture of β -glucuronidase and aryl sulfatase

enzymes), ($\pm$)-3,4-MDMA.HCl, ($\pm$)-3,4-MDA.HCl and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were obtained from Sigma Chemical Co. Ltd (Poole, UK). ($\pm$)-HMMA solution (1.0 mg ml^{-1} , Radian International, TX, USA) was obtained from Promochem Ltd (Welwyn Garden City, UK). Worldwide Monitoring XTRACT[®] mixed-mode SPE cartridges were obtained from Anachem (Luton, UK).

Drug administration, collection and storage of biofluids

After overnight fasting, naturally voided blank urine samples were collected from two castrated thoroughbred male racehorses (age 10 and 12 years, bodyweight 425 and 515 kg, respectively). The horses were fed ($\pm$)-MDMA.HCl ($75.0\text{ mg} = 63.1\text{ mg free base}$). All post-administration urine samples were collected for the first 24 h and three samples per day were collected between 08:00 and 17:00 h for the next 2 days. The void times and volumes were recorded and aliquots were stored at -20°C until required for analysis.

Hydrolysis, extraction, derivatization and analysis of urine

Pre- and sequential post-administration urine samples (5 ml) were adjusted to pH 5.0, incubated overnight at 37°C (or at 50°C for 2 h) with *H. pomatia* enzymes (50 μl), readjusted to pH 6.0, centrifuged (2000g, 15 min) and extracted using XTRACT[®] mixed-mode SPE columns (Dumasia and Houghton 1991). The residues were derivatized as TMS ethers by the addition of 50% MSTFA in toluene (50 μl ; 60°C , 0.5 h), the reagents removed under nitrogen at 40°C , the derivatized residues dissolved in toluene (50 μl) and analysed by GC-MS in the EI+ mode.

*Synthesis of *N*-hydroxyamphetamine and *N*-hydroxymethamphetamine*

The hydroxylamines were synthesized using the method described by Cashman *et al.* (1999). The products were characterized by ^1H -NMR spectroscopy and also analysed by GC-MS as their TMS-ether derivatives (Lindeke *et al.* 1973).

GC-MS analysis

EI+/GC-MS at 70 eV was performed on a Fisons MD800 bench-top mass spectrometer interfaced to a Thermoquest Trace 2000 series GC. A BPX5 column (about $25\text{ m} \times 0.25\text{ mm i.d.}$, $0.25\text{ }\mu\text{m}$ film thickness) was used with helium as the carrier gas. The initial column temperature, 90°C , was maintained for 1 min. The oven was then programmed at $15^\circ\text{C min}^{-1}$ to 320°C and maintained at 320°C for 5 min. The injector, transfer line and the ion source temperatures were 260, 280 and 150°C , respectively. An aliquot (1 μl) of the derivatized isolate was injected in splitless mode (1 min) using an autosampler (CTC model A200S, Zwingen, Switzerland). Full-scan EI+ mass spectra were recorded from 5 to 18 min of run time by scanning from m/z 40 to 550 (1.5 scans s^{-1}). The reference compounds were derivatized and analysed together with the samples.

Results

The total ion current (TIC) and mass chromatograms of the ions m/z 44 and 58 obtained by GC-MS analysis of a TMS-derivatized urine isolate (figure 1) show positive responses for the presence of MDMA and related compounds (M1 $\rightarrow$ M5). The full-scan EI+ mass spectra of these compounds are shown in figure 2. The EI+ spectrum of authentic MDMA showed a weak molecular ion $[\text{M}^+]$ at m/z 193 with fragment ions at m/z 58 (base peak), m/z 135 $[\text{M}^+-58]$ and m/z 136 $[\text{M}^+-57]$. By comparison of its retention time (RT) and mass spectrum with those of authentic MDMA, compound A (figure 2) was identified as the unchanged parent drug.

M1 $\rightarrow$ M3 (figure 2) showed the diagnostic ion m/z 44, indicating *N*-demethylation to form the primary amine and a major ion at M^+-43 (loss of $\text{C}_2\text{H}_5\text{N}$). M1 (figure 2) was identified as MDA by comparison of its RT and the EI+ mass spectrum with those of authentic methylenedioxyamphetamine. The presence of the ions m/z 136 $[\text{M}^+-43]$ and 135 $[\text{M}^+-44]$ in the spectrum indicated losses from the alkylamine side chain. M3 (figure 2) showed a molecular ion $\text{M}^+ m/z$ 311, the α -cleavage ion m/z 44, fragment ions m/z 296 $[\text{M}^+-15]$ and m/z 268 $[\text{M}^+-43]$ and the TMS ion at m/z 73. It was identified as the *bis*-O-TMS derivative of the

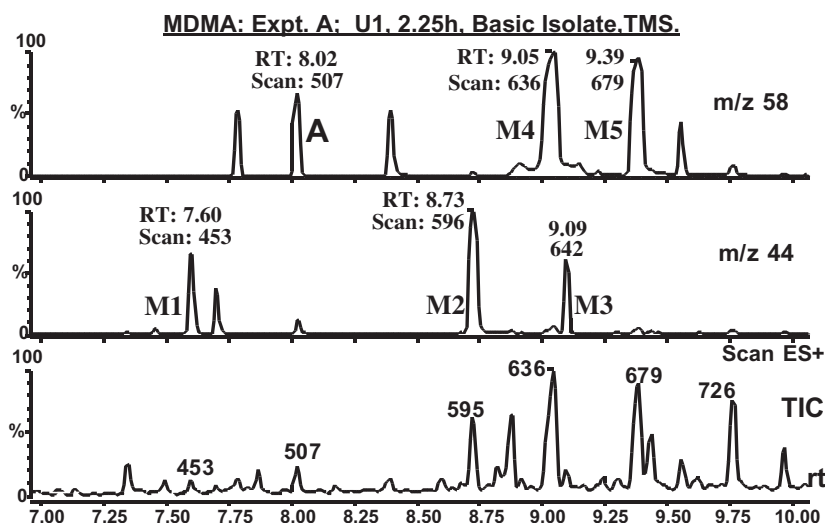


Figure 1. TIC and mass chromatograms of the α -cleavage ions m/z 44 and 58 obtained by EI+/GC-MS analysis of the TMS-derivatized isolate of a hydrolysed horse urine sample, showing the presence of MDMA (A) and its known metabolites M1→M5.

catechol metabolite 3,4-dihydroxyamphetamine. M2 (figure 2) showed ions at m/z 238 [$M^+ - 15$, base peak], m/z 210 [$M^+ - 43$] and m/z 44. It was identified as the *mono-O*-TMS derivative of the guaiacol, 4-hydroxy-3-methoxyamphetamine (HMA), formed presumably by the action of catechol *O*-methyl transferase, from M3. M2 and M3 could be formed either from initial demethylenation of M1 or by *N*-dealkylation of M4 and M5.

The spectra of M4 and M5 (figure 2) showed the base peak at m/z 58 indicating the unchanged secondary amine and fragment ions at [$M^+ - 15$] and [$M^+ - 57$; C_3H_7N]. From a comparison with the RT and mass spectrum of the *O*-TMS-derivative of authentic HMMA, M4 was identified as the *mono-O*-TMS derivative of the guaiacol, 4-hydroxy-3-methoxymethamphetamine. It was the major metabolite of MDMA in horse urine and could be detected for up to 72 h. From an interpretation of the mass spectral fragmentation, M5 was characterized as the *bis-O*-TMS derivative of the catechol 3,4-dihydroxymethamphetamine.

The TIC, mass chromatograms of the α -cleavage ions m/z 132 and 146 and the EI+ spectra of the TMS derivatives of the synthesized *N*-hydroxyamphetamine and *N*-hydroxymethamphetamine are shown in figures 3 and 4. The ions at m/z 73, 75 are TMS species and the ion at m/z 91 represents the tropylium species (C_7H_7) formed after benzylic cleavage of the side chain.

The TIC, mass chromatograms of the ions m/z 132 and 146 and the full-scan EI+ spectra of five hydroxylamine metabolites (M6→M10), obtained from the derivatized urine isolate, are shown in figures 5 and 6. From the molecular weights of the TMS derivatives and their mass spectral fragmentations, M6 was identified as *N*-hydroxy-MDA and M7 as *N*,4-dihydroxy-3-methoxyamphetamine (*N*-hydroxy-HMA). Although the *N*,3,4-trihydroxyamphetamine metabolite was detected in the early urine samples, the level was not sufficient to obtain an acceptable full-scan mass spectrum. Similarly, M8–10 were identified as *N*-hydroxy-MDMA, *N*,4-dihydroxy-3-methoxymethamphetamine (*N*-hydroxy-HMMA) and *N*,3,4-trihydroxymethamphetamine, respectively. The structures

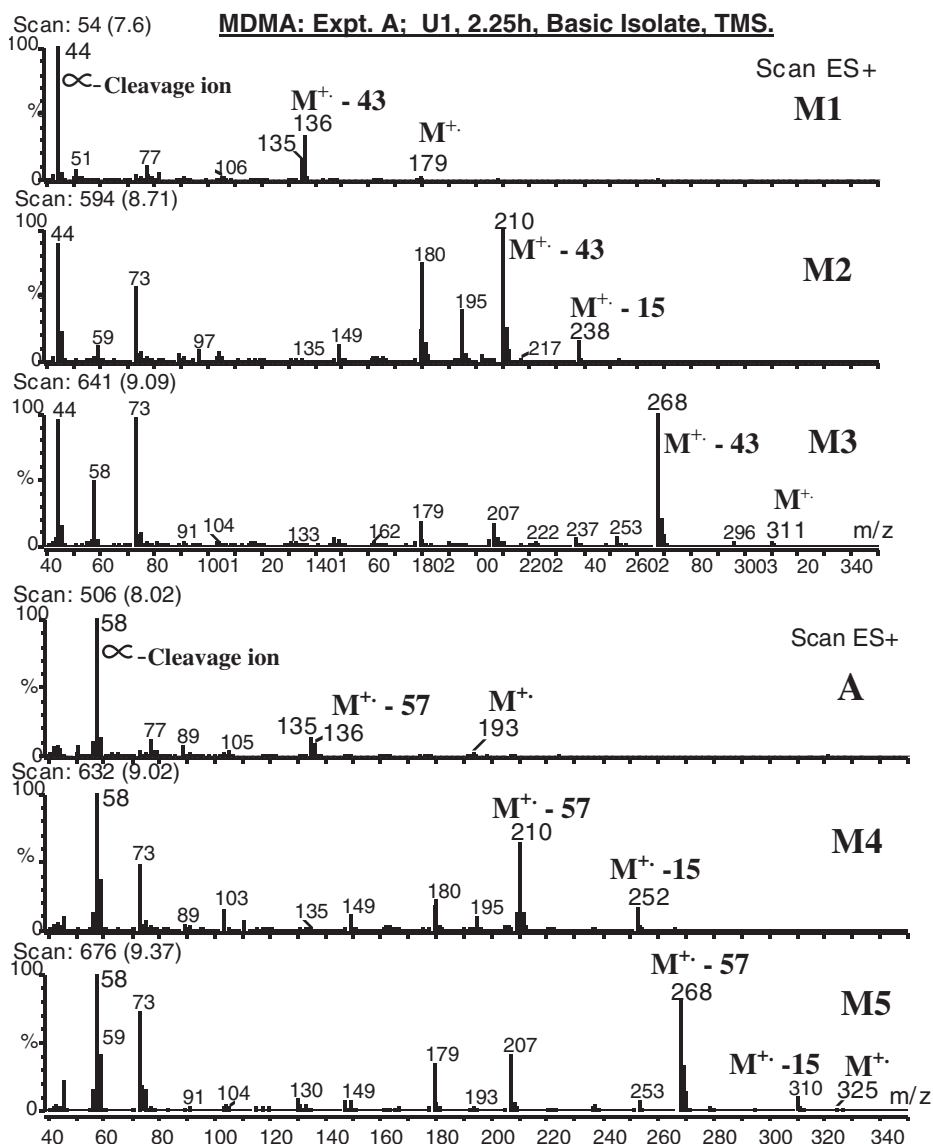


Figure 2. Full-scan EI+ mass spectra of underivatized MDA (M1), 4-hydroxy-3-methoxyamphetamine [M2; (HMA), as *mono*-TMS derivative], 3,4-dihydroxyamphetamine (M3; as *bis*-TMS derivative), underivatized MDMA (A), 4-hydroxy-3-methoxymethamphetamine [M4; (HMMA), as *mono*-TMS derivative] and 3,4-dihydroxymethamphetamine (M5; as *bis*-TMS derivative) obtained from the hydrolysed urine sample.

of MDMA and the putative metabolites identified in horse urine are shown in figure 7.

Discussion

Following oral administration of (±)-MDMA to the thoroughbred horse, several Phase I and II metabolites were excreted in urine. After enzyme hydrolysis, the aglycones were isolated by a targeted SPE method for basic drugs using mixed-mode

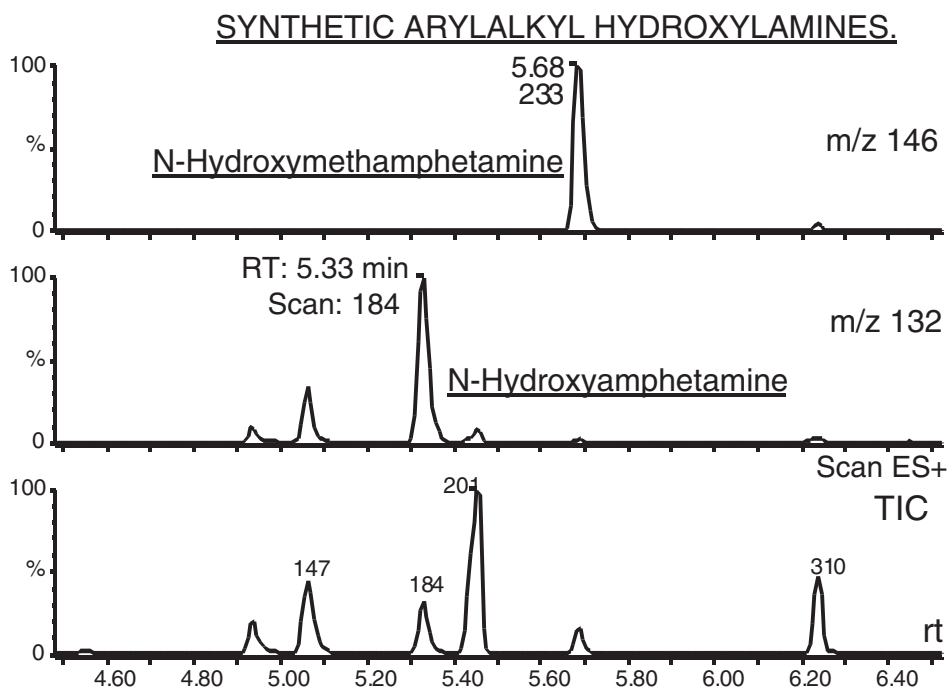


Figure 3. TIC and mass chromatograms showing the charge retained α -cleavage ions m/z 132 and 144 obtained from analysis of the *O*-TMS-derivatives of the synthesized compounds *N*-hydroxyamphetamine and *N*-hydroxymethamphetamine.

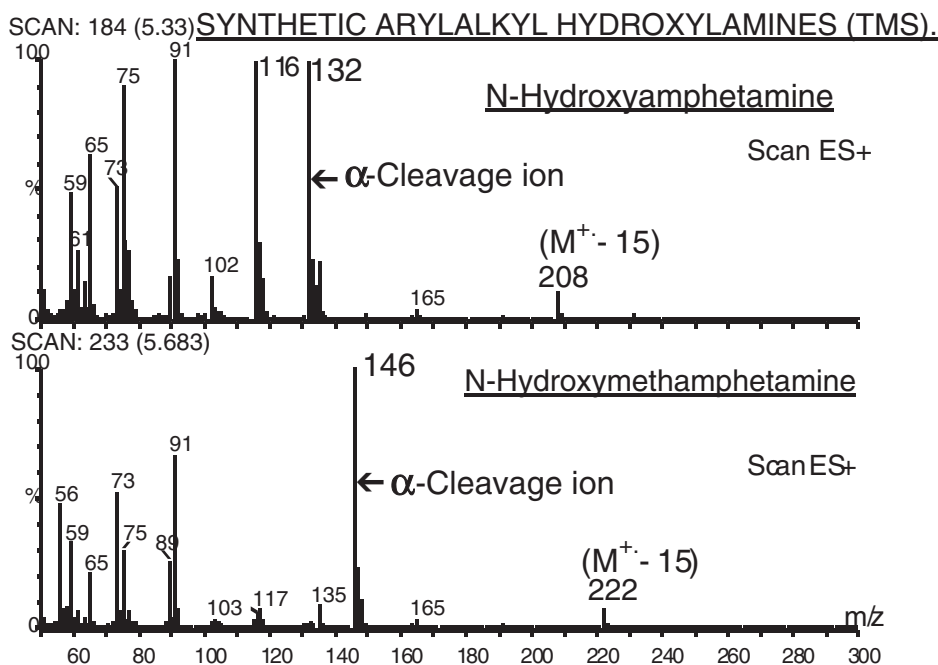


Figure 4. Full-scan EI+ mass spectra of the *O*-TMS-derivatives of *N*-hydroxyamphetamine and *N*-hydroxymethamphetamine.

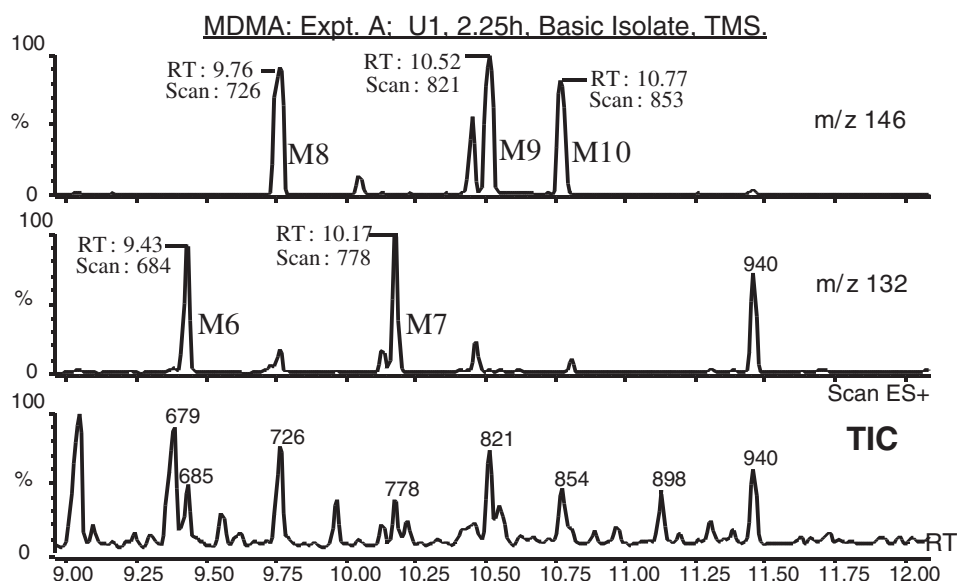


Figure 5. TIC and mass chromatograms of the diagnostic ions m/z 132 and 146 obtained from the TMS-derivatized isolate of a hydrolysed urine sample showing the presence of five *N*-oxygenated (hydroxylamine) metabolites M6→M10.

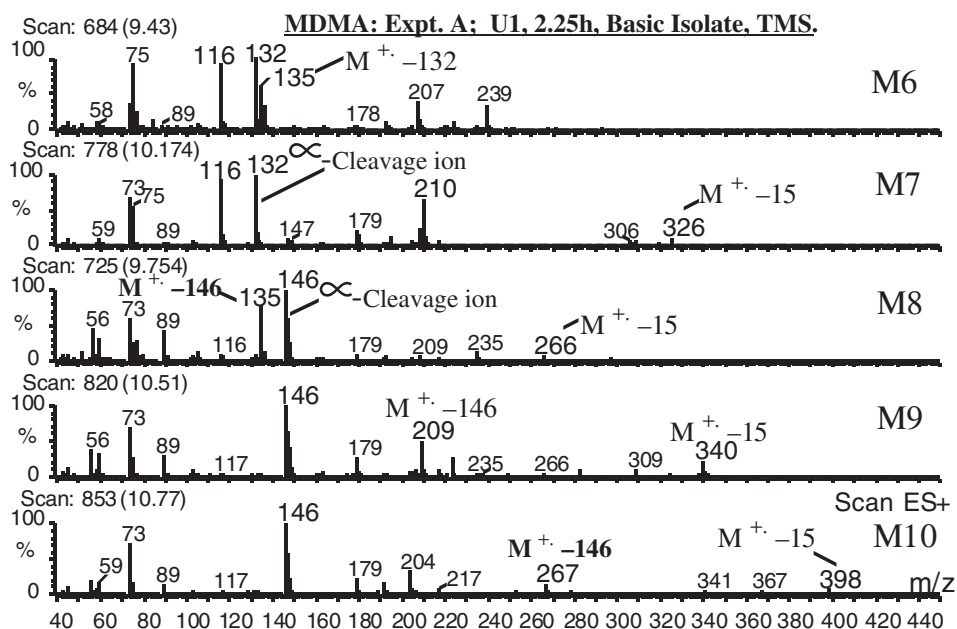


Figure 6. Full-scan EI+ spectra of *N*-hydroxy-MDA (M6; as *mono-O*-TMS derivative), *N*,4-dihydroxy-3-methoxyamphetamine (M7; *N*-hydroxy-HMA as *bis-O*-TMS derivative), *N*-hydroxy-MDMA (M8; as *mono-O*-TMS derivative), *N*,4-dihydroxy-3-methoxymethamphetamine (M9; *N*-hydroxy-HMMA as *bis-O*-TMS derivative) and *N*,3,4-trihydroxymethamphetamine (M10 as *tris-O*-TMS derivative) obtained from hydrolysed horse urine.

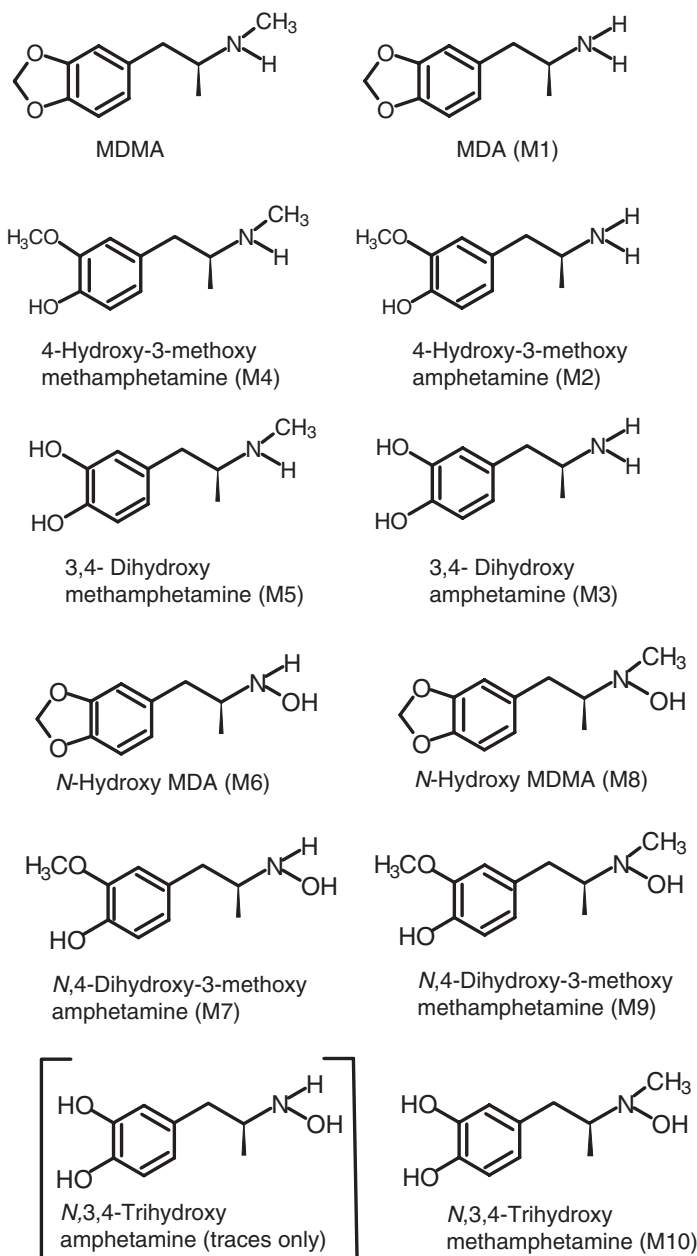
In Vivo Metabolism of Methylenedioxymethamphetamine in the Horse.

Figure 7. Structures of the primary and secondary metabolites of (±)-MDMA identified in horse urine by GC-MS.

cartridges (Dumasia and Houghton 1991). At pH six, basic compounds containing the secondary and primary amino groups exist in solution as their conjugate acid form and are retained by the cation-exchange sorbent (benzenesulphonic acid). Other metabolites formed *in vivo* by the oxidative deamination of MDMA,

such as the neutral, catecholic and guaiacolic 3,4-substituted phenyl-2-propanones, phenyl-2-propanols and their acidic metabolites are retained on the C_{18} phase by non-polar and polar interactions. These compounds are removed at the methanol wash step. In this study, only the basic metabolites were selectively recovered by elution with basified solvents, derivatized and analysed by EI+/GC-MS.

EI+ mass spectra of mono- and bifunctional arylisopropylamines such as amphetamines, phentermines and ephedrine were dominated by the diagnostic charge-retained α -cleavage ions. Depending on the substituent present on the amine nitrogen these compounds show α -cleavage ions at m/z 44 (primary amine), 58 or 72 (secondary amine; methyl or ethyl groups). In most cases, the molecular ion (M^+) is of very low intensity or not observed. The mass spectra of MDMA and its metabolites also show characteristic charge retained ions at [$M^+ - 43$] and [$M^+ - 57$], probably formed by a proton transfer on to the 1,3-benzodioxole moiety with concomitant loss of the $CH_3-CH=N-H$ or $CH_3-CH=N-CH_3$ groups. Following oxidation of the aromatic carbocycle, the α -cleavage ion remains unchanged but an increase in the molecular weight of the compounds (and their TMS derivatives) and a shift in the mass of some of the fragment ions are observed. However, *N*-hydroxylation and TMS-derivatization of the hydroxylamine metabolite results in an increase of 88 mass units of the α -cleavage ions. Thus, for the primary amine, the ion at m/z 44 exhibits a shift to m/z 132 and for the secondary *N*-methylamine, the ion at m/z 58 shifts to m/z 146.

In the thoroughbred horse, a number of *in vivo* Phase I biotransformation pathways were observed for the detoxification and elimination of ($\pm$)-MDMA in urine. In addition to the parent drug A, 10 compounds (M1 $\rightarrow$ M10; figure 7) were detected and identified. The main pathways leading to the formation of the basic metabolites were *N*-demethylation to form the primary amine MDA, demethylenation of the benzodioxole moiety to form the catechols, catechol *O*-methylation to form the guaiacols and *N*-oxygenation of both primary and secondary metabolites to form hydroxylamines. Both phenolic and *N*-hydroxylated metabolites were excreted as Phase II conjugates (glucuronides and/or sulphates). M1 $\rightarrow$ M5 (figure 7) were identified in man and rat (Yousif *et al.* 1990, Ortuno *et al.* 1999, Pizarro *et al.* 2002). 3,4-Dihydroxymethamphetamine (M5) is reported to be a major metabolite of MDMA in human urine (Segura *et al.* 2001). *N*-Hydroxy-MDA (M6) has previously been identified in human urine as an *N*-oxygenated metabolite of MDMA (de Boer *et al.* 1997). In the present study, four other *N*-hydroxy metabolites of MDMA (M7 $\rightarrow$ M10; figure 7) are recorded for the first time in horse urine.

Many other classes of drugs such as the β -adrenergic mimetics, phenothiazines and tricyclic antidepressants also contain secondary and tertiary *N*-alkylamine substructures. The *in vitro* formation of primary and secondary hydroxylamines of phenothiazines and tricyclic antidepressants has been reported and also implicated as intermediates in the retro reduction or further transformations to oximes, nitroso and nitrones (Beckett *et al.* 1988, Clement *et al.* 1993). However, formation of stable hydroxylamine metabolites has not been observed during previous studies on the *in vivo* metabolism of β -adrenergic mimetics (Dumasia 1992) and the phenothiazines and tricyclic antidepressants in the horse (Dumasia, unpublished data).

Hydroxylamines, especially of arylalkylamines where α -hydrogens are present, are unstable and converted by non-enzymatic chemical oxidation (under alkaline

conditions) as well as enzymatically to the corresponding oximes (Lindeke *et al.* 1978, Beckett *et al.* 1979, Valaer *et al.* 1990). *N*-Hydroxylation is reported as being catalysed by both microsomal flavin-containing monooxygenases (FMO; Cashman 2000, Ziegler 2002) and P450 enzymes (Hlavica 2002). However, the fundamental oxidation mechanism of FMO using hydroperoxyflavoprotein as oxidant is quite distinct from that of the P450 enzymes using hypervalent iron-oxene species as the oxidant (Coon *et al.* 1998). Using adult human cDNA expressed FMO3 maltose binding fusion protein (FMO3-MBP), Cashman *et al.* (1999) reported the *in vitro* *N*-oxygenation of amphetamines to their corresponding hydroxylamine intermediates and retro reduction with both stereoselective oxime formation and oxidative *N*-deamination. The authors also suggested that the presence of the methyl group on the α -carbon adjacent to the nitrogen heteroatom causes a substantial change in the way human FMO3-MBP stereoselectively *N*-oxygenates amphetamines and other phenethylamines.

From the results of the present study and taking into account the previously identified hydroxylamine metabolites of amphetamines, ephedrine and selegiline (Dumasia 2001), it appears that in the horse, *N*-oxygenation of phenethylamines containing an alkyl substituent on the α -carbon is a major metabolic pathway.

Conclusion

A number of *in vivo* Phase I pathways were observed for the detoxification and elimination of ($\pm$)-MDMA in the thoroughbred horse. Only the isolation and GC-MS characterization of the basic metabolites containing the arylisopropylamine group is reported here. *N*-oxygenation was a major metabolic pathway. In addition to MDMA and the six previously reported metabolites, four other *N*-hydroxylated compounds are recorded for the first time in horse urine.

Acknowledgements

The author thanks Mr J. Fox for the synthesis of the hydroxylamines of amphetamine and methamphetamine and Mr B. Williams, MRCVS, and the stable staff for help with animal administrations.

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Metabolism and disposition of gemfibrozil in Wistar and multidrug resistance-associated protein 2-deficient TR⁻ rats

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Received 28 February 2003

1. The roles of multidrug resistance-associated protein (Mrp) 2 deficiency and Mrp3 up-regulation were evaluated on the metabolism and disposition of gemfibrozil.

2. Results from *in vitro* studies in microsomes showed that the hepatic intrinsic clearance (CL_{int}) for the oxidative metabolism of gemfibrozil was slightly higher (1.5-fold) in male TR⁻ rats, which are deficient in Mrp2, than in wild-type Wistar rats, whereas CL_{int} for glucuronidation was similar in both strains.

3. The biliary excretion of intravenously administered [¹⁴C]gemfibrozil was significantly impaired in TR⁻ rats compared with Wistar rats (22 versus 93% of the dose excreted as the acyl glucuronides over 72 h). Additionally, the extent of urinary excretion of radioactivity was much higher in TR⁻ than in Wistar rats (78 versus 2.6% of the dose).

4. There were complex time-dependent changes in the total radioactivity levels and metabolite profiles in plasma, liver and kidney, some of which appeared to be related to the up-regulation of Mrp3.

5. Overall, it was demonstrated that alterations in the expression of the transporters Mrp2 and Mrp3 significantly affected the excretion as well as the secondary metabolism and distribution of [¹⁴C]gemfibrozil.

Introduction

The transporter multidrug resistance-associated protein (Mrp) 2 was originally recognized in rat hepatocyte canalicular membranes and has been shown to mediate the ATP-dependent transport of glutathione, glucuronide and sulfate conjugates of lipophilic compounds, and several other endogenous and xenobiotic compounds (Jansen *et al.* 1985, Oude Elferink and Jansen 1994, Gerk and Vore 2002). The protein has been cloned from rat and human liver (Mayer *et al.* 1995) and also shown to be expressed in the renal proximal tubules, small intestinal villi and brain, as well as in the placenta (Schaub *et al.* 1997, St-Pierre *et al.* 2000, Mottino *et al.* 2001, Kusuhara and Sugiyama 2002).

Mrp2 deficiency has been implicated in the aetiology of Dubin–Johnson syndrome, a disease phenotype characterized by chronic conjugated hyperbilirubinemia and impaired hepatobiliary transport of many endogenous and

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xenobiotic compounds which, normally, are excreted into the bile via Mrp2 (Oude Elferink and Jansen 1994, Paulusma and Oude Elferink 1997). Several mutations in the Mrp2 gene have been characterized in patients with Dubin–Johnson syndrome (Paulusma *et al.* 1997, Wada *et al.* 1998, Mor-Cohen *et al.* 2001).

TR⁻ rats, a mutant strain that originated from albino Wistar rats, are characterized by a phenotype similar to the Dubin–Johnson syndrome. Hereditary chronic conjugated hyperbilirubinemia, caused by defective hepatic anion transport, has been reported in these rats (Jansen *et al.* 1985). Uptake of organic anions from plasma to liver is normal in the TR⁻ rats; however, transport of conjugated bilirubin, tetrabromosulphophthalein, glutathione conjugates and other organic anions from liver to bile is impaired severely (Jansen *et al.* 1985). A single-nucleotide deletion in the Mrp2 gene has been characterized in the TR⁻ rats (Paulusma *et al.* 1996).

Mrp3, another organic anion transporter, is expressed exclusively in the intestine but with much lower levels found in other tissues in Sprague–Dawley rats (Cherrington *et al.* 2002); however, the inducible nature of hepatic Mrp3 has been well characterized (Ogawa *et al.* 2000). In the normal rat, the expression of Mrp3 in the liver is induced by bile duct ligation (Hirohashi *et al.* 1998) as well as under cholestatic conditions in human (Konig *et al.* 1999). Furthermore, a large expression of Mrp3 was observed in Mrp2-deficient Dubin–Johnson patients (Konig *et al.* 1999) and a similar effect was observed in Eisai hyperbilirubinemic rats which also lack hepatic Mrp2 (Hirohashi *et al.* 1998). Mrp3 is localized to the basolateral membrane, where it is involved in the excretion of various organic anions from cells into the sinusoidal blood (Hirohashi *et al.* 1999, Renes *et al.* 2000). Therefore, it seems likely that Mrp3 is up-regulated to compensate for a deficiency of Mrp2.

Gemfibrozil (figure 1) is a fibric acid derivative widely used as a lipid-regulating agent. Even though its mechanism of action is not well established, gemfibrozil is believed to activate the peroxisome proliferator activated receptor- α resulting in an increase in lipoprotein lipase activity and a decrease in hepatic triglyceride production (Todd and Ward 1988).

Gemfibrozil undergoes extensive phase I and II metabolism in rats, hamsters, and humans (Okerholm and Keely 1976, Dix *et al.* 1999, Thomas *et al.* 1999). Phase I metabolism occurs primarily by hydroxylation of the phenyl ring (*para* to the side chain) and the meta-methyl group to give the *meta*-hydroxy methyl derivative, which is further oxidized to a benzoic acid derivative. Extensive glucuronidation reactions result in the formation of various ether and acyl glucuronide conjugates. The disposition of gemfibrozil is species and gender dependent. Biliary excretion as mainly acyl glucuronides is predominant in rats (50–90% of the dose) and dogs (62% of the dose), whereas in hamsters, monkeys and humans, most of the excretion occurs via the kidney (about 80, 62 and 66% of the dose, respectively). A significant gender difference has been reported in hamsters, where biliary excretion was about twofold higher in males than females (Okerholm *et al.* 1976, Dix *et al.* 1999). Enterohepatic circulation has been shown to play a significant role in the elimination of gemfibrozil in rats (Curtis *et al.* 1985).

The present investigation evaluated the role of the transporters Mrp2 and Mrp3 in the disposition of gemfibrozil in rats.

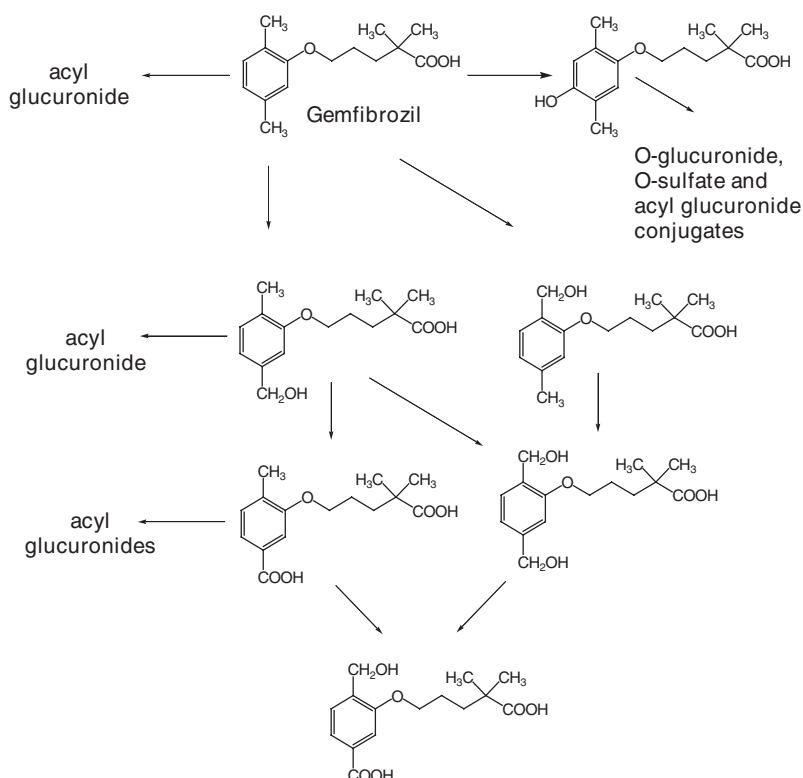


Figure 1. Structures of gemfibrozil and its metabolites (Dix *et al.* 1999, Thomas *et al.* 1999).

Materials and methods

Chemicals

[Carboxy- ^{14}C]gemfibrozil, with a radiochemical purity of 99.2% and specific activity of 28 mCi mmol^{-1} , was purchased from Moravak BioChemicals, Inc. (Mercury Lane, CA, USA). Gemfibrozil, alamethicin, saccharolactone, UDPGA, glucose 6-phosphate (G6P), G6P dehydrogenase and NADP were purchased from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA). All other reagents were of analytical or HPLC grade.

Animals

Male Wistar and TR^- rats, weighing between 300 and 400 g, were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were housed in cages and maintained on a 12-h light/dark cycle. They were allowed free access to food (Purina rodent chow 7012 meal, Purina, St Louis, MO, USA) and water for the duration of the experiment. All procedures for animal experiments were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

Preparation of microsomes

Livers and kidney tissues were collected from 10 Wistar and 10 TR^- rats after gaseous CO_2 euthanasia. Microsomes were prepared as described by Raucy and Lasker (1991) and protein concentrations were determined using the bicinchoninic acid method (Smith *et al.* 1985).

In vitro CYP-mediated metabolism

Liver microsomal samples (0.05 mg) were incubated with [^{14}C]gemfibrozil (1, 5, 10, 20, 50, 100, 200, and $400\text{ }\mu\text{M}$) in a total volume of 0.2 ml. The reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 6 mM MgCl_2 , and an NADPH-regenerating system consisting of G6P, G6P dehydrogenase, and NADP (10 mM glucose 6-phosphate, 1 mM NADP, and 0.7 units ml^{-1} G6P dehydrogenase). Incubations were carried out at 37°C for 30 min and reactions were quenched by adding 0.2 ml acetonitrile containing 2% formic acid. Samples were then spun in a centrifuge at 16 000 g

for 10 min, and the supernatants were analysed by HPLC (see below). Gemfibrozil was added in methanol (final volume 1%). The incubation conditions were linear with respect to microsomal protein concentration and the rate of metabolism was determined based on the formation of two hydroxylated metabolites. Kinetic parameters were calculated by fitting the experimental data from duplicate incubations to the Michaelis–Menten equations using SigmaPlot software (Enzyme Kinetics Module™ 1.1).

In vitro uridine diphosphate-glucuronosyltransferase (UGT)-mediated metabolism

For the incubation of [^{14}C]gemfibrozil with UDPGA-fortified Wistar and TR $^{-}$ rat liver microsomes, the final mixtures (200 μl) contained 100 mM Tris buffer, pH 7.5, 0.025 mg ml $^{-1}$ alamethicin, 5 mM MgCl $_2$, 5 mM saccharolactone, and 5 mM UDPGA. The incubations were carried out at protein concentrations of 0.05 ml $^{-1}$ for liver and kidney, respectively. The microsomal preparations were first incubated with alamethicin, saccharolactone, and MgCl $_2$ in Tris buffer (pH 7.5) at 4°C for 15 min followed by the addition of [^{14}C]gemfibrozil (1, 5, 10, 20, 50, and 100 μM). The reactions were started by the addition of UDPGA and allowed to proceed at 37°C for 10 min for liver and kidney microsomes, respectively. The reactions were terminated by adding 200 μl acetonitrile containing 2% formic acid. Samples were centrifuged at 16 000 g for 10 min. The supernatants were injected directly onto an HPLC column and analysed using a radiometric detector. The incubation conditions were linear with respect to microsomal protein concentration and the rate of metabolism was determined based on the formation of the acyl glucuronide of gemfibrozil. Kinetic parameters were calculated by fitting the experimental data as described previously (see above).

Studies in bile duct-cannulated rats

Rats were anaesthetized by intramuscular administration of a cocktail consisting of 4 ml ketamine (100 mg ml $^{-1}$), 2 ml xylazine (20 mg ml $^{-1}$) and 1 ml atropine (1 mg ml $^{-1}$), at 100 μl /100 g body weight, and bile ducts were cannulated surgically. After a full day of recovery from surgery, [^{14}C]gemfibrozil (specific activity 62.5 $\mu\text{Ci mg}^{-1}$) was dosed intravenously via the femoral vein, at 1 mg kg $^{-1}$ (2 ml kg $^{-1}$) to four male Wistar and TR $^{-}$ rats. The dosing vehicle consisted of ethanol: PEG 400: water, 10:40:50, by volume, and each rat was given between 20 and 25 μCi radioactivity. Bile samples were collected at predose, 0–4, 4–8, 8–24, 24–48 and 48–72 h post-dose and acidified with 5 ml 0.5 M formate buffer, pH 3.0, during sample collection to stabilize labile acyl glucuronide metabolites. Urine and faeces were collected at 24 h intervals up to 72 h post-dose. Urine samples also were acidified with 20 ml 0.5 M formate buffer, pH 3.0. The radioactive contents of bile and urine samples were determined by direct liquid scintillation counting. Twenty and 100 μl bile and urine samples, respectively, were mixed with 6 ml scintillant (ScintiSafe Gel™, Fisher Scientific) and counted using a liquid scintillation analyser (Tri-Carb, Packard, Downers Grove, IL, USA). An equal volume of acetonitrile, containing 2% formic acid, was added to bile and urine samples and spun in a centrifuge at 16 000 g for 10 min. The supernatants were drawn off and transferred into clean glass tubes and evaporated to dryness under a stream of N $_2$. Residues were reconstituted in the mobile phase and analysed using an HPLC system coupled with a radiometric detector (see below).

Metabolite profiles and radioactivity levels in plasma, liver and kidney

Three Wistar and TR $^{-}$ rats received an intravenous dose of [^{14}C]gemfibrozil (specific activity 55.6 $\mu\text{Ci mg}^{-1}$) at 1 mg kg $^{-1}$ via a lateral tail vein. The dosing vehicle used was the same as in the studies in bile duct-cannulated rats (see above). Blood samples were collected into heparinized containers by terminal cardiocentesis after gaseous CO $_2$ euthanasia at 1, 4, and 8 h post-dose ($n=1/\text{time point}$). Plasma samples were obtained from the blood by centrifugation at 1600 g and acidified with 0.5 M formate buffer (pH 3.0; 0.3 ml buffer/ml plasma). Livers and kidneys also were collected at the same time points. The radioactive content of the plasma (50 μl) was determined directly by liquid scintillation counting (see above). Livers and kidneys were blotted dry, weighed and homogenized in 4 vols 0.25 M formate buffer using a Polytron homogenizer (PT3100, Brinkmann, Westbury, NY, USA). Aliquots (about 300–400 mg) were dispensed into paper cones, air-dried overnight and combusted using an oxidizer (Oximate 80, Model 307, Packard), followed by liquid scintillation counting. Other portions of plasma, liver and kidney homogenates were extracted with 3 vols acetonitrile containing 2% formic acid and centrifuged at 1600 g for 30 min. The supernatants were transferred into clean glass tubes and evaporated to dryness under a stream of N $_2$. The residues were reconstituted in the mobile phase and analyzed by HPLC column (see below).

HPLC analysis

The HPLC system consisted of a Shimadzu LC-10ADVP a Shimadzu SIL-10ADVP (Columbia, MD, USA), and a β -Ram scintillation detector (IN/US Systems, Inc., Tampa, FL, USA). Samples were injected onto a Zorbax XDB-C8 column (250 $\times$ 4.6 mm; Agilent Technologies, Palo Alto, CA, USA), which was eluted with 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). Two linear gradients were used: gradient 1, which consisted of 30% B for 3 min,

increased to 100% B in 12 min, and then held for an additional 5 min, at a flow rate of 1.5 ml min^{-1} , was used for the samples from the microsomal incubation; gradient 2 was used for all the other samples. This latter gradient consisted of 15% B for 2 min, which was increased to 50% in 20 min, then to 95% over a further 10 min, followed by isocratic conditions for an additional 5 min, at a flow rate of 1.5 ml min^{-1} . In both cases, radiochromatographic peaks were monitored using a β -Ram scintillation detector.

Metabolite identification using LC-MS/MS

The LC-MS/MS system consisted of a Shimadzu SCL-10Avp system controller, Shimadzu LC-10ADvp pumps, a Perkin-Elmer series 200 autosampler (Norwalk, CT, USA), a Packard flow scintillation analyser, and a ThermoFinnigan Deca XP ion trap mass spectrometer (San Jose, CA, USA). A Zorbax XDB-C8 column ($250 \times 4.6 \text{ mm}$, $5.0 \mu\text{m}$) and gradient 2 (see above) were used for chromatographic separation. The eluent (1.0 ml min^{-1}) was split between the mass spectrometer (MS) and the flow scintillation analyser. One-quarter of the flow was directed into the MS and the remaining three-quarters were mixed with 3 vols Packard Ultima Flo-M scintillation fluid for the determination of radioactivity. The MS was operated in the electrospray negative-ion mode. The source voltage was maintained at 4 kV. The source temperature was set to 350°C and the sheath gas was set to 40. The instrument was set to acquire three microscans with a maximum injection time of 300 ms/microscan. Ultrahigh-purity helium was used as the buffer gas. For data-dependent MS^n analysis, the most abundant ions exceeding the threshold of 5×10^4 were isolated using an isolation width of 3 amu and the predominant monoisotopic peak was selected for collision-induced dissociation experiments. The MS^2 and MS^3 data were obtained using a relative collision energy of 25%. Data acquisition and reduction were carried out using Xcalibur software (v.1.2). The structures of the metabolites were based on the MS^2 and MS^3 data.

Results

In vitro CYP- and UGT-mediated metabolism of gemfibrozil

The CYP- and UGT-mediated metabolism of gemfibrozil was evaluated in liver microsomes prepared from Wistar and TR^- rats. The kinetic parameters, V_{max} and K_{m} of CYP- and UGT-mediated metabolism of gemfibrozil are summarized in table 1 and figure 2. CL_{int} was calculated from $V_{\text{max}}/K_{\text{m}}$. The V_{max} of phase I metabolism of [^{14}C]gemfibrozil in TR^- liver microsomes was less than twofold higher than that in Wistar liver microsomes, whereas the K_{m} values were comparable. The resulting CL_{int} for oxidative metabolism was about 50% higher in liver microsomes from TR^- rats when compared with that obtained from Wistar rat liver microsomes. However, the V_{max} and K_{m} values of glucuronidation

Table 1. Kinetic parameters for CYP- and UGT-mediated metabolism of gemfibrozil in Wistar and TR^- rat liver microsomes.

Reactions	Wistar			TR^-		
	K_{m} (μM)	V_{max} (nmol min^{-1} mg^{-1})	CL_{int} ($\mu\text{l min}^{-1}$ mg^{-1})	K_{m} (μM)	V_{max} (nmol min^{-1} mg^{-1})	CL_{int} ($\mu\text{l min}^{-1}$ mg^{-1})
CYP-mediated ^a	41, 77	1.5, 2.2	37, 29	45, 65	2.9, 3.3	64, 46
UGT-mediated ^b	7.2, 10	9.2, 11.2	1278, 1120	7.8, 8.4	10.3, 10.9	1397, 1298

Values from two separate experiments using pooled microsomes ($n=10$) are listed.

^a Rate of metabolism was determined based on the formation of two hydroxylated metabolites. Kinetic parameters were calculated by fitting the experimental data from duplicate incubations to the Michaelis–Menten equations using SigmaPlot software (Enzyme Kinetics ModuleTM 1.1).

^b Rate of metabolism was determined based on the formation of an acyl glucuronide of gemfibrozil. Kinetic parameters were calculated by fitting the experimental data from duplicate incubations to the Michaelis–Menten equations using SigmaPlot software (Enzyme Kinetics ModuleTM 1.1).

of gemfibrozil were similar in both rat strains. Overall, the hepatic CL_{int} for glucuronidation was about 30-fold higher than that for Phase I oxidation (table 1).

Studies in bile duct-cannulated rats

The *in vivo* disposition of [^{14}C]gemfibrozil was evaluated in Wistar and TR⁻ rats after a bolus IV administration of 1 mg kg⁻¹. The extent of biliary and urinary excretion of radioactivity is summarized in table 2 and figure 3. The mean biliary excretion of radioactivity was considerably lower in TR⁻ rats compared with Wistar rats (22 versus 93% of the dose in 72 h). In both strains, the majority of the excretion into bile occurred within 4 h: 17 and 91% in TR⁻ and Wistar rats, respectively. Despite the notable decrease in the biliary excretion of radioactivity, the acyl glucuronide of gemfibrozil remained the major component observed in the bile samples from both TR⁻ rats and Wistar rats (figure 4). The urinary excretion of radioactivity was strikingly higher in TR⁻ rats than in Wistar rats (78 versus 2.6%) (table 2). The acyl glucuronides of gemfibrozil and its benzoic acid derivative were identified as major metabolites in the urine samples from TR⁻ rats, whereas no metabolites could be identified in the urine samples from Wistar rats because of the low levels of radioactivity (figure 4).

Metabolite profiles and radioactivity levels in plasma, liver and kidney

The metabolite profiles of [^{14}C]gemfibrozil in the plasma, liver, and kidney are shown in figures 5–7. In plasma, the acyl glucuronide of gemfibrozil and its benzoic acid derivative, a glucuronide of hydroxylated gemfibrozil, along with gemfibrozil itself, were identified as major entities in both rat strains. However, in the plasma from TR⁻ rats, a major radioactive metabolite was observed which was particularly evident at 4 and 8 h post-dose. Based on LC-MS/MS analysis, this putative metabolite was identified as an acyl glucuronide of a sulfate conjugate of hydroxylated gemfibrozil. This metabolite produced a molecular ion at m/z 521 $[M - H]^-$ and the major MS/MS fragment was observed at m/z 345, formed by a neutral loss of 176 Da, which is characteristic of a glucuronide conjugate. Further fragmentation of m/z 345 (MS³ data) gave rise to m/z 265, 216 and 137, respectively. The loss of 80 Da (SO₃) from m/z 345 to 265 was suggestive of a sulfate conjugate. The fragment ion at m/z 265 was 16 amu higher than that of gemfibrozil itself, suggesting a hydroxylated gemfibrozil structure. Further fragmentation of m/z 265 gave rise to m/z 137, which confirmed that the hydroxylation occurred on the dimethylphenyl moiety. Therefore, based on the mass spectrometry data, it was speculated that this metabolite was an acyl glucuronide of a hydroxylated gemfibrozil sulfate. In a previous study, 4'-hydroxygemfibrozil

Table 2. Recoveries of radioactivity in bile and urine over 72 h following intravenous administration of [^{14}C]gemfibrozil to male Wistar and TR⁻ rats at 1 mg kg⁻¹.

Species	Per cent radioactive dose recovered ^a		
	Bile ^b (mean ± SD)	Urine ^b (mean ± SD)	Total (mean ± SD)
Wistar	93 ± 3.2	2.6 ± 1.2	96 ± 2.5
TR ⁻	22 ± 5.5	78 ± 28	99 ± 32

^a $n = 4$ male rats.

^bBile and urine were collected between 0 and 72 h.

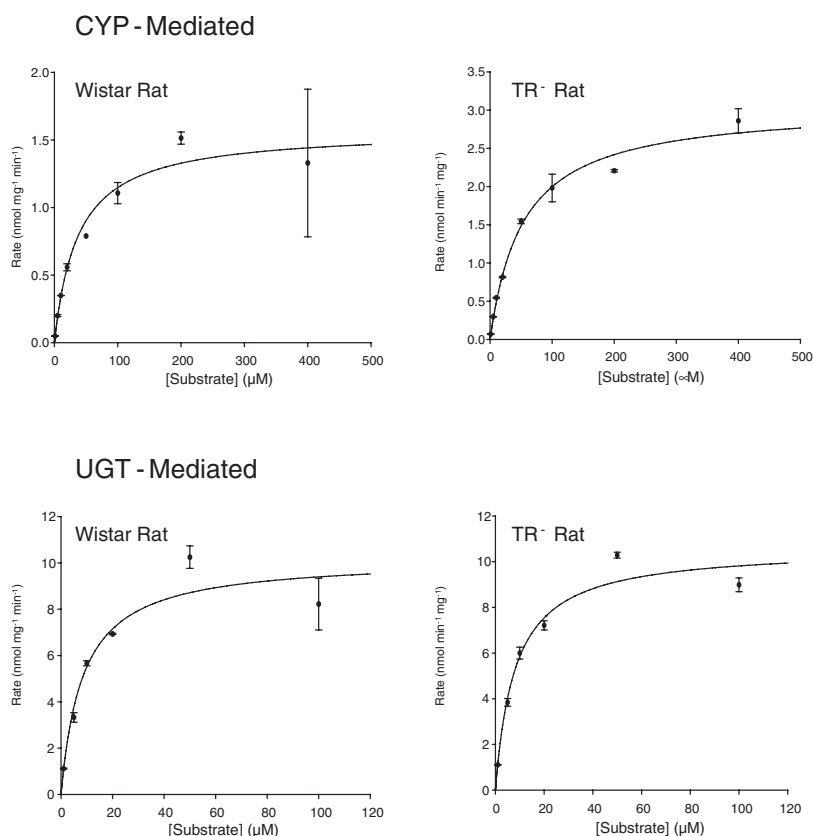


Figure 2. Michaelis–Menten plots of the CYP- and UGT-mediated metabolism of gemfibrozil in Wistar and TR⁻ rat liver microsomes.

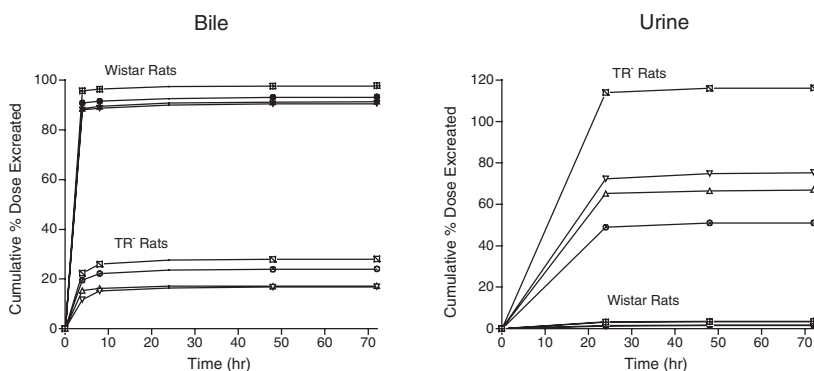


Figure 3. Cumulative recoveries of radioactivity in bile and urine over 72h following intravenous administration of [¹⁴C]gemfibrozil to male Wistar and TR⁻ rats at 1 mg kg⁻¹ from individual animals ($n = 4$).

sulfate was identified as one of the metabolites in rats and its MS³ data are identical to the metabolite identified herein (Thomas *et al.* 1999). Thomas *et al.* also observed the fragment ion at m/z 216, which could be assigned as a radical anion. Further evaluation by NMR and by synthesis is necessary to confirm

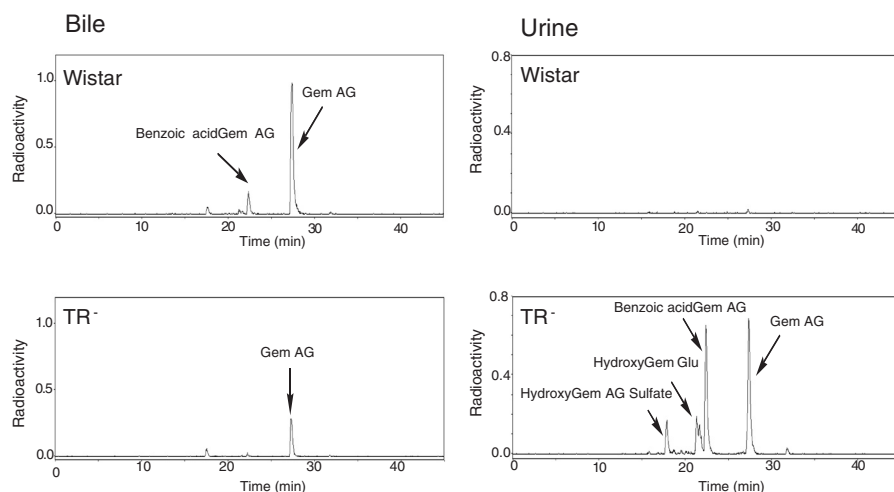


Figure 4. Metabolite profiles of bile and urine following intravenous administration of [^{14}C]gemfibrozil to male Wistar and TR^- rats at 1 mg kg^{-1} . Samples from individual animals ($n=4/\text{time point}$) were pooled based on the volume collected at different time points (0–72 h) and pooled again by the same volume across animals. AG, acyl glucuronide.

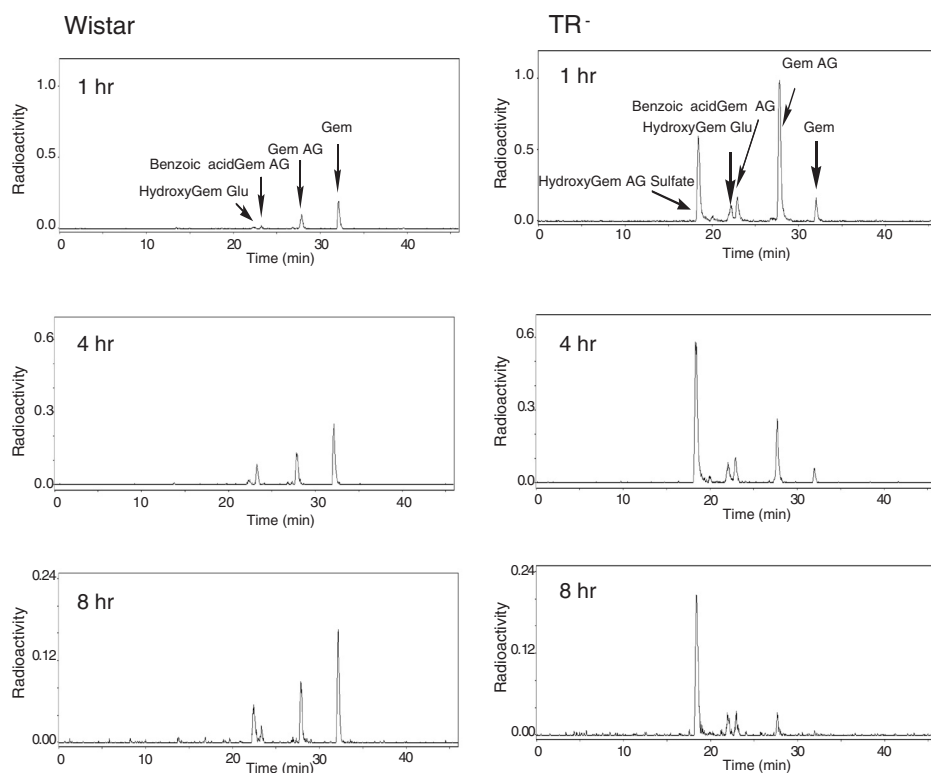


Figure 5. Metabolite profiles of plasma following intravenous administration of [^{14}C]gemfibrozil to male Wistar and TR^- rats at 1 mg kg^{-1} . Samples from individual animals ($n=1/\text{time point}$). AG, acyl glucuronide.

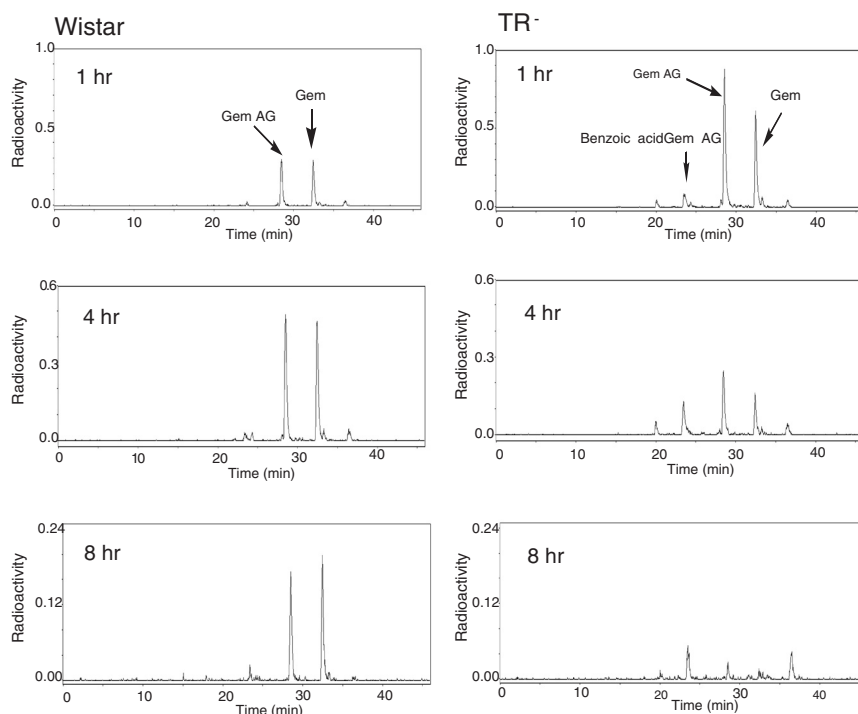


Figure 6. Metabolite profiles of liver following intravenous administration of [^{14}C]gemfibrozil to male Wistar and TR^- rats at 1 mg kg^{-1} . Samples from individual animals ($n = 1/\text{time point}$). AG, acyl glucuronide.

Table 3. Radioactivity of [^{14}C]gemfibrozil and [^{14}C]gemfibrozil acyl glucuronide in plasma, liver and kidney following intravenous administration to male Wistar and TR^- rats at 1 mg kg^{-1} .^a

Time point (h):		Wistar			TR^-		
		1	4	8	1	4	8
[^{14}C]Gemfibrozil	plasma	0.22	0.28	0.17	0.13	0.05	<0.01
	liver/plasma	3.2	3.0	3.5	7.9	5.6	<3
	kidney/plasma	0.9	0.5	0.6	12.4	7.6	<7
[^{14}C]gemfibrozil acyl glucuronide	plasma	0.08	0.11	0.06	0.65	0.15	0.02
	liver/plasma	5.0	4.7	5.5	1.4	2.0	2.0
	kidney/plasma	1.0	1.18	1.3	0.5	0.5	1.0

^a Expressed as $\mu\text{g eq. ml}^{-1}$ and estimated based on the specific activity of [^{14}C]gemfibrozil and the percent of radioactivity associated with gemfibrozil and its acyl glucuronide in the HPLC radiochromatogram. Data are derived from individual animals ($n = 1/\text{time point}$).

fully the structure of this metabolite. Gemfibrozil and its acyl glucuronide were identified in the liver and kidney from both strains as the major components. However, the acyl glucuronide of gemfibrozil benzoic acid derivative and a glucuronide of hydroxylated gemfibrozil were also observed at notable levels in TR^- rats.

The ratios of [^{14}C]gemfibrozil and [^{14}C]gemfibrozil acyl glucuronide in liver or kidney to those in plasma from Wistar and TR^- rats are summarized in table 3.

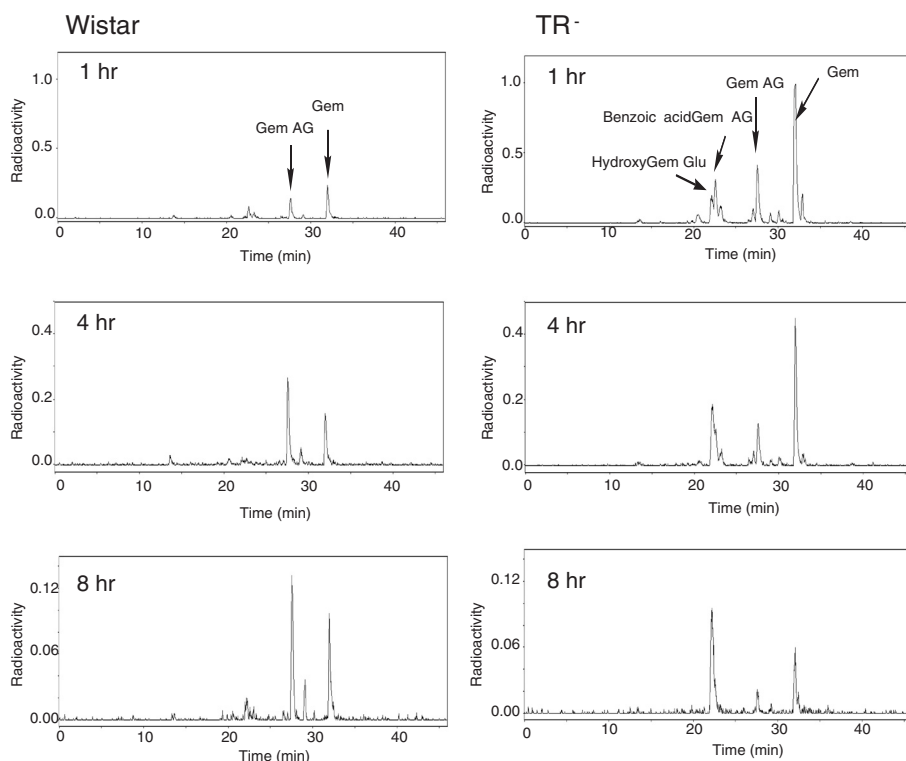


Figure 7. Metabolite profiles of kidney following intravenous administration of [^{14}C]gemfibrozil to male Wistar and TR $^{-}$ rats at 1 mg kg^{-1} . Samples from individual animals ($n = 1/\text{time point}$). AG, acyl glucuronide.

In the case of [^{14}C]gemfibrozil, the ratios were higher in TR $^{-}$ rats, particularly at 1 and 4 h post-dose. However, the ratios for [^{14}C]gemfibrozil acyl glucuronide were significantly lower in TR $^{-}$ rats than in Wistar rats at all time points, which is most likely attributable to the upregulation of Mrp3 in TR $^{-}$ rats. Total amounts of radioactivity levels in the plasma at 1 and 4 h post-dose were higher in TR $^{-}$ than Wistar rats; however, amounts at 8 h were the same in the two strains. Analysis by LC-MS/MS of plasma extracts, accompanied by radiometric detection, revealed that parent gemfibrozil accounted for a lower percentage of circulating radioactivity in TR $^{-}$ than Wistar rats, particularly at the 4 and 8 h time points (figure 5). Similarly, the percentage of plasma radioactivity accounted for by gemfibrozil acyl glucuronide was lower in TR $^{-}$ than in Wistar rats at 4 and 8 h. By way of contrast, the percentage of radioactivity due to conjugates of oxidative metabolites was two- to ninefold higher in TR $^{-}$ than that in Wistar rats (figure 8). Similar results were obtained for liver (figures 6 and 8) and kidney (figures 7 and 8), except that the liver levels of acyl glucuronide in TR $^{-}$ rats were higher at 1 and 4 h, but lower at 8 h than in Wistar rats.

Discussion

In studies with several carboxylic acid-containing drug candidates that exclusively undergo acyl glucuronidation, significant species differences in circulating

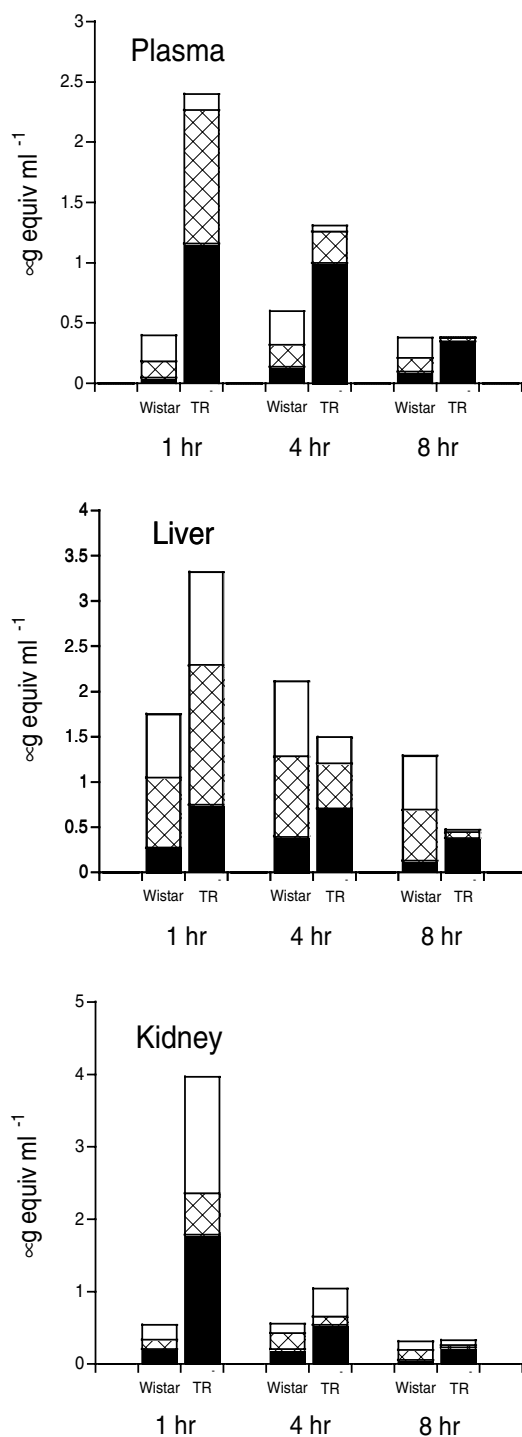


Figure 8. Concentrations of radioactivity ($\mu\text{g eq. ml}^{-1}$) associated with gemfibrozil (open bars), its acyl glucuronide (hatched bars) and other metabolites (closed bars) in plasma, liver and kidney following intravenous administration of [^{14}C]gemfibrozil to male Wistar and TR⁻ rats at 1 mg kg^{-1} . Samples from individual animals ($n=1/\text{time point}$).

levels of their acyl glucuronides were encountered. In most cases, plasma levels of acyl glucuronide were lower in rats than in monkeys and humans. Since some acyl glucuronides have been shown to bind covalently to proteins, causing altered activities and possibly toxicities (Spahn-Langguth and Benet 1992, Sallustio *et al.* 2000), it is important to examine possible factors that could contribute to these species differences. The species differences in plasma levels of acyl glucuronides may be indicative of different efficiencies of glucuronidation and/or hepatic transport mechanisms, such as biliary excretion by Mrp2 and sinusoidal efflux by Mrp3. The expression level of Mrp3 is very low in normal rat liver (Cherrington *et al.* 2002), but relatively higher MRP3 levels in human liver have been reported (Konig *et al.* 1999). To evaluate these factors, metabolism and excretion studies were conducted with carboxylic acid-containing drugs under conditions of induced metabolism by 1,7-phenanthroline in Sprague–Dawley rats (Kim *et al.* in preparation) and impaired excretion in TR⁻ rats. Results from studies in TR⁻ rats are reported herein.

The Mrp2-deficient TR⁻ rat has been used as an animal model for Dubin–Johnson syndrome and has greatly contributed to the characterization of the function of Mrp2. Substrate specificity and transport mechanisms of Mrp2 have been characterized in several different experimental systems including isolated perfused livers and canalicular membrane vesicles from TR⁻ rats (de Vries *et al.* 1989, Xiong *et al.* 2000). Few studies, however, have been conducted *in vivo* in TR⁻ rats. Dietrich *et al.* (2001a, b) extensively characterized the metabolism and disposition of the food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). After oral administration, the bioavailability of PhIP increased (about two- to threefold) in TR⁻ rats, indicating that Mrp2 may have played a role in the defence mechanism against PhIP by direct extrusion of the carcinogen from the gut mucosa. Biliary excretion of PhIP was severely impaired in TR⁻ rats. In addition, the levels of glucuronides and 4'-OH-PhIP at 2 h after intravenous administration were lower in TR⁻ than in Wistar rats.

The present study evaluated the role of Mrp2 deficiency and Mrp3 up-regulation on the metabolism and disposition of gemfibrozil, which was chosen as a model substrate since it is known to undergo both phase I and II metabolism to hydroxylated metabolites and acyl glucuronides. *In vitro* phase I oxidation and glucuronidation reactions were conducted in liver microsomes and kinetic parameters $V_{\max}$ and K_m were determined. The K_m values for phase I oxidation were comparable, whereas the $V_{\max}$ in TR⁻ rat liver microsomes was greater (about twofold) than in Wistar liver microsomes (table 1). These results suggested higher activities of the cytochrome P450 enzymes catalysing gemfibrozil metabolism in TR⁻ liver microsomes. Different expressions of cytochrome P450 enzymes and their activities in TR⁻ compared with Wistar rats have been reported (Jäger *et al.* 1998). The cytochrome P450 content and expression of CYP2B1/2 and CYP3A1/2 were higher in Wistar rat liver microsomes, whereas the expression levels of CYPs1A2, 2D and 2E1 were comparable in both rat strains. Newton and colleagues (personal communication) have demonstrated significantly higher formation of 7 α -hydroxylated testosterone (catalysed by CYP2A1) and 2 α - and 16 α -hydroxylated testosterone (catalysed by CYP2C11) in TR⁻ liver microsomes than in Wistar rat liver microsomes. Acyl glucuronidation is probably the major elimination pathway for gemfibrozil in rats, as indicated by the higher intrinsic clearance in liver microsomes compared with that of oxidative metabolism (table 1).

Relatively comparable $V_{\max}$ and K_m values of glucuronidation of gemfibrozil were observed in liver microsomes prepared from both strains. However, note that the results from these kinetic studies might have little statistical significance and can only be qualitative. Even though the rat uridine diphosphate-glucuronosyltransferase (UGT) enzymes catalysing the phase 2 metabolism of gemfibrozil are not known, human recombinant UGT1A1 and UGT1A3 were identified as major enzymes capable of catalysing the glucuronidation of gemfibrozil (Prueksaritanont *et al.* 2002). Further studies are needed to characterize the activities of UGT enzymes in Wistar and TR⁻ rats.

The effect of Mrp2 deficiency on the disposition of [¹⁴C]gemfibrozil was examined *in vivo* using bile duct-cannulated Wistar and Mrp2-deficient TR⁻ rats. Biliary excretion was extensively impaired in TR⁻ rats, although about 20% of the radioactive dose was still excreted into the bile. The acyl glucuronide of gemfibrozil was the major component detected in the bile samples from both strains, suggesting that multiple mechanisms, including Mrp2-mediated active transport, may be involved in the biliary excretion of gemfibrozil acyl glucuronide. Two distinct canalicular transport mechanisms, ATP-dependent and membrane potential-dependent transport of non-bile acid organic anions and glutathione *S*-conjugates, have been reported (Nishida *et al.* 1992, Ballatori and Truong 1995). Biliary excretion of bilirubin diglucuronide has also been shown to be mediated by these two mechanisms in normal rats. However, in TR⁻ rats, ATP-dependent transport of bilirubin diglucuronide was absent, whereas the membrane potential-dependent transport was intact (Nishida *et al.* 1992). Furthermore, the presence of multiple ATP-dependent biliary excretion mechanisms for conjugates has also been proposed (Niinuma *et al.* 1997). Additional studies are needed to clarify which transport mechanisms other than Mrp2 may be operative in the biliary excretion of gemfibrozil glucuronide. The defect in biliary excretion seemed to be compensated by a substantially enhanced urinary excretion in TR⁻ rats. The glucuronides of gemfibrozil, and its hydroxy and benzoic acid derivatives were identified as major metabolites in the TR⁻ rat urine samples. Obviously, the deficiency of Mrp2 did not seem to impair the urinary excretion of gemfibrozil derived glucuronides and this result is consistent with previous findings (de Vries *et al.* 1989, Dietrich *et al.* 2001a). The renal proximal tubule is the major site of carrier-mediated transport of various organic anions from blood to urine (Van Aubel *et al.* 2000). Several uptake transporters for organic anions were identified in basolateral membrane including organic anion transporter (OAT1). Various transport systems for the efflux of organic anions into the lumen or reabsorption from lumen into the cell are also present in the apical membrane. Mrp2, the primary organic anion efflux transporter and the kidney specific OAT-K1 and OAT-K2, as well as male rat-specific oatp1, which play a role in efflux and reabsorption have been identified in the apical membrane of renal proximal tubules (Van Aubel *et al.* 2000, Kudo *et al.* 2002). Whether OAT-K1 or OAT-K2, along with glomerular filtration, play a role in urinary excretion of gemfibrozil and its metabolites remains to be determined.

To understand better the impact of Mrp2 deficiency, and possibly Mrp3 up-regulation, in the metabolism and overall disposition of gemfibrozil, the total radioactivity levels and metabolite profiles in the plasma, liver and kidney obtained from Wistar and TR⁻ rats were determined. The notably higher levels of radioactivity in the liver and kidney from TR⁻ rats compared with Wistar rats

at 1 h post-dose reflected the extensive impairment of biliary excretion and compensatory urinary excretion of gemfibrozil glucuronides. The different metabolite profiles shown in the liver indicate that not only the disposition, but also the secondary metabolism of gemfibrozil in these two strains are markedly different. The formation of an acyl glucuronide of a hydroxylated gemfibrozil sulfate in TR⁻ rats indicates possible induction of sulfate conjugation. Further studies are needed to investigate whether this is the case. The significantly higher radioactivity and different metabolite profiles in the plasma from TR⁻ than Wistar rats can, most likely, be attributed to basolateral secretion of metabolites of gemfibrozil from the liver into the plasma via Mrp3. The lower contribution of unchanged gemfibrozil at the later time points, in spite of higher total radioactivity in the plasma in TR⁻ compared with Wistar rats may be due to diminished enterohepatic recirculation as a result of the impaired biliary excretion of acyl glucuronide, as well as increased formation and efflux into plasma of phase I metabolites and their conjugates. Not surprisingly, metabolite profiles in plasma samples from TR⁻ rats were reasonably representative of the urine profiles.

In conclusion, it has been demonstrated that alterations in Mrp2 and Mrp3 expression substantially affected the disposition and tissue distribution of gemfibrozil, using the TR⁻ rat as an animal model. Also observed were additional changes in the secondary metabolism of gemfibrozil in the TR⁻ rat compared with in the Wistar rat. The expression levels and function of Mrp2 and Mrp3 can be altered by a number of factors (Cherrington *et al.* 2002, Gerk and Vore 2002) and polymorphism of MRP2/cMOAT in healthy Japanese subjects has been reported (Ito *et al.* 2001). Given the role of Mrp2 and Mrp3 as transporters of many conjugates, as well as cancer chemotherapeutics and antibiotics, alterations in expression and/or activity of these transporters will affect the pharmacokinetic, pharmacodynamic and toxicologic aspects of drugs, and thus have significant clinical impact. Further studies are needed to evaluate whether altered expression and/or activity of MRP2 and MRP3 will affect the excretion and distribution as well as the metabolism of drugs in humans.

Acknowledgements

The authors thank Mr Chris Kochansky for expert technical assistance in the determination of radioactivity in liver and kidney tissues.

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Disposition of flurbiprofen in man: influence of stereochemistry and age

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Received 20 February 2003

1. The stereoselective metabolism and pharmacokinetics of the enantiomers of flurbiprofen were investigated following the oral administration of the racemic drug (100 mg) to four young and four elderly healthy volunteers (two males and two females per group).

2. The stereochemical composition of the drug and the 4'-hydroxy- metabolite in serum and the drug, 4'-hydroxy- and 3'-hydroxy-4'-methoxy- metabolites, both free and conjugated, in urine were determined by a direct chromatographic method of enantiomeric analysis.

3. Modest enantioselectivity in clearance (CL *S/R*: young, 0.86; elderly, 0.88) was largely responsible for the apparent elimination half-life of (*S*)-flurbiprofen being significantly greater ($p < 0.01$) than that of the *R*-enantiomer in both age groups (young, *S*: 5.2 ± 0.7 versus *R*: 4.5 ± 0.6 h; elderly, *S*: 9.6 ± 1.2 versus *R*: 7.1 ± 1.0 h). The serum concentrations of 4'-hydroxyflurbiprofen were five- to 20-fold lower than those of the corresponding drug enantiomers, stereoselective disposition being evident in the significantly greater ($p < 0.05$) apparent half-lives of the *S*- compared with the *R*-enantiomer in both groups (young, *S*: 10.6 ± 2.4 versus *R*: 6.7 ± 1.1 h; elderly, *S*: 13.7 ± 1.7 versus *R*: 10.2 ± 1.2 h).

4. Some 60 and 72% of the dose was excreted in 24-h urine in elderly and young volunteers, respectively, a significantly greater ($p < 0.05$) proportion of which was of the *R*-configuration in both age groups (*S/R*: young, 0.87; elderly, 0.81). The major urinary excretion products were flurbiprofen and 4'-hydroxyflurbiprofen, and their acyl-conjugates in both groups.

5. Age-associated differences in the pharmacokinetics of flurbiprofen occurred in a non-stereoselective manner and were primarily as a consequence of a significant ~40% decrease ($p < 0.01$) in clearance of both enantiomers in the elderly due to reduced metabolic activity. Consequently, the elderly had greater exposure to both enantiomers, as reflected by the AUC_{0-inf} being significantly higher ($p < 0.05$), by 60%, in this age group compared with the young.

6. The findings suggest that age-related alterations in the disposition of flurbiprofen could have significant implications for the use of the drug in the elderly.

Introduction

Racemic flurbiprofen [(±)-(*R,S*)-2-(2-fluoro-4-biphenyl)propionic acid] (figure 1), a chiral 2-arylpropionic acid (2-APA) derivative, is commonly used as an analgesic non-steroidal anti-inflammatory drug (NSAID). The anti-inflammatory activity, mediated via inhibition of the cyclooxygenases, is predominantly associated with the *S*-enantiomer (Carabaza *et al.* 1996, Geisslinger *et al.*

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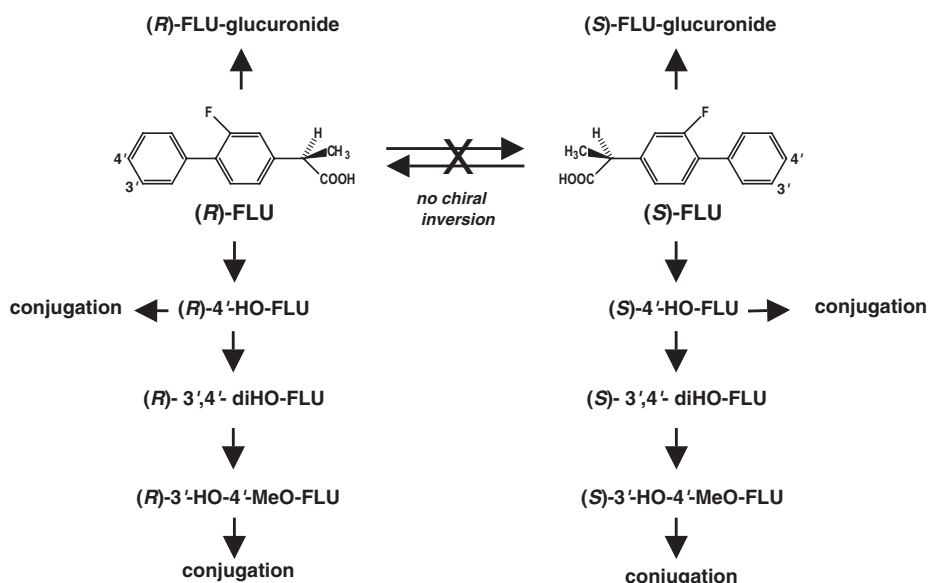


Figure 1. Metabolism of the enantiomers of flurbiprofen (FLU) in humans via glucuronidation and oxidation to 4'-hydroxyflurbiprofen (4'-HO-FLU), 3',4'-dihydroxyflurbiprofen (3',4'-diHO-FLU) and methylation to 3'-hydroxy-4'-methoxyflurbiprofen (3'-HO-4'-MeO-FLU).

2000); but both enantiomers reduce zymosan-induced rat paw inflammation (Tegeder *et al.* 2001a) and *(R)*-flurbiprofen is slightly more potent than the *S*-enantiomer in the inhibition of nuclear factor kappa B (Tegeder *et al.* 2001a, b). The antinociceptive activity is associated with both enantiomers of flurbiprofen (Brune *et al.* 1991, Geisslinger and Schaible 1996). The activity of the *R*-enantiomer being within the central nervous system (Geisslinger and Schaible 1996, Buritova and Besson 1998) and recent animal studies have indicated that the antinociceptive activity of both enantiomers is in part mediated by inhibition of spinal prostaglandin E_2 release (Geisslinger *et al.* 2000).

Flurbiprofen has also been shown to exhibit antiproliferative effects both *in vivo* (McCracken *et al.* 1996) and *in vitro* (Dodhia *et al.* 2002), in which the enantiomers show similar activity. The activity of the *R*-enantiomer has also been examined in APC^{Min/+} and TRAMP mice, animal models of colon and prostate cancer, respectively, and reported to reduce both tumour formation and progression (Wechter *et al.* 2000a, b). The above data, together with the reduced gastrointestinal effects of *(R)*-flurbiprofen, in comparison with either the racemate or the *S*-enantiomer (Wechter *et al.* 1993, Jerussi *et al.* 1998, Mahmud *et al.* 1998), have resulted in its clinical evaluation in patients suffering from prostate cancer (Keegan and Loughman 2001).

NSAID use has recently been reported to be associated with a reduced risk of Alzheimer's disease (in't Veld *et al.* 2001), which is thought to be via the suppression of β -amyloid ($A\beta$) peptide accumulation (Weggen *et al.* 2001). Some of the compounds initially examined for their effects on $A\beta$ peptide accumulation were racemates of the 2-APA group and recent *in vitro* investigations, using human embryonic kidney cells, have indicated that the *R*-enantiomers of both ibuprofen and flurbiprofen inhibit the production of $A\beta_{42}$, with *(R)*-flurbiprofen showing greater activity (Moriyama *et al.* 2002). This latter observation, together with the

potential for reduced cyclooxygenase associated adverse effects, resulted in the suggestion that (*R*)-flurbiprofen may be a useful candidate for clinical development (Moriyama *et al.* 2002).

The use of NSAIDs for the prevention of such age-associated diseases is an area of potential concern as the adverse reactions and toxicity of these agents, particularly gastrointestinal, but also hepatic and renal damage, together with the development of congestive heart failure (Wolfe *et al.* 1999, Page and Henry 2000, Tramèr *et al.* 2000), are a major cause of drug related hospital admissions in the elderly (Cunningham *et al.* 1997).

Studies on the influence of age on the disposition and pharmacokinetics of racemic flurbiprofen in man are scarce and suggest that age has a minimal influence on drug handling (Hamdy *et al.* 1980, 1990, Kean *et al.* 1992). However, as these latter investigations were based on non-stereospecific analytical methodologies such conclusions may be misleading and may mask age-associated differences in enantiomeric disposition. Tan *et al.* (2003) reported on the influence of age on the stereoselectivity of ibuprofen disposition following administration of the racemic drug. The elderly (age range 66–84 years) exhibited a significant stereoselective increase in unbound fraction and reduction in unbound clearance of (*S*)-ibuprofen, resulting from reduced clearance via both glucuronidation and oxidation, in comparison with young (age range 20–36 years) healthy volunteers. In contrast, no significant age-associated differences were observed in the disposition of the *R*-enantiomer (Tan *et al.* 2003). It was therefore of interest to examine the disposition of flurbiprofen, following administration of the racemate to young and elderly volunteers in order to determine the influence of ageing on the stereoselectivity of drug handling.

Materials and methods

Chemicals

The individual flurbiprofen enantiomers (enantiomeric purity of each $\geq 99.6\%$), (*R,S*)-flurbiprofen, (*R,S*)-4'-hydroxyflurbiprofen and (*R,S*)-3'-hydroxy-4'-methoxyflurbiprofen were the generous gifts of Boots Co. Ltd (Nottingham, UK). (*R,S*)-Benoxaprofen was kindly supplied by Lilly Research Centre Ltd (Windlesham, UK), (*S*)-naproxen was obtained from Aldrich (Gillingham, UK) and β -glucuronidase (EC 3.2.1.31) type H-5, with 530 units β -glucuronidase and 30 units sulphatase activity/mg solid, was purchased from Sigma Chemicals (Poole, UK). HPLC solvents were purchased from Rathburn (Walkerburn, UK); and all other reagents and solvents were purchased from BDH (Poole, UK).

Chromatographic column

The chiral stationary phase (CSP) was a Chiralpak AD (amylose tris(3,5-dimethylphenyl-carbamate)) column (250 $\times$ 4.6 mm, 10 μ m) used with a matching guard column (50 $\times$ 4.6 mm, 10 μ m) and was supplied by HPLC Technology Ltd (Macclesfield, UK).

Instrumentation

High-performance liquid chromatography (HPLC) was performed using an LDC Constametric 3000 pump linked to a Kontron SFM-25 fluorescence detector (Watford, UK) and a LDC Spectromonitor 3100 UV detector (Stone, UK). Data from both detectors was acquired using LDC CI-4000 and LDC CI-4100 computing integrators. Samples were injected on column using a Perkin Elmer ISS-100 autosampler fitted with a 100- μ l injection loop (Beaconsfield, UK).

Analytical methods

Full details of the analytical methodologies employed, including their development, validation and preliminary application to samples of biological origin, have been reported by Patel *et al.* (2002, 2003). Brief details of each method are presented below.

Serum assay. The enantiomeric composition of flurbiprofen and 4'-hydroxyflurbiprofen in serum was determined by HPLC using a direct approach to enantiomeric analysis (Patel *et al.* 2003). Following the addition of the internal standard, (*R,S*)-benoxaprofen (0.25 µg; 50 µl of a 5.0 µg ml⁻¹ solution in acetonitrile) to 1 ml serum samples, the analytes were isolated by extraction using hexane-ethyl acetate (90:10 v/v; 6 ml) at pH 3.8. Following phase separation, the organic layer was removed, evaporated to dryness and the residue reconstituted in 150 µl mobile phase and 100 µl injected on column. Chromatographic analysis was performed using the Chiralpak AD CSP with a mobile phase of hexane-ethanol (87:13 v/v) containing trifluoroacetic acid (TFA, 0.05% v/v) at a flow rate of 1.0 ml min⁻¹. Quantification was carried out using the fluorescence detector with excitation and emission wavelengths of 288 and 340 nm, respectively. Under these conditions, recoveries were typically greater than 85% and (*R*)- and (*S*)-flurbiprofen and (*R*)- and (*S*)-4'-hydroxyflurbiprofen eluted at 5.2, 6.4, 8.6 and 11.8 min, respectively, the enantiomers of the internal standard eluting at 14.8 and 16.7 min. The enantiomers of both analytes were quantified by reference to calibration curves constructed over the range 0.1–6.0 and 0.01–0.6 µg ml⁻¹ for flurbiprofen and the 4'-hydroxy-metabolite respectively; the within- and between-day coefficient of variation over the respective calibration ranges were less than 5%.

Urine assay. Urinary analysis was performed to determine total (post-β-glucuronidase and alkaline hydrolytic treatments), free plus acyl-glucuronide (post-alkaline hydrolytic treatment) and free (non-treated) levels of the enantiomers of flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen (Patel *et al.* 2003). Following the addition of the internal standard, (*S*)-naproxen (2.5 µg; 50 µl of a 50 µg ml⁻¹ solution in acetonitrile), the non-treated or treated urine samples were extracted under acidic conditions (pH 3.8) with hexane-isopropanol (95:5 v/v; 6 ml). Following phase separation and evaporation of the organic layer, the residue was reconstituted in 150 µl mobile phase and 100 µl injected on column. Chromatographic analysis was performed using the Chiralpak AD CSP with a mobile phase consisting of hexane-ethanol (90:10 v/v) containing TFA (0.05% v/v) at a flow rate of 1.0 ml min⁻¹. Column eluate was monitored using a dual detection approach: the spectrofluorometer was set at excitation and emission wavelengths of 288 and 340 nm, respectively, to quantify lower concentrations of the 3'-hydroxy-4'-methoxy- metabolite (0.01–1.0 µg ml⁻¹) and UV detection at 254 nm was used for monitoring flurbiprofen (0.05–10 µg ml⁻¹), 4'-hydroxyflurbiprofen (0.1–20 µg ml⁻¹) and higher concentrations of 3'-hydroxy-4'-methoxyflurbiprofen (0.1–2.0 µg ml⁻¹). Under these conditions, the retention times were: flurbiprofen: *R*, 5.8 min; *S*, 7.6 min; 4'-hydroxyflurbiprofen: *R*, 12.9 min; *S*, 19.0 min; 3'-hydroxy-4'-methoxyflurbiprofen: *R*, 28.5 min; *S*, 39.6 min and internal standard: 10.1 min. The within- and between-day coefficient of variation for each analyte enantiomer over the respective calibration ranges were less than 12%; the extraction recovery was 75% or greater for the enantiomers of all three analytes.

Volunteer study

Four healthy young volunteers (two male and two female) between the ages of 24 and 29 years (mean ± SD; 26.8 ± 2.2 years, weight 67.8 ± 4.1 kg), and four healthy elderly volunteers (two male and two female) aged from 72 to 87 years (mean ± SD; 78.5 ± 7.2 years, weight 65.8 ± 10.6 kg) gave written informed consent and participated in the study which was approved by the Local Research Ethics Committee of King's College Hospital. Volunteers were judged to be in good health based on case history, a thorough physical examination and routine biochemical tests. None of the subjects had been involved in investigations of a similar nature for at least 3 months. The volunteers were required to abstain from any alcohol, caffeine-based products and medication for at least 24 h before the study and to fast overnight. On the morning of the study, each subject swallowed a single 100-mg tablet of racemic flurbiprofen (Froben® tablet) with approximately 150 ml water. Blood samples (10 ml) were collected immediately before drug administration and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10, 12 and 24 h post-dosing in plain silicone coated vacutainer tubes. The venous blood samples were drawn for the initial 12 h via an indwelling forearm cannula and thereafter by venepuncture. Samples were allowed to clot for 2 h and serum obtained after centrifugation (10 min at 2000g). Urine samples were collected every 2 h for the first 12 h and then between 12–22 and 22–24 h post-drug administration. The individual urine volumes were recorded and a pooled 24 h sample was prepared from the individual collections. Serum and urine samples were frozen at –20°C until required for analysis. All analyses were carried out in duplicate.

Data analysis

Determination of pharmacokinetic parameters was performed by conventional non-compartmental analysis of the enantiomeric serum concentration–time profiles of flurbiprofen and the 4'-hydroxy-metabolite. Maximum serum concentrations (*C*_{max}) and the corresponding times (*t*_{max}) were determined by examination of the individual serum concentration–time curves for each volunteer. The terminal disposition constant (*λ*) and the apparent elimination half-life (*t*_{1/2}) for each profile were calculated by

linear regression of the log-linear phase of the serum concentration–time curve. The area under the concentration–time curves from time zero to 24 h (AUC_{0-24}) was calculated using the linear–trapezoidal method and extrapolated to infinity (AUC_{0-inf}) by dividing the last measurable serum concentration by λ . The average extrapolated area accounted for 8 and 16% of the total area for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen, respectively. The apparent total clearance (CL/F) and volume of distribution (V_D/F) of flurbiprofen were calculated from the following expressions:

$$CL/F = D/AUC_{0-inf}$$

$$V_D/F = D/(AUC_{0-inf}\lambda),$$

where F is the fraction of the dose (D) reaching the systemic circulation. Renal clearance (CL_{ren}) was calculated from:

$$CL_{ren} = A_{24}/AUC_{0-24},$$

where A_{24} is the amount of material recovered unchanged over the 24-h urinary collection period.

The fraction of each flurbiprofen enantiomer converted to oxidation products (F_{ox}), namely 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, was calculated from an examination of the total recoveries (free and conjugated) of the individual enantiomers of flurbiprofen and metabolites in urine over 24 h using:

$$F_{ox} = \frac{\text{oxidative metabolites (\%)}}{\text{total recovery (\%)}}$$

where oxidative metabolites (%) is the sum of the molar amounts of the 4'-hydroxy- and 3'-hydroxy-4'-methoxy metabolites excreted, both free plus conjugated, expressed as a percentage of the administered dose and total recovery (%) is the percentage of the dose recovered in urine. The clearance of 4'-hydroxyflurbiprofen (CL_m) was estimated from:

$$CL_m = F_{ox} \cdot (F \cdot D) / AUC_m,$$

where AUC_m is the area under the 4'-hydroxyflurbiprofen serum concentration–time curve to infinity, corrected for molar equivalency to the parent drug.

Assuming the major metabolic products (figure 1) are cleared exclusively via renal elimination, the associated formation clearances (CL_f) may be determined by:

$$CL_f = A_p / AUC_{parent},$$

where A_p is the total amount of product recovered in urine over 24 h either unchanged or conjugated, and in the case of the 4'-hydroxy product includes the amount undergoing further metabolism to the 3'-hydroxy-4'-methoxy metabolite; and AUC_{parent} is the AUC_{0-24} of the compound from which the product was derived (Rowland and Tozer 1995). Previous reports have indicated the extensive urinary recovery of flurbiprofen following oral administration to man (70–100%), thereby supporting the above approach (Risdall *et al.* 1978, Szpunar *et al.* 1987).

Statistical comparisons of pharmacokinetic data derived from an examination of individual enantiomers may prove problematic unless their systemic availability is known to be equivalent. In the case of flurbiprofen previous investigations, following oral administration of the racemic drug (Risdall *et al.* 1978) and comparison of solution doses with formulated products (Spzunar *et al.* 1987), have indicated a bioavailability close to unity and therefore limited enantioselectivity. Statistical analysis of the data was carried out using Student's *t*-test for paired and independent samples.

Results

Enantioselective differences

The mean serum concentration–time profiles for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following the oral administration of 100 mg of the racemate to both young and elderly healthy volunteers are shown in figure 2. The corresponding pharmacokinetic parameters derived from analysis of the individual data and the magnitude of the enantiomeric differences, expressed as *S/R* ratios, are presented in table 1. The serum concentrations of (*S*)-flurbiprofen were consistently greater than those of the *R*-enantiomer from 3-h post-drug administration, resulting in a greater area under the curve for the *S*-enantiomer in both age groups (AUC_{0-inf} *S/R*: young, 1.17; elderly, 1.14), although with the small number

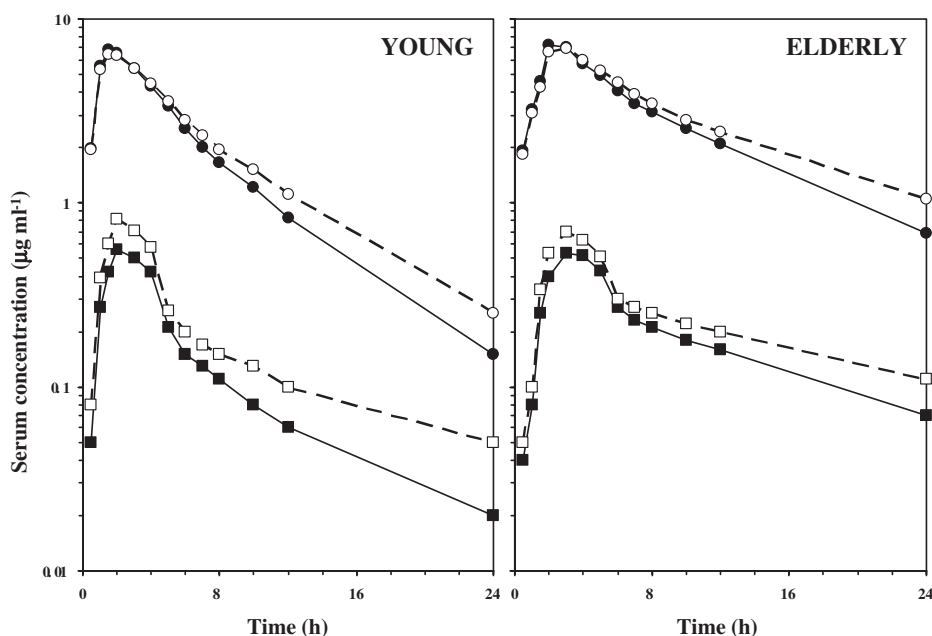


Figure 2. Mean serum concentration versus time profiles of (*R*)-flurbiprofen (●), (*S*)-flurbiprofen (○), (*R*)-4'-hydroxyflurbiprofen (■) and (*S*)-4'-hydroxyflurbiprofen (□) following oral administration of racemic flurbiprofen (100 mg) to four young and four elderly volunteers.

of subjects studied (two male and two female per group) this difference only attained statistical significance in the elderly. The progressive enrichment of (*S*)-flurbiprofen with respect to the total drug serum concentration in both volunteer groups is reflected in the mean apparent elimination half-life of (*R*)-flurbiprofen being significantly shorter than that of the *S*-enantiomer. In the elderly, other statistically significant differences were observed between the individual enantiomers with (*S*)-flurbiprofen displaying a reduced clearance and a greater volume of distribution than (*R*)-flurbiprofen. It is noteworthy that the magnitude of enantioselectivity for clearance was comparable between the two groups, but did not attain statistical significance ($p=0.07$) in the young with one volunteer having similar clearance values for both enantiomers.

The enantiomers of 4'-hydroxyflurbiprofen were present in serum at concentrations five- to 20-fold lower than those of the corresponding drug enantiomers and their serum versus time profiles showed a more prominent biphasic characteristic (figure 2) with apparent terminal half-lives longer than those of the parent drug enantiomers in both age groups (table 1). The serum concentrations of (*S*)-4'-hydroxyflurbiprofen always exceeded those of the *R*-enantiomer, the area under the curve and maximum serum concentration being significantly greater in both volunteer age groups. The apparent terminal half-life and total clearance of 4'-hydroxyflurbiprofen, calculated from the fraction (F_{ox}) of the enantiomeric dose undergoing oxidative metabolism, showed significant differences in both groups with (*S*)-4'-hydroxyflurbiprofen having a longer half-life and a lower clearance compared with the *R*-enantiomer.

The urinary recovery of flurbiprofen and its metabolites showed stereoselectivity with a significantly greater amount of material recovered in 24 h having the

Table 1. Mean pharmacokinetic parameters (±SD) of the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following oral administration of racemic flurbiprofen (100 mg) to four young and four elderly volunteers.

Parameter (units)	Young			Elderly		
	R-enantiomer	S-enantiomer	S/R	R-enantiomer	S-enantiomer	S/R
Flurbiprofen						
$C_{\max}$ ($\mu\text{g ml}^{-1}$)	6.9 ± 1.8	6.5 ± 1.6	0.96	8.4 ± 2.2	7.8 ± 2.1	0.93
$t_{\max}$ (h) [†]	1.5 (1.5–2.0)	2.5 (1.5–3.0)		2.5 (1.0–3.0)	2.5 (1.0–3.0)	
$t_{1/2}$ (h)	4.5 ± 0.6	5.2 ± 0.7**	1.15	7.1 ± 1.0 ^{††}	9.6 ± 1.2** ^{††}	1.35
AUC _{0–24} ($\mu\text{g h ml}^{-1}$)	41.3 ± 3.1	46.5 ± 3.6	1.13	63.3 ± 12.5 [†]	69.3 ± 15.2** [†]	1.09
AUC _{0–inf} ($\mu\text{g h ml}^{-1}$)	42.3 ± 2.6	49.5 ± 5.3	1.17	69.5 ± 11.8 [†]	79.3 ± 15.0** [†]	1.14
CL/F (ml min ^{–1})	19.7 ± 1.2	17.0 ± 1.9	0.86	12.3 ± 2.1 ^{††}	10.8 ± 2.0** ^{††}	0.88
CL _{ren} (ml min ^{–1})	1.22 ± 0.62	0.99 ± 0.64	0.81	1.56 ± 1.08	1.19 ± 0.88	0.76
V_{D}/F (L)	7.8 ± 1.4	7.6 ± 0.9	0.99	7.6 ± 1.6	8.9 ± 1.9 [*]	1.18
F_{ox}	0.71 ± 0.05	0.71 ± 0.05	1.01	0.65 ± 0.07	0.65 ± 0.07	1.00
4'-Hydroxyflurbiprofen						
$C_{\max}$ ($\mu\text{g ml}^{-1}$)	0.56 ± 0.28	0.82 ± 0.41 [*]	1.45	0.58 ± 0.11	0.73 ± 0.15**	1.27
$t_{\max}$ (h) [†]	2.0 (2.0–3.0)	2.0 (2.0–2.0)		3.5 (3.0–5.0)	3.5 (2.0–5.0)	
$t_{1/2}$ (h)	6.7 ± 1.1	10.6 ± 2.4 [*]	1.58	10.2 ± 1.2 ^{††}	13.7 ± 1.7**	1.35
AUC _{0–24} ($\mu\text{g h ml}^{-1}$)	3.05 ± 0.87	4.54 ± 1.06**	1.49	4.63 ± 0.81 ^{††}	6.06 ± 0.96* [†]	1.31
AUC _{0–inf} ($\mu\text{g h ml}^{-1}$)	3.25 ± 0.85	5.37 ± 0.94**	1.68	5.67 ± 0.92 ^{††}	8.08 ± 1.35* [†]	1.44
CL/F (ml min ^{–1})	200 ± 39	120 ± 22**	0.60	104 ± 21 ^{††}	73.3 ± 16.2* [†]	0.71
CL _{ren} (ml min ^{–1})	37.0 ± 18.4	22.7 ± 11.0	0.61	38.6 ± 29.3	23.4 ± 15.9	0.61

$C_{\max}$, maximum observed serum concentration; $t_{\max}$, time to $C_{\max}$; $t_{1/2}$, terminal elimination half-life; AUC_{0–24}, area under the curve from time zero to 24 h; AUC_{0–inf}, area under the curve from time zero to infinity; CL/F, oral clearance; CL_{ren}, renal clearance; V_{D}/F , volume of distribution; F_{ox} , oxidative metabolite fraction. Data are mean ± SD; significant differences between enantiomers (* p < 0.05, ** p < 0.01) or between age groups ([†] p < 0.05, ^{††} p < 0.01); ^{*}median (range).

R-configuration in both age groups (*S/R*: young, 0.87; elderly, 0.81; table 2). About 21% of the dose was recovered as flurbiprofen, both free and conjugated, in both groups, with a small but significant enantioselective preference for the excretion of the *R*-enantiomer. The major urinary product was 4'-hydroxyflurbiprofen, both free and conjugated, accounting for approximately 47 and 36% of the dose in the young and elderly groups, respectively, and, as for the parent drug, was predominantly of the *R*-configuration (table 2). Conjugation of the 4'-hydroxy-metabolite displayed both regio- and stereoselectivity and favoured the formation of the acyl glucuronide rather than phenolic conjugates (phenol/acyl: young, 0.07; elderly, 0.10) and (*R*)-4'-hydroxyflurbiprofen acyl glucuronide rather than (*S*)-4'-hydroxyflurbiprofen acyl glucuronide (*S/R*: young, 0.83; elderly, 0.76). The 3'-hydroxy-4'-methoxy metabolite was a minor component accounting for 4.2 and 2.9% of the dose, almost exclusively as conjugates, in the young and elderly, respectively. Interestingly, conjugation shows opposing regioselectivity to that of the 4'-hydroxy metabolite favouring the formation of phenolic conjugates (phenol/acyl: young, 80; elderly, 140) and, although not statistically significant in either case, opposing enantiomeric preference between the two volunteer groups (*S/R*: young, 1.17; elderly, 0.91).

The partial metabolic clearances for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen are presented in table 3. Clearance via the different metabolic pathways was always greater for the *R*-enantiomer of both the drug and the 4'-hydroxy metabolite, with mean *S/R* ratios ranging from 0.55 to 0.77 in both groups. However, the enantiomeric differences did not reach statistical significance in all cases (table 3).

Age-related differences

Comparison between the young and elderly groups indicated age-related differences with respect to the serum pharmacokinetics of (*R*)- and (*S*)-flurbiprofen

Table 2. Urinary recoveries (per cent of administered dose in 24 h) of flurbiprofen and its metabolites following the oral administration of racemic flurbiprofen (100 mg) to four young and four elderly volunteers.

Analyte	Young			Elderly		
	<i>R</i> -enantiomer	<i>S</i> -enantiomer	<i>S/R</i>	<i>R</i> -enantiomer	<i>S</i> -enantiomer	<i>S/R</i>
Flurbiprofen						
Free	2.99 ± 1.55	2.67 ± 1.55*	0.89	6.03 ± 4.59	5.00 ± 3.86*	0.83
Acyl-conjugate	8.43 ± 1.51	7.26 ± 1.03*	0.86	5.93 ± 2.02	4.66 ± 1.86*	0.79
Total	11.42 ± 2.34	9.92 ± 2.19*	0.87	11.96 ± 4.23	9.66 ± 3.29*	0.81
4'-Hydroxyflurbiprofen						
Free	5.86 ± 2.48	5.40 ± 2.19	0.92	9.23 ± 6.20	7.82 ± 5.07	0.85
Acyl-conjugate	18.18 ± 1.84	15.05 ± 1.60*	0.83	9.73 ± 4.38 [†]	7.42 ± 3.46* [†]	0.76
Phenol-conjugate	1.30 ± 1.34	1.01 ± 1.18	0.78	1.03 ± 1.01	0.73 ± 0.57	0.71
Total	25.33 ± 1.45	21.46 ± 0.65*	0.85	19.99 ± 3.40 [†]	15.98 ± 2.14* [†]	0.80
3'-Hydroxy-4'-methoxyflurbiprofen						
Free	0.021 ± 0.030	0.024 ± 0.004	1.14	0.016 ± 0.031	0.013 ± 0.027	0.81
Acyl-conjugate	0.029 ± 0.034	0.022 ± 0.027	0.76	0.011 ± 0.022	0.009 ± 0.018	0.82
Phenol-conjugate	1.87 ± 0.27	2.20 ± 0.33	1.18	1.51 ± 0.42	1.38 ± 0.31 [†]	0.91
Total	1.92 ± 0.30	2.24 ± 0.37	1.17	1.53 ± 0.44	1.40 ± 0.31 [†]	0.91
Total recovery	38.67 ± 2.63	33.63 ± 2.03*	0.87	33.49 ± 6.32	27.03 ± 4.42*	0.81

Data are mean ± SD; significant differences ($p < 0.05$) between enantiomers* or between age groups[†].

Table 3. Formation clearances (ml min^{-1}) of flurbiprofen and 4'-hydroxyflurbiprofen metabolites following the oral administration of racemic flurbiprofen (100 mg) to four young and four elderly volunteers.

Metabolite	Young			Elderly		
	<i>R</i> -enantiomer	<i>S</i> -enantiomer	<i>S/R</i>	<i>R</i> -enantiomer	<i>S</i> -enantiomer	<i>S/R</i>
Flurbiprofen						
4'-Hydroxyflurbiprofen	11.2 $\pm$ 0.68	8.5 $\pm$ 0.56*	0.77	5.8 $\pm$ 1.3 ^{††}	4.4 $\pm$ 1.14 ^{**††}	0.76
Acyl-glucuronide	3.5 $\pm$ 0.87	2.6 $\pm$ 0.40	0.74	1.6 $\pm$ 0.40 [†]	1.1 $\pm$ 0.38 ^{**††}	0.69
4'-Hydroxyflurbiprofen						
3'-Hydroxy-4'-methoxyflurbiprofen	11.7 $\pm$ 3.06	8.9 $\pm$ 0.96	0.68	5.9 $\pm$ 1.32 [†]	4.2 $\pm$ 1.16 ^{*††}	0.71
Conjugate(s)	118.8 $\pm$ 32.3	65.7 $\pm$ 19.9 ^{**}	0.55	40.9 $\pm$ 8.2 ^{††}	24.4 $\pm$ 10.0 ^{**††}	0.60

Data are mean $\pm$ SD; significant differences between enantiomers (* $p < 0.05$, ** $p < 0.01$) or between age groups ([†] $p < 0.05$, ^{††} $p < 0.01$).

(table 1). The elderly volunteers exhibited significantly longer elimination half-lives, larger AUCs and reduced clearance for both enantiomers of the drug in comparison with the young subjects (table 1). Age-associated differences were also observed in the pharmacokinetic parameters determined for 4'-hydroxyflurbiprofen, both enantiomers displaying significantly larger AUCs and reduced clearance in the elderly (table 1). The medians and ranges of t_{max} for both enantiomers of the metabolite were also greater in the elderly in comparison with the young (table 1). An additional difference between the two age groups was seen in the case of the *R*-enantiomer with the elderly exhibiting a significantly greater apparent half-life for (*R*)-4'-hydroxyflurbiprofen. These age-related changes in the pharmacokinetics of the enantiomers of the drug and 4'-hydroxy- metabolite were associated with only modest alterations in enantioselectivity with *S/R* ratios generally displaying good agreement between the two age groups (table 1).

Metabolite formation clearances were significantly reduced by two- to three-fold in the elderly compared with the young, but with limited effect on enantioselectivity (table 3). This pattern is reflected in a lower, but not statistically significant, amount of the dose recovered in the elderly (60.5 versus 72.3%), which was primarily due to a significant reduction in the urinary recovery of both enantiomers of 4'-hydroxyflurbiprofen and (*S*)-3'-hydroxy-4'-methoxyflurbiprofen (table 2). Furthermore, the proportions of flurbiprofen and 4'-hydroxyflurbiprofen recovered as acyl glucuronides were reduced in the elderly group such that elimination of these components in the free form becomes quantitatively as important as conjugation in this age group (table 2). The recovery of both (*R*)- and (*S*)-3'-hydroxy-4'-methoxyflurbiprofen phenolic conjugates were lower in the elderly, but the decrease was only significant for the *S*-enantiomer which resulted in a reversal of the prominent enantiomer in the total recovered material, i.e. *S/R*: young = 1.17 and elderly = 0.91 (table 2).

Discussion

Enantioselectivity was observed in the disposition of flurbiprofen following oral administration of the racemate to both young and elderly volunteers. In agreement with previous reports, in both patients and healthy volunteers, the extent of the enantioselectivity was relatively modest (Jamali *et al.* 1988, 1991, Small *et al.* 1990,

Cefali *et al.* 1991, Young *et al.* 1991, Knadler *et al.* 1992a, b, Geisslinger *et al.* 1994, Oelkers *et al.* 1997, Suri *et al.* 1997) and comparable between the two volunteer groups. The stereochemical aspects of flurbiprofen disposition are not as complex as other agents in the 2-arylpropionic acid group, e.g. ibuprofen, as in man, unlike other species (Menzel-Soglowek *et al.* 1992), (*R*)-flurbiprofen does not undergo chiral inversion (figure 1), which is presumably due to its inability to form a coenzyme-A thioester, as has been observed in *in vitro* studies using rat tissues (Knihinicki *et al.* 1989, Knadler and Hall 1990, Porubek *et al.* 1991).

As the systemic availability of racemic flurbiprofen is reported to be 95–100% (Risidall *et al.* 1978, Szpunar *et al.* 1987), the observed enantioselective disposition of the drug, resulting in the more rapid systemic clearance of the *R*-enantiomer, is presumably associated with stereoselectivity in intrinsic clearance and/or protein binding.

In vitro studies, using human liver microsomes, have indicated enantiomeric differences in the oxidation of flurbiprofen to the 4'-hydroxy metabolite, enzyme affinity (K_M) of the *R*-enantiomer being 1.6-fold greater than of (*S*)-flurbiprofen, whereas the maximal velocities (V_{max}) were comparable on incubation of the individual enantiomers (Tracy *et al.* 1995). Calculation of the intrinsic clearance from these data results in a twofold difference favouring the *S*-enantiomer. A more limited study using tissue from two human livers, and incubation of the racemate, indicated enantiomeric interactions in metabolism resulting in a reduction in both affinity and maximal velocity for both enantiomers and a decrease in the ratio of intrinsic clearance (*S/R*) to about 1.1 (Tracy *et al.* 1995).

The more rapid clearance of (*R*)-flurbiprofen observed in the present study has been reported previously following administration of either the racemate (Jamali *et al.* 1988, Small *et al.* 1990, Cefali *et al.* 1991, Knadler *et al.* 1992a, b) or the individual enantiomers (Geisslinger *et al.* 1994). However, the unbound clearance of the drug showed no stereoselectivity on administration to healthy volunteers (Knadler *et al.* 1992a) or uraemic patients (Knadler *et al.* 1992b). The formation clearance of the major metabolite, 4'-hydroxyflurbiprofen, was stereoselective in both volunteer groups (enantiomeric ratio *S/R*: young, 0.77; elderly, 0.76), with a selectivity similar to that reported by Knadler *et al.* (1992a) based on unbound formation clearance (*S/R*: total, 0.61; unbound, 0.72). Thus, from the information currently available the *in vitro* and *in vivo* data show opposing stereoselectivity with respect to the 4'-oxidation of the drug.

Glucuronidation of flurbiprofen has been examined *in vitro* using human liver microsomes and exhibits a threefold selectivity for conjugation of the *R*-enantiomer (Hamdoun *et al.* 1995). However, the application of such data to the *in vivo* situation should be carried out with some caution due to the instability of acyl glucuronides and their stereoselective hydrolysis *in vivo* (Knadler and Hall 1991). The partial metabolic formation clearances of the glucuronide conjugates showed stereoselectivity for the *R*-enantiomer in both age groups, with enantiomeric ratios (*S/R*) of 0.74 and 0.69 for the young and elderly, respectively, values similar to those reported by Knadler *et al.* (1992a) based on unbound formation clearance (*S/R*, 0.69).

Previous *in vitro* studies have highlighted the extensive nature of the protein binding of flurbiprofen but present an inconsistent picture with regard to enantioselectivity, suggesting a modest preference for the opposing enantiomer (Knadler *et al.* 1989, Blouin *et al.* 1993). However, *ex vivo* determinations for

healthy volunteers and uraemic patients both indicate that (*S*)-flurbiprofen is more highly bound, with a mean enantiomeric ratio (*S*/*R*) of the free fraction of 0.87 and 0.81 in the two groups, respectively (Knadler *et al.* 1992a, b). These enantiomeric differences did not attain statistical significance in either group, but the free fractions of both enantiomers were significantly greater in the uraemic patients (Knadler *et al.* 1992b). These data were used to rationalize differences in the individual enantiomer clearances between the groups which, while greater in the uraemic patients, did not attain statistical significance on comparison of the unbound clearances. Similarly, Blouin *et al.* (1993) showed the opposite stereoselectivity, i.e. the free fraction of (*S*)-flurbiprofen was greater than that of the *R*-enantiomer, the differences again did not achieve statistical significance in control subjects.

From the above, it is obvious that at present the underlying cause of the greater clearance of (*R*)-flurbiprofen with respect to either hepatic metabolism or protein binding is unclear. However, Knadler *et al.* (1992a) rationalized their observed enantioselectivity in terms of metabolite formation and protein binding.

There are few reports concerned with the influence of age on the pharmacokinetics of flurbiprofen (Hamdy *et al.* 1980, 1990, Kean *et al.* 1992), only one of which examines the metabolism of the drug (Kean *et al.* 1992), and all suggest that age has minimal effects on drug disposition. In contrast, the current study has shown significant age-related differences in the disposition of both enantiomers of flurbiprofen with a reduction in systemic clearance of 38% for each (table 1), resulting in significant increases in both the AUCs and terminal half-lives for both enantiomers in the elderly. Similarly, age-associated differences were observed in the disposition of the major metabolite, 4'-hydroxyflurbiprofen, the elderly showing significant reductions in systemic clearance, and increased AUC and half-life of both enantiomers in comparison with the young. Examination of the urinary drug recovery indicated that the elderly excreted less 4'-hydroxyflurbiprofen and acyl-conjugates of both this metabolite and the parent drug, whereas total drug recovery was comparable between the groups. Calculation of the metabolite formation clearances of both major metabolites from the enantiomers of flurbiprofen and for the pathways of 4'-hydroxyflurbiprofen, showed significant reductions, between 48 and 66%, in the elderly (table 3).

Such differences in drug and metabolite disposition could arise from changes in protein binding in the elderly as ageing is reported to be associated with decreased albumin concentrations (Wallace and Verbeeck 1987, Woodhouse and Wynne 1987). However, while we did not determine the albumin concentrations in these volunteers, a previous study with ibuprofen found no differences in albumin concentration with age (Tan *et al.* 2003) and other studies with flurbiprofen have reported that the binding of the racemate does not alter with age (Knadler *et al.* 1989). Additionally, an increase in the free fraction of this low hepatic extraction drug would be expected to result in an increase in systemic clearance, rather than a reduction, as has been observed following administration of the racemate to uraemic (Knadler *et al.* 1992b) and end-stage renal disease patients (Cefali *et al.* 1991). Thus, the reduced clearance of flurbiprofen enantiomers observed in the elderly is likely to be associated with a reduction in hepatic metabolic activity rather than changes in protein binding.

Previous studies have indicated that clearance via cytochrome P450 (CYP) mediated pathways is generally reduced in the elderly (Sotaniemi *et al.* 1997,

Hammerlein *et al.* 1998), whereas conjugation pathways, e.g. glucuronidation, are not greatly influenced by age (Mooney *et al.* 1985). However, as for the reduced conjugation clearance of flurbiprofen and 4'-hydroxyflurbiprofen observed in the present study, the decreased clearance of the related agent ketoprofen, a drug predominantly metabolized by glucuronidation, has been reported in the elderly (Advenier *et al.* 1983, Dennis *et al.* 1985) and Rudy *et al.* (1995) and Tan *et al.* (2003) reported a stereoselective reduction in the formation clearance of the glucuronide of (*S*)-ibuprofen with age.

The oxidative metabolism of the enantiomers of flurbiprofen to 4'-hydroxyflurbiprofen has been reported to be mediated by CYP2C9 (Tracy *et al.* 1995, 1996). The enantiomers of ibuprofen also undergo predominantly CYP2C9-mediated oxidation, with contributions from CYP2C8, such that the stereo- and regioselectivity of oxidation are influenced by the isozymes involved (Hamman *et al.* 1997). *In vivo* studies have indicated that the clearance of the enantiomers of ibuprofen by oxidative pathways are differentially affected by age, with a reduction in formation clearance of metabolites arising from the *S*-enantiomer in the elderly, whereas corresponding values for (*R*)-ibuprofen are essentially unchanged (Tan *et al.* 2003). Such differential effects imply that different CYP enzymes are predominantly involved in the clearance of the individual enantiomers. In the present investigation, the reduction in formation clearance of 4'-hydroxyflurbiprofen with age was 48% for both enantiomers, indicative that the same enzyme(s) are involved in the biotransformation(s). The observation that the oxidation of flurbiprofen is mediated specifically by CYP2C9 (Tracy *et al.* 1995, 1996) lead to the suggestion that a urinary recovery ratio (4'-hydroxyflurbiprofen/[4'-hydroxyflurbiprofen + flurbiprofen]) could be used as an index of CYP2C9 activity in man (Frye *et al.* 2000, Hutzler *et al.* 2000). As the CYP2C9 content of human liver microsomes has been reported to decline with age, but the correlation was weak (Schmucker *et al.* 1990), it was of interest to examine the application of the suggested flurbiprofen metabolic index to the data obtained in the present study. However, for the small number of volunteers studied, determination of this ratio, whether in a stereospecific or non-stereospecific manner and with or without taking into consideration the further metabolism to the 3'-hydroxy-4'-methoxy-metabolite, showed a poor correlation with the fractional clearance of 4'-hydroxyflurbiprofen and as such, revealed no age-related differences (data not shown).

In conclusion, modest, but significant, stereoselective differences were observed in the pharmacokinetics of flurbiprofen following the administration of the racemic drug to healthy young and elderly volunteers. Age-related differences in the disposition of flurbiprofen arise from a reduction in clearance resulting in the elderly having a greater exposure to both enantiomers of the drug with enantiomeric AUCs typically 1.6-fold larger than the young. Such alterations in drug disposition could contribute to the incidence of adverse effects to NSAIDs in the elderly and has potential significance for the use of such agents in the treatment of age-associated diseases such as prostate cancer and Alzheimer's disease.

Acknowledgements

The authors gratefully acknowledge the award of a University of London Triangle Trust Postgraduate Studentship to B. K. P. and a King's College Research Strategy Fund award to S. H. D. J., C. G. S. and A. J. H.

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Oral absorption, metabolism and excretion of 1-phenoxy-2-propanol in rats

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Received 11 April 2003

1. This study was designed to determine the absorption, metabolism and excretion of 1-phenoxy-2-propanol in Fischer 344 rats following oral administration in an effort to bridge data with other propylene glycol ethers.

2. Rats were administered a single oral dose of 10 or 100 mg kg⁻¹ ¹⁴C-1-phenoxy-2-propanol as a suspension in 0.5% methyl cellulose ether in water (w/w). Urine was collected at 0–12, 12–24 and 24–48 h and faeces at 0–24 and 24–48 h post-dosing and the radioactivity was determined. Urine samples were pooled by time point and dose level and analysed for metabolites using LC/ESI/MS and LC/ESI/MS/MS.

3. The administered doses were rapidly absorbed from the gastrointestinal tract and excreted. The major route of excretion was via the urine, accounting for 93 ± 5% of the low and 96 ± 3% of the high dose. Most of the urinary excretion of radioactivity occurred within 12 h after dosing; 85 ± 2% of the low and 90 ± 1% of the high dose. Total faecal excretion remained < 10%. Rats eliminated the entire administered dose within 48 h after dosing; recovery of the administered dose ranged from 100 to 106%. Metabolites tentatively identified in urine were conjugates of phenol (sulphate, glutathione) with very low levels (< 2%) of hydroquinone (glucuronide), conjugates of parent compound (glucuronide, sulphate) and a ring-hydroxylated metabolite of parent. There was no free parent compound or phenol in non-acid-hydrolysed urine. In acid-hydrolysed urine, 61% of the dose was identified as phenol and 13% as 1-phenoxy-2-propanol. Although the parent compound was stable to acid hydrolysis, some of the phenol in acid hydrolysed urine may have arisen from degradation of acid-labile metabolite(s) as well as hydrolysis of phenol conjugates.

4. Rapid oral absorption, metabolism and urinary excretion of 1-phenoxy-2-propanol in rats were similar to other propylene glycol ethers.

Introduction

1-Phenoxy-2-propanol is a clear and colourless liquid with a slight odour. It is a slow evaporating and very hydrophobic glycol ether, more hydrophobic than would be expected from its molecular weight. 1-Phenoxy-2-propanol is an excellent coating and lining agent and coalescent for acrylic-based latexes. It is also used extensively in dyeing applications, where it functions as a dye solubilizer and carrier. Its other applications include solvent for inks in ballpoint and felt tip pens, stamp pads, textile printing pastes, and paint removers. In 1993, the annual production of 1-phenoxy-2-propanol was reported to be 1–5 million lbs (Boatman 2001).

The acute oral LD₅₀ of 1-phenoxy-2-propanol for male and female Fischer 344 rats was determined as 2830 and 3730 mg kg⁻¹ body weight, respectively (Boatman

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2001). No death or signs of overt toxicity resulted in rats after a 7-h exposure via inhalation to saturated 1-phenoxy-2-propanol vapours generated at room temperature. However, exposure to saturated vapours generated at 100°C showed significant nasal, respiratory, and eye irritation during exposure without any death. Some pathological changes in liver, lung and kidney were reported 24 h after the termination of exposure, all of which were resolved by the end of the study (14 days after the termination of exposure) (Boatman 2001).

Data on the metabolism and pharmacokinetics of 1-phenoxy-2-propanol are not available. This study, therefore, was designed to determine the oral absorption, metabolism and excretion of 1-phenoxy-2-propanol in Fischer 344 rats. If the metabolism is similar to other propylene glycol ethers (microsomal *O*-dealkylation), this study could serve as a bridge for data extrapolation from structurally related compounds. Furthermore, by understanding the metabolism of 1-phenoxy-2-propanol, we may be able to understand better its kinetics and toxicological properties, as its likely metabolite(s) include conjugates of the test material, propylene glycol and/or phenol. Both propylene glycol and phenol have been extensively studied for their kinetics, metabolism and toxicological properties (Capel *et al.* 1972, Kao *et al.* 1979, Miller *et al.* 1983, Edwards *et al.* 1986, Hughes and Hall 1995).

Materials and methods

Chemicals and dose formulation

Uniformly phenyl ring-radiolabelled ^{14}C -1-phenoxy-2-propanol (MW 159.2, specific activity 6.8mCi mmol^{-1} , 96% radiochemical purity) was purchased from Sigma Chemical Co. (Milwaukee, WI, USA) and used without further purification. Non-radiolabelled 1-phenoxy-2-propanol (purity > 93%) was added to adjust the concentration of the dosing suspensions. About 5% of the impurity in non-radiolabelled 1-phenoxy-2-propanol consisted of two isomers of dipropylene glycol ether. The remaining 2% impurity consisted of 16 minor chromatographic components ranging from 0.06 to 0.53%. Methyl cellulose ether was obtained from The Dow Chemical Co. (Midland, MI, USA). All other chemicals used were of the purest grade available and obtained from standard sources.

Dose suspensions were prepared in 0.5% methyl cellulose ether in water (w/w) by adding appropriate amounts of ^{14}C -labelled and non-radiolabelled 1-phenoxy-2-propanol to methyl cellulose ether and stirring for 24 h at 4°C. The concentration of 1-phenoxy-2-propanol in the dose suspensions was 2 and 20mg g^{-1} and the radioactivity $50\mu\text{Ci g}^{-1}$ methyl cellulose ether. The dose suspensions were continuously mixed for homogeneity with a magnetic stirrer until dosed.

The concentration of 1-phenoxy-2-propanol in each of the dose suspensions was determined in aliquots taken immediately before dosing. Concentration in dose suspensions was determined by using high-performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) absorbance detector. The amount of radioactivity in the dose suspensions was determined by liquid scintillation counting (LSC) using a Packard 2900TR liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL, USA) in Hionic Fluor scintillation cocktail (Packard, Meriden, CT, USA). Similarly, homogeneity of the dose suspensions was determined by LSC in aliquots of the ^{14}C -labelled dosing suspensions taken from various locations in the suspension containers.

Animals (care and dosing)

The Institutional Animal Care and Use Committee of The Dow Chemical Company approved the animal care and experimental protocols. Animal care and treatment were conducted in accordance with the established guidelines. The study followed good laboratory practice guidelines and was inspected by the Quality Assurance Unit, Toxicology & Environmental Research and Consulting, The Dow Chemical Company.

Adult male Fischer 344 rats (10 weeks old, 200–208 g) were purchased from Charles River Laboratories, Inc. (Raleigh, NC, USA) and allowed to acclimatize to the animal facility for 1 week before dosing. Animals were housed two per cage in stainless steel cages, in rooms designed to maintain adequate conditions ($22 \pm 3^\circ\text{C}$, 40–70% relative humidity, 12-hour light/dark photocycle, air exchange 12–15 times/h). The stainless steel cages had wire-mesh floors suspended above catch pans. Cages

contained hanging feeders and pressure activated, nipple-type watering systems. Animals were provided LabDiet® Certified Rodent Diet #5002 (PMI Nutrition International, St Louis, MI, USA) in pelleted form. Feed and municipal water were provided *ad libitum*. Acclimatization to glass metabolism cages was performed by placing one rat per cage for 2 days before administering the dose. After acclimatization, animals were randomly selected for dosing. Groups of three rats were gavaged with a single dose of either 10 or 100 mg kg⁻¹ 1-phenoxy-2-propanol in methyl cellulose ether suspension using a glass disposable syringe and disposable stainless steel feeding needle. The dose suspensions were administered at 5 g kg⁻¹ body weight. After the completion of dosing, animals were housed individually in glass metabolism cages. Animals were fasted for 16 h before dosing and had free access to food 4 h after dosing.

Sample collection and analysis

All urine voided by individual animals at 0–12, 12–24 h and 24–48 h post-dosing were collected in dry-ice cooled traps. Cages were rinsed with water and rinsings collected at the time of each urine collection. Urine specimens and cage rinses were weighed, and weighed aliquots of each sample were analysed for radioactivity by LSC. Radioactivity found in cage rinses was added to the corresponding urine samples. Urine samples of each dose and time were pooled using equal volume aliquots and stored at –80°C until chemical analyses.

Faeces were collected in dry ice-chilled containers for 24-h intervals. An aqueous homogenate (about 25% w/w) was prepared and weighed aliquots of homogenates were placed in scintillation vials, solubilized with Soluene®-350 (Packard) and quantified for radioactivity after decolourization and pH adjustment. Samples were decolourized with 30% hydrogen peroxide and the pH was adjusted with glacial acetic acid. In addition, equal volume aliquots of faecal homogenates from each animal were taken from the 0–24 h collection interval, pooled and stored at –80°C for chemical analysis. Since < 10% of the administered dose was excreted in faeces, no chemical analysis was performed on pooled faecal samples.

Forty-eight hours post-dosing, the animals were euthanized with CO₂. Rat carcasses were stored at –20°C for possible measurement of radioactivity for mass balance of the administered dose. Following the sacrifice of the animals, a final cage wash was performed and collected. A weighed aliquot of the final cage wash was analysed for radioactivity for mass balance. The minor residual radioactivity left in carcasses was not determined as 103 ± 2% of the administered radioactivity was recovered in excreta.

Control samples

Control urine and faeces were collected in dry ice cooled traps from three rats not dosed with 1-phenoxy-2-propanol to use as blanks in the LSC and chemical analysis.

¹⁴C analysis

The scintillation counter was calibrated to correct for quenching by establishing a curve using quench standards. The rate of chemiluminescence was also monitored and samples were counted until chemiluminescence completely decayed. The counting efficiency of the scintillation counter was > 95% for ¹⁴C-labelled compounds. Counts per min (cpm) were corrected for background and converted to disintegrations per min (dpm) before calculating concentrations.

Metabolite identification and quantitation

As most of the administered ¹⁴C-1-phenoxy-2-propanol (87–93%) was eliminated in urine within the first 24 h after dosing, only samples collected between 0–12 and 12–24 h were used for metabolite profiling. Urine samples collected between 24 and 48 h were not analysed for metabolite(s). Pooled urine samples from 0–12 and 12–24 h time points of the 10 and 100 mg kg⁻¹ doses were centrifuged to remove solid particles and analysed by HPLC with ¹⁴C-detection to obtain radiochemical profiles of urinary metabolites. There was no loss of radioactivity during centrifugation (data not shown).

Attempts to identify components of the chromatographic peaks that contained > 5% of the administered dose was performed on 0–12-h pooled urine samples from rats dosed with 100 mg kg⁻¹ 1-phenoxy-2-propanol using HPLC separations, electrospray ionization (ESI) and detected by mass spectrometry (MS). In addition, identification of conjugated hydroquinone (a possible reactive metabolite) was conducted even though this metabolite represented < 5% of the dose. Instrumental conditions for these analyses are presented in table 1. Urine samples were analysed by two different mass spectrometers using HPLC/ESI/MS and HPLC/ESI/MS/MS to identify metabolites. HPLC conditions were identical for both of the mass spectrometric analyses (table 1). Metabolite identification was accomplished by full-scan ESI/MS, both in the positive- and negative-ion modes on both instruments. Using the apparent molecular ions from the full-scan analyses, the sample was then reanalysed on an ion-trap mass spectrometer in the MS/MS mode to obtain product ions from these molecular ions.

Table 1. Analytical instrumentation and conditions for the analysis of rat urine samples.

HPLC System:	Agilent Model 1100 (Agilent Technologies, Palo Alto, CA, USA)
Column:	YMC Basic, 4.6 × 250 mm w/ 4 × 20 mm YMC Basic guard column (Waters, Milford, MA, USA)
Injection volume:	10 µl
Eluent A:	water with 4 mM ammonium formate and 0.1% formic acid
Eluent B:	methanol with 4 mM ammonium formate and 0.1% formic acid
Gradient:	0–1 min, 98%A; 1–16 min, 62%A; 16–25 min 50% A; 25–37 min 0% A; 37–45 min, 98% A
<i>Analysis conditions for ¹⁴C and UV profiles</i>	
Wavelength:	254 nm
¹⁴ C detector:	Berthold Model LB 507A (Berthold, Wildbad, Germany)
H range 1 and 2:	100 K cpm
Cell:	YG 150 S4D
<i>Analysis conditions for mass spectrometry identification (HPLC/MSD)</i>	
Mass spectrometer:	Agilent 1100 LC/MSD
Ionization:	electrospray in positive- and negative-ion modes
HPLC system/gradient:	same as above
Gas temperature:	300°C
Drying gas:	8.01 min ⁻¹
Neb pressure:	45 psig
Capillary voltage:	3300 V
Fragmentor:	70
Scan range:	50–500 amu (negative and positive modes)
<i>Analysis conditions for mass spectrometry identification (Finnigan LCQ)</i>	
Mass spectrometer:	Finnigan LCQ Duo (Thermo Finnigan, San Jose, CA, USA)
Ionization:	electrospray in positive- and negative-ion modes
HPLC system/gradient:	same as above
Capillary temperature:	200°C
Capillary voltage:	5 V (positive polarity); –10 V (negative polarity)
Sheath gas:	N ₂ at flow of 50 (arbitrary units)
Aux gas:	N ₂ at flow of 10 (arbitrary units)
Collision energy:	25–35%
Full-scan conditions:	positive ions, 80–500 amu; negative ions, 80–500 amu positive ion product ion scans, <i>m/z</i> 100–810 from precursor <i>m/z</i> 400 negative ion product ion scans, <i>m/z</i> 50–300 from precursor <i>m/z</i> 173, <i>m/z</i> 65–300 from <i>m/z</i> 245, <i>m/z</i> 65–300 from <i>m/z</i> 247, <i>m/z</i> 90–375 from <i>m/z</i> 327, <i>m/z</i> 60–300 from <i>m/z</i> 231

Pooled urine samples from the 0–12-h time point, 100 mg kg⁻¹ dosed animals, were also hydrolysed with 1 N HCl (1:1, v/v) at 90°C for 22 h to cleave acid-labile conjugated metabolites. Control urine samples were fortified with 1-phenoxy-2-propanol and acid hydrolysed in the same manner to verify the stability of the parent compound under these hydrolysis conditions. Hydrolysed urine samples were analysed as above for quantitation and identification of acid-labile conjugated metabolites.

A standard solution containing 1-phenoxy-2-propanol and two of the possible metabolites (phenol and hydroquinone) were separated by HPLC equipped with UV and radiochemical detectors. These chromatograms were used for retention time match of phenol and hydroquinone in unchanged (non-acid-hydrolysed) and acid-hydrolysed urine samples.

Results

Absorption and excretion

Table 2 presents the recovery of the administered 1-phenoxy-2-propanol in urine, faeces and final cage wash. The administered doses were absorbed relatively rapidly from the gastrointestinal tract, with 83–91% (85 ± 2% and 90 ± 1% of the low and high doses, respectively) of the dose absorbed and excreted in urine within 12 h after administration. Almost all the administered dose was excreted in urine

Table 2. Recovery of the administered ^{14}C -1-phenoxy-2-propanol in urine, faeces and final cage wash after a single oral dose of 10 and 100 mg kg $^{-1}$ to rats.

Time (h)	Urine (% dose)		Faeces (% dose)		Final cage wash (% dose)	
	10 mg kg $^{-1}$	100 mg kg $^{-1}$	10 mg kg $^{-1}$	100 mg kg $^{-1}$	10 mg kg $^{-1}$	100 mg kg $^{-1}$
0-12	85.40 $\pm$ 2.06	89.86 $\pm$ 1.26	—	—	—	—
0-24	—	—	6.34 $\pm$ 1.69	5.12 $\pm$ 0.19	—	—
12-24	6.85 $\pm$ 3.86	3.31 $\pm$ 2.21	—	—	—	—
24-48	1.03 $\pm$ 0.54	2.71 $\pm$ 1.71	0.74 $\pm$ 0.39	0.54 $\pm$ 0.12	—	—
Total	93.29 $\pm$ 5.27	95.87 $\pm$ 2.76	7.08 $\pm$ 1.35	5.65 $\pm$ 0.13	2.56 $\pm$ 3.29	1.50 $\pm$ 0.75

Data are mean $\pm$ standard deviation, derived from three animals.

—, Sample not collected.

within 48 h, accounting for 93 $\pm$ 5% of the low and 96 $\pm$ 3% of the high dose (table 2). Faecal excretion accounted for <10% of the administered dose (7 $\pm$ 1% of the low, 6 $\pm$ 0.1% of the high dose). Rats eliminated virtually all the administered dose within 48 h. Mean recovery of the administered dose was 103 $\pm$ 1% for the low dose group and 103 $\pm$ 3% for the high dose group (table 2).

Radiochemical profile of unchanged (non acid-hydrolysed) urine

Figure 1A shows a representative HPLC radiochromatogram of 0-12-h urine sample from the 100 mg kg $^{-1}$ dosed animals. The distribution of radiolabelled metabolites present in pooled urine samples (as per cent of the administered dose) is presented in table 3. A total of 10 and 11 radioactive peaks were found in the chromatograms of 0-12-h urine samples from animals administered 10 and 100 mg kg $^{-1}$ 1-phenoxy-2-propanol, respectively (table 3). Only four of these chromatographic peaks (Group E/F, I, K and L) contained > 5% of the administered dose. The major chromatographic peak(s), labelled Group E/F, was found to contain several components via mass spectral analysis. However, these components were not resolvable with the separation conditions employed here. The per cent dose found in this group of chromatographic peaks was therefore expressed as one value (figure 1A and table 3). The recovery of radioactivity injected onto the HPLC system was 103 $\pm$ 10% (data not shown).

Radiochemical profile of acid-hydrolysed urine

A representative HPLC ^{14}C -chromatogram of acid hydrolysed urine (0-12 h, 100 mg kg $^{-1}$ dosed rats) and an HPLC/UV chromatogram of a standard containing 1-phenoxy-2-propanol, phenol and hydroquinone are shown in figure 1B, C. Upon acid hydrolysis, an average of 1.5% of the administered dose was tentatively identified as hydroquinone, 61% as phenol and 13% as parent 1-phenoxy-2-propanol (confirmed by chromatographic retention times only). The remaining radioactivity in the acid-hydrolysed urine was distributed among seven other minor chromatographic peaks ranging from 0.8 to 3.1% of the administered dose (figure 1B). 1-Phenox-2-propanol was stable under the conditions of acid hydrolysis employed here (data not shown).

Identification of metabolites

The 0-12- and 12-24-h, non-acid-hydrolysed urine samples were analysed by LC/ESI/MS and LC/ESI/MS/MS to characterize the metabolites

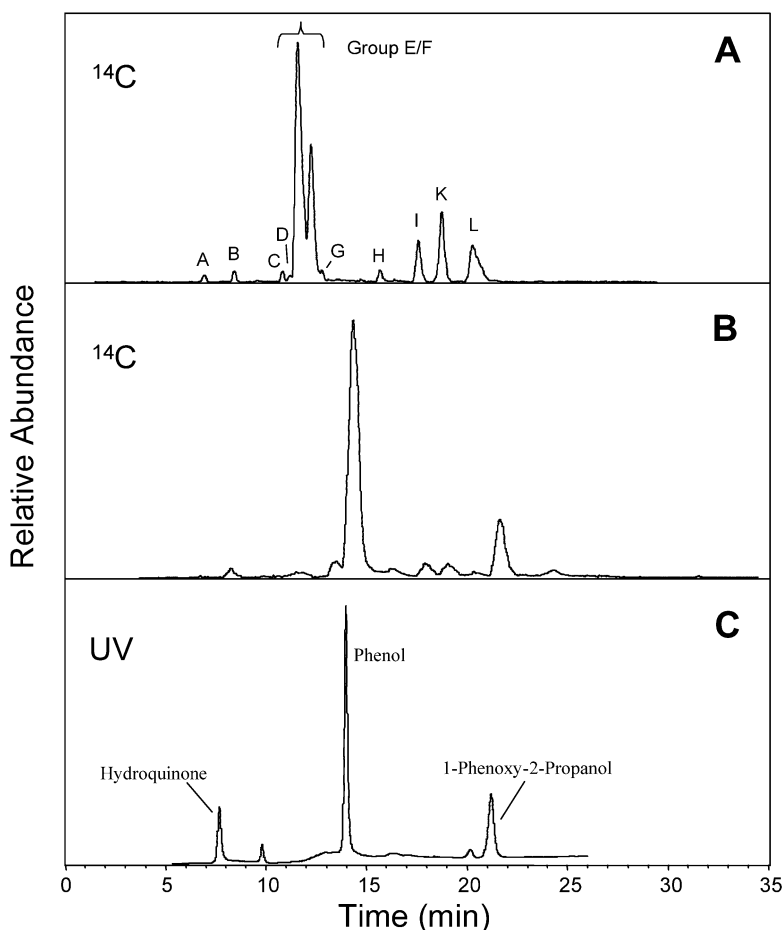


Figure 1. Representative HPLC radiochromatograms of 0–12-h pooled urine collected from rats administered a single oral dose of 100 mg kg^{-1} ^{14}C -1-phenoxy-2-propanol before (A) and after (B) acid hydrolysis. (C) HPLC/UV chromatogram of a standard solution containing 1-phenoxy-2-propanol, phenol and hydroquinone.

of 1-phenoxy-2-propanol. The ESI positive-ion mass spectrum of peak A afforded an $[\text{M} - \text{H}]^-$ molecular ion of m/z 285 (data not shown). This peak did not afford a useable product ion spectrum upon ESI-LC/MS/MS analysis. However, based on the molecular weight of 286, the structure of this metabolite was tentatively assigned to be the glucuronide conjugate of hydroquinone. The region labelled Group E/F, representing 61–65% of the dose with a retention time of 12–14 min (figure 1A), contained four metabolites. The sulphate conjugate of phenol was tentatively identified by an $[\text{M} - \text{H}]^-$ molecular ion at m/z 173 and product ions at m/z 93 and 80, via negative-ion LC/ESI/MS/MS analysis (figure 2). The glutathione conjugate of phenol was tentatively identified by positive-ion LC/ESI/MS/MS analysis (figure 3) with quasi-molecular ion at m/z 400 ($[\text{M} + \text{H}]^+$) and product ions at m/z 383 and 209. The sulphate conjugate of a ring-hydroxylated 1-phenoxy-2-propanone was tentatively identified by negative-ion LC/ESI/MS/MS analysis, with the quasi-molecular ion at m/z 245 ($[\text{M} - \text{H}]^-$) and a

Table 3. Distribution of radioactivity (per cent dose in chromatographic peaks) in non-acid-hydrolysed rat urine after a single oral dose of 10 and 100 mg kg⁻¹ 1-phenoxy-2-propanol.

Peak	Per cent of administered dose/peak			
	10 mg kg ⁻¹		100 mg kg ⁻¹	
	0–12 h	12–24 h	0–12 h	12–24 h
A	0.43	ND	0.74	ND
B	1.52	ND	1.03	ND
C	ND	ND	0.87	ND
D	ND	ND	0.46	ND
Group E/F	55.94	4.21	61.11	2.89
G	0.91	ND	0.86	ND
H	0.94	ND	1.24	ND
I	3.86	0.34	5.40	ND
J	0.83	ND	ND	ND
K	7.25	0.63	9.38	ND
L	9.31	0.71	8.78	0.42
Sum	80.99	5.88	89.86	3.31

ND, not detected above the detection limit of 0.3% of the dose.

Data are derived from pooled urine samples from three rats.

Variations in the total per cent dose recovered as metabolites in urine were within the expected range of the instrumental (HPLC) recovery (see Results for details).

major product ion at m/z 188, which corresponds to loss of the propanone moiety (figure 4). The sulphate conjugate of a ring-hydroxylated 1-phenoxy-2-propanol was tentatively identified by negative-ion LC/ESI/MS/MS analysis (figure 5), with the quasi-molecular ion at m/z 247 ($[M - H]^-$) and a major product ion at m/z 188, corresponding to loss of propanol moiety.

Peaks I and K (4–5 and 8–9% of the dose, respectively) had comparable full-scan and product-ion mass spectra. A representative negative product ion mass spectrum of peak I is shown in figure 6. A quasi-molecular ion at m/z 327 ($[M - H]^-$) is present along with several fragments resulting from loss of various groups from the glucuronide ring. The corresponding mass spectra of peak K (not shown) were indistinguishable from those of peak I shown in figure 6. These data support tentative identification of peaks I and K as isomers of the glucuronide conjugate of 1-phenoxy-2-propanol.

Peak L accounted for 9–10% of the administered dose. The product ion spectrum, in negative-ion mode, shown in figure 7, contains a quasi-molecular ion at m/z 231 ($[M - H]^-$), with major product ions present at m/z 93 and 137. These data support tentative identification of peak L as the sulphate conjugate of 1-phenoxy-2-propanol.

Analysis of the unchanged urine by LC/ESI/MS and LC/ESI/MS/MS, together with the results from analysis of acid hydrolysed urine, suggests that the majority of urinary excreted radioactivity was either conjugated parent 1-phenoxy-2-propanol or phenol (figure 8). In addition, some ring-hydroxylated parent was also present in urine. No free 1-phenoxy-2-propanol or phenol were detected in any of the non-hydrolysed urine samples. Analysis of the acid-hydrolysed urine indicated that majority of the metabolites excreted in urine were conjugates of phenol.

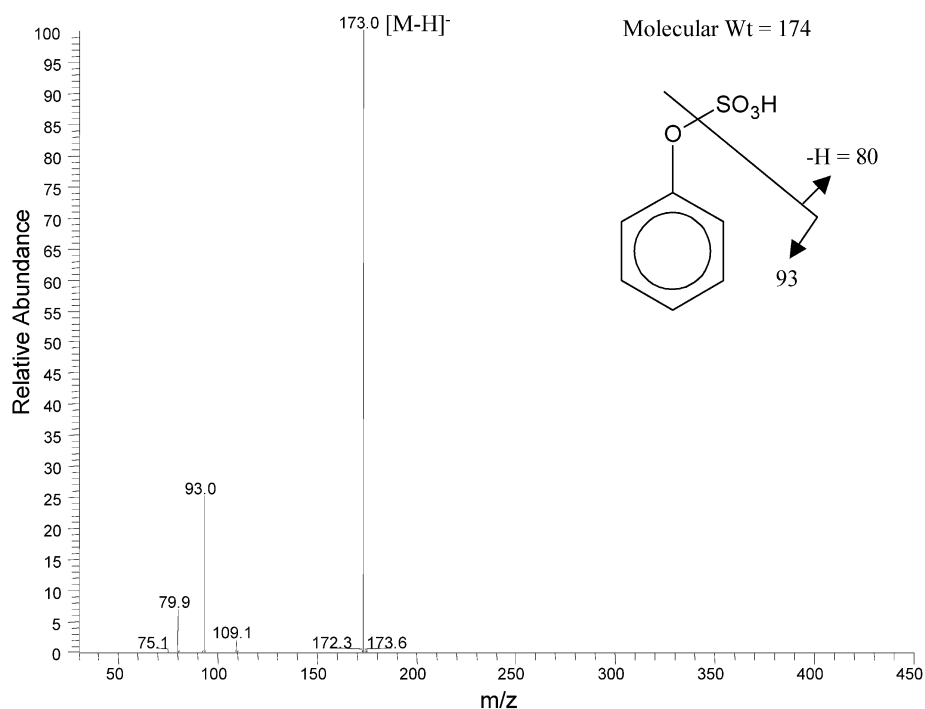


Figure 2. Negative-ion MS/MS product ion spectrum of a component of Group E/F, tentatively identified as the sulphate conjugate of phenol (product ions of $[M - H]^-$ m/z 173).

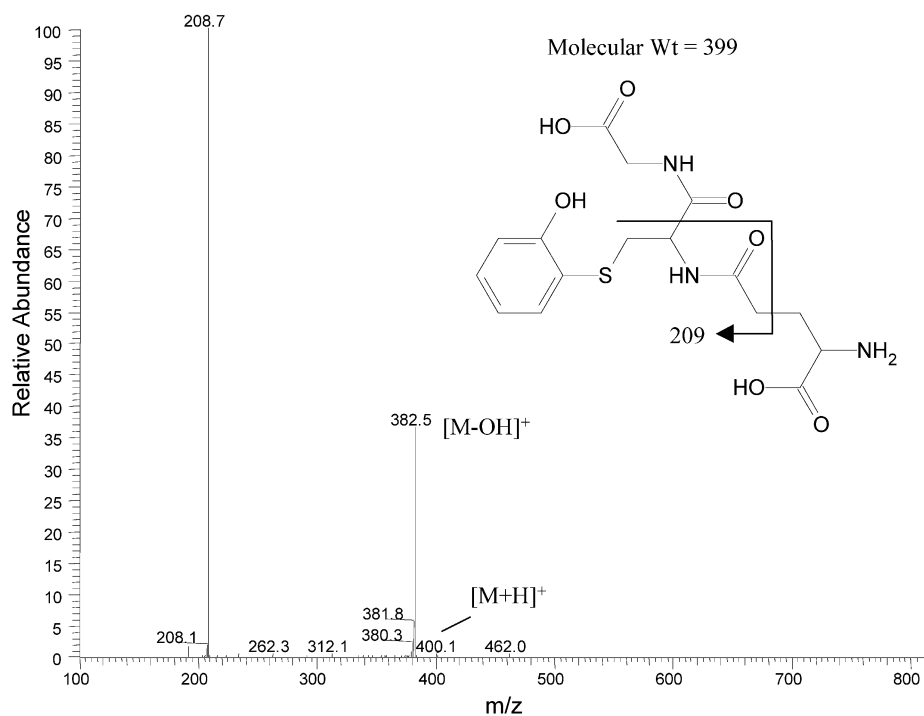


Figure 3. Positive-ion MS/MS product ion spectrum of a component of Group E/F, tentatively identified as the glutathione conjugate of phenol (product ions of $[M + H]^+$ m/z 400).

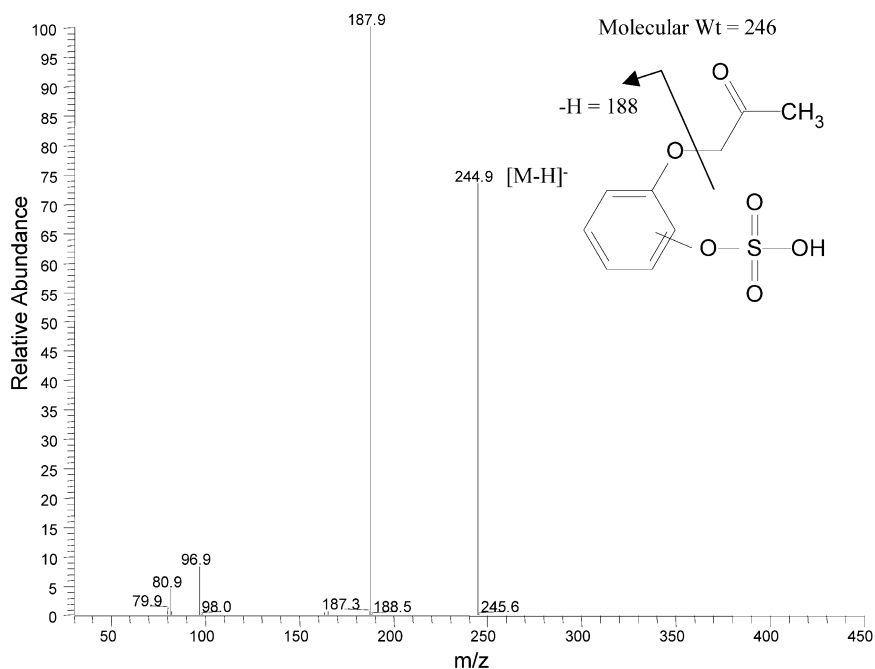


Figure 4. Negative-ion MS/MS product ion spectrum of a component of Group E/F tentatively identified as the sulphate conjugate of a ring-hydroxylated 1-phenoxy-2-propanone (product ions of $[M - H]^-$ m/z 245).

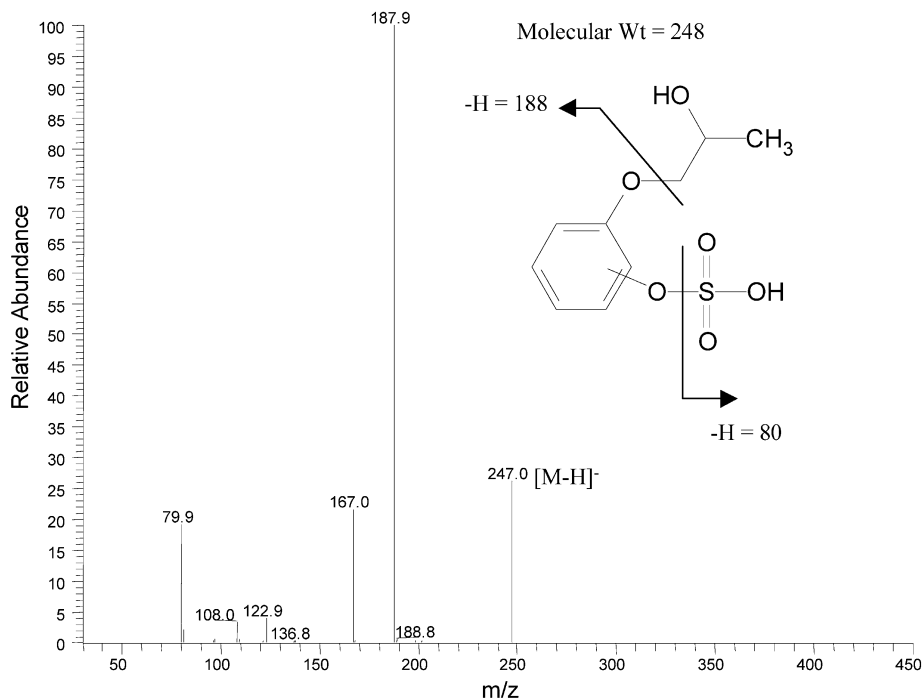


Figure 5. Negative-ion MS/MS product ion spectrum of Group E/F tentatively identified as the sulphate conjugate of a ring-hydroxylated 1-phenoxy-2-propanol (product ions of $[M - H]^-$ m/z 247).

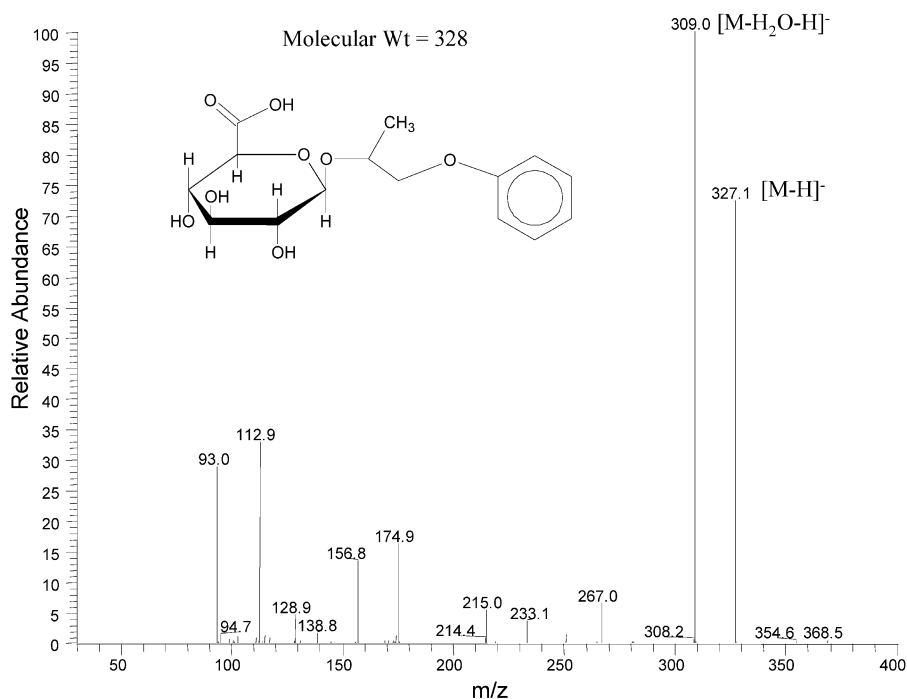


Figure 6. Negative-ion MS/MS product ion spectrum of peak I (also representative of the corresponding mass spectrum of peak K) tentatively identified as the glucuronide conjugate of 1-phenoxy-2-propanol (product ions of $[M - H]^-$ m/z 327).

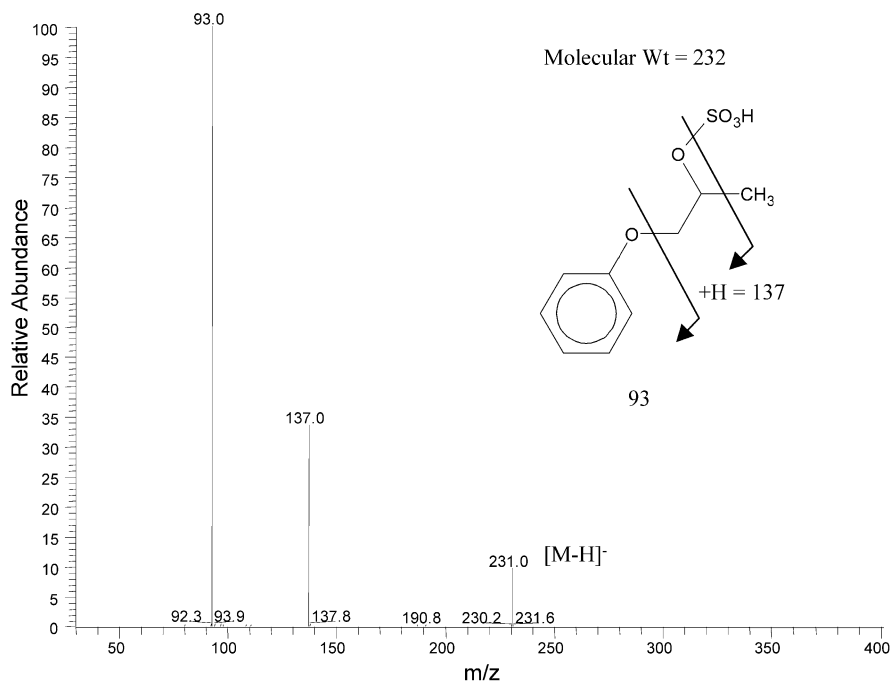


Figure 7. Negative-ion MS/MS product ion spectrum of peak L tentatively identified as the sulphate conjugate of 1-phenoxy-2-propanol (product ions of $[M - H]^-$ m/z 231).

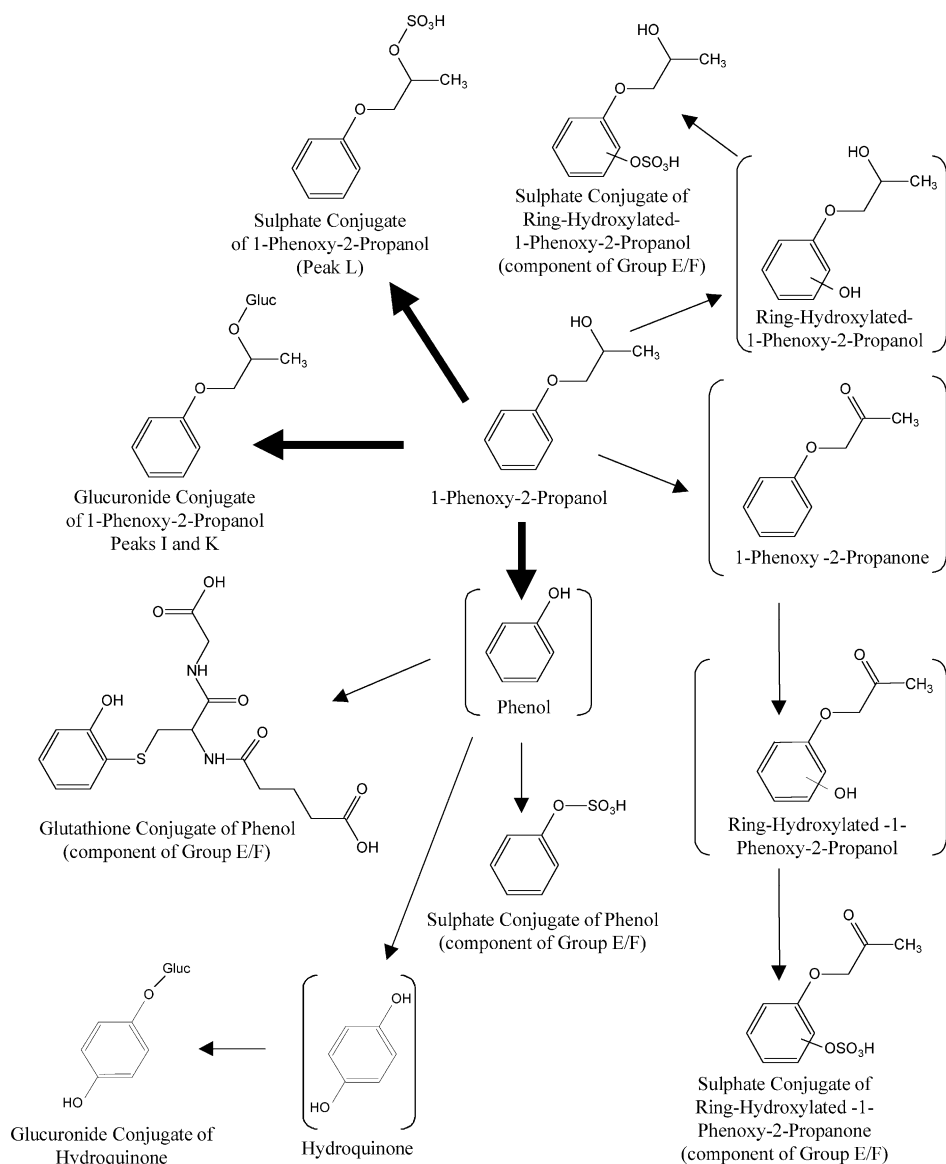


Figure 8. Proposed pathway of metabolism of 1-phenox-2-propanol in rats.

Discussion

1-Phenox-2-propanol was rapidly absorbed and eliminated after oral administration to rats. Within 12-h post-exposure, more than 80% of the administered radioactivity was excreted in urine. Initial O-dealkylation affords primarily phenol, which was almost completely conjugated before excretion. The pattern of rapid urinary excretion was similar to what has been reported for phenol (Capel *et al.* 1972, Kao *et al.* 1979, Edwards *et al.* 1986, Hughes and Hall 1995). Although not determined, residual accumulation of the administered 1-phenox-2-propanol in tissues would be extremely low as virtually all

($103 \pm 2\%$) of the administered radioactivity was recovered in excreta within 48 h after dosing. Faecal excretion of 1-phenoxy-2-propanol was between 6 and 8% of the administered dose. Most of the faecal excretion occurred within first 24 h after dosing.

Much of the absorbed 1-phenoxy-2-propanol was rapidly *O*-dealkylated to form phenol and propylene glycol. The generated phenol was rapidly conjugated, primarily with sulphate and glutathione; similar to the earlier reported major metabolites of phenol (Capel *et al.* 1972, Hughes and Hall 1995). Although somewhat novel, the tentative identification of an intact glutathione conjugate of phenol as a urinary metabolite is consistent with previous reports of intact glutathione conjugates of other xenobiotics (Domoradzki *et al.* 1996, Mutlib *et al.* 1999, Bartels *et al.* 2003). A glucuronide conjugate of hydroquinone was also observed, consistent with further hydroxylation and conjugation of metabolic phenol (Capel *et al.* 1972, unpublished data). The amount of conjugated hydroquinone formed after oral administration of 1-phenoxy-2-propanol was significantly lower than the amount formed after oral dosing of phenol to rats; twofold lower than at 25 mg kg^{-1} (Capel *et al.* 1972) and more than 10-fold lower than at 150 mg kg^{-1} (unpublished data). Conjugation of the 1-phenoxy-2-propanol and metabolically generated phenol was complete as no free 1-phenoxy-2-propanol or phenol were found in urine. As there was no radiolabel tracer on the propylene glycol moiety of the compound, the fate of that metabolite could not be tracked. However, elimination of radiolabelled propylene glycol has been reported to be 50–60% in exhaled air as $^{14}\text{CO}_2$ and 10–20% in urine (Miller *et al.* 1983). Approximately 10% of the administered radioactivity was found in urine in the form of sulphate and glucuronide conjugates of parent compound. Two other metabolites, sulphate conjugates of ring-hydroxylated 1-phenoxy-2-propanol and 1-phenoxy-2-propanone, were also found in urine. The propanone metabolite of 1-phenoxy-2-propanol is proposed to arise from oxidation of the secondary alcohol moiety to form the corresponding ketone metabolite (1-phenoxy-2-propanone), which was further metabolized through aromatic hydroxylation, followed by conjugation with sulphate.

In summary, 1-phenoxy-2-propanol was extensively absorbed after oral administration. 1-Phenoxy-2-propanol was predominantly metabolized to phenol (about 60% of the administered dose), which was subsequently conjugated with sulphate and glutathione and the conjugates rapidly excreted in the urine. The remainder of the administered 1-phenoxy-2-propanol was primarily conjugated with sulphate and glucuronic acid or underwent aromatic hydroxylation and subsequent conjugation. 1-Phenoxy-2-propanol was rapidly absorbed, metabolized and excreted in urine within 48 h and tissue accumulation was negligible. Overall, the oral administration of 1-phenoxy-2-propanol to rats resulted in its rapid absorption, metabolism and excretion in urine, similar to other propylene glycol ethers (Miller *et al.* 1983, Breslin *et al.* 1991, Boatman 2001).

Acknowledgements

The authors gratefully acknowledge the contributions of T. J. Bell, T. L. Card, A. J. Clark, R. S. Drury, J. W. Lacher, and E. Mullen for help with the study and report.

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Almokalant glucuronidation in human liver and kidney microsomes: evidence for the involvement of UGT1A9 and 2B7

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Received 11 April 2003

1. Almokalant, a class III antiarrhythmic drug, is metabolized to form isomeric glucuronides identified in human urine. Synthesis of the total glucuronide was studied in human liver and kidney microsomes. Recombinant UDP-glucuronosyltransferases (UGTs) were screened for activity and kinetic analysis was performed to identify the isoform(s) responsible for the formation of almokalant glucuronide in man.

2. From a panel of recombinant isoforms used, both UGT1A9 and 2B7 catalysed the glucuronidation of almokalant. The K_m values in both instances were similar with 1.06 mM for the 1A9 and 0.97 mM for the 2B7. V_{max} for 1A9 was fourfold higher than that measured for UGT2B7, 92 compared with 21 pmol min⁻¹ mg⁻¹, respectively, but UGT1A9 was expressed at approximately twofold higher level than the UGT2B7 in the recombinant cell lines. Therefore, the contribution of UGT2B7 to almokalant glucuronidation could be as significant as that of UGT1A9 in man.

3. Liver and kidney microsomes displayed similar K_m values to the cloned expressed UGTs, with the liver and kidney microsomes at 1.68 and 1.06 mM almost identical to the 1A9.

4. The results suggest a significant role for UGT1A9 and 2B7 in the catalysis of almokalant glucuronidation.

Introduction

The class III antiarrhythmic drug Almokalant [(*RS*)-4-[3-ethyl[3-propylsulphonyl]-propyl]amino]-2-hydroxypropoxybenzonitrile] was removed from drug development due to adverse side-effects. Much is known about its pharmacokinetics and metabolism, and 69% of an oral dose is excreted as stereo-specific glucuronides designated M18a and M18b (figure 1), although the UDP-glucuronosyl transferase (UGT) isoforms responsible for almokalant glucuronidation have not been identified (Hoffmann and Gabrielsson 1992, Bäärnhielm and Sothell 1993, Andersson *et al.* 2001).

UGTs constitute a superfamily of microsomal membrane-bound enzymes that catalyse the transfer of a glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to an aglycone acceptor. This reaction is the major step in the metabolism of thousands of endogenous (steroids and bilirubin) and exogenous substrates (drugs and carcinogens) that contain hydroxyl, carboxyl, amine and sulfhydryl groups, enhancing their hydrophilicity and facilitating excretion

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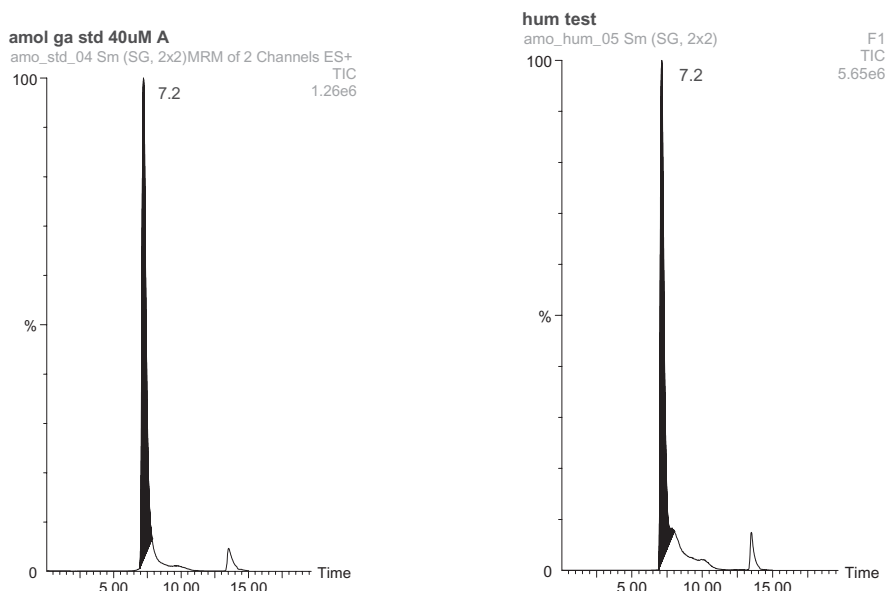
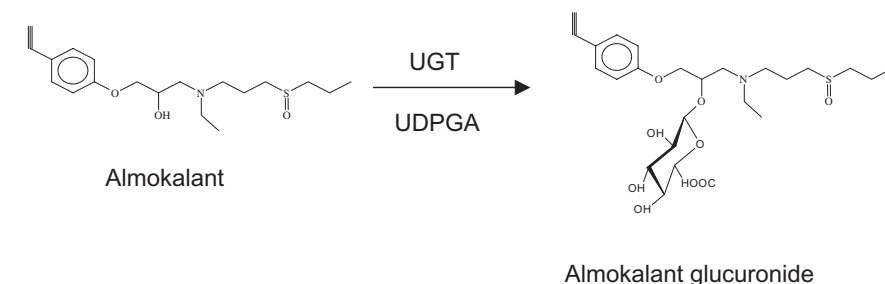


Figure 1. Glucuronidation of almokalant (upper) and (lower) comparison of the LC trace of almokalant glucuronide standard (left) with the product of almokalant incubation with human liver microsomes (right). Note that the isomeric glucuronides M18a and M18b were not separated under these LC conditions and eluted as one peak.

(Burchell *et al.* 1997). To date, 18 functional UGTs have been cloned, sequenced and divided into two subfamilies based on evolutionary divergence of their gene structure: subfamily 1 is responsible for the glucuronidation of bilirubin and phenols and subfamily 2 for that of steroids and bile acids (Mackenzie *et al.* 1997). Both subfamilies also catalyse the metabolism of a large variety of drugs (Miners and Mackenzie 1991, Burchell *et al.* 1997, Burchell 2003).

Many new chemicals fail in early development in humans because of unsatisfactory pharmacokinetic parameters (Prentis *et al.* 1988, Kennedy 1997). Gathering knowledge about the metabolism of substances that can facilitate the prediction of these parameters for newly developed drugs is therefore essential. Multiple human UGT isoforms have been cloned and expressed in various recombinant systems, and probe substrates that can be used to test the activity of individual isoforms have been identified (Burchell *et al.* 1995). This has provided a means to identify the UGT isoform(s) responsible for drug glucuronidation.

The aim was to identify and determine the kinetic parameters of the recombinant human UGT isoform(s) involved in the glucuronidation of almokalant.

Various human UGT isoforms stably expressed in Chinese hamster V79- and baculovirus-transfected SF9 cell lines were tested with a high-performance liquid chromatography (HPLC) assay using radioactive ^{14}C -UDPGA (Coughtrie *et al.* 1986, Ethell *et al.* 1998). For UGT1A9 and UGT2B7, the isoforms capable of catalysing the reaction, the K_m and $V_{\max}$ were determined and compared with those found in human liver and kidney microsomes.

Materials and methods

Chemicals and reagents

Bilirubin, 2-aminobiphenyl, hyodeoxycholic acid, 4-methyl umbelliferone, 1-naphthol, octylgallate, propofol and UDPGA were from Sigma-Aldrich (Gillingham, UK). ^{14}C -UDP-glucuronic acid, UDPGA ($\geq 303 \text{ mCi mmol}^{-1}$, radiochemical purity $> 97\%$) was purchased from NEN Dupont (Hounslow, UK). Almokalant [(*RS*)-4-[3-ethyl[3-propylsulphonyl]-propyl]amino]-2-hydroxypropoxybenzonitrile] and its metabolites ($> 94\%$ pure) were kindly provided by AstraZeneca R&D (Mölnådal, Sweden). Ammonium acetate and acetonitrile (HPLC grade) were used as the mobile phase, and all other chemicals were from Merck Eurolab (Poole, UK) and of the highest grade available.

Human samples

Human liver and kidney samples were purchased from the UK Human Tissue Bank, Leicester Royal Infirmary, UK. The local ethical committees approved the collection and use of all human tissues in this study. The liver ($n = 2$) and kidney ($n = 2$) samples were obtained from middle aged, male donors and placed in liquid nitrogen within 6 h post mortem. Wherever possible, details of medication and diagnosis were obtained for each sample (table 1). Microsomes were prepared as described by Burchell *et al.* (1989). Aliquots were stored at -80°C until required and used within 2 months.

Tissue culture

Cloning and expression of the UGT1A1, 1A3, 1A6, 1A9 and 2B7 in V79 cell lines were previously described (Fournel-Gigleux *et al.* 1991, Wooster *et al.* 1991, Sutherland *et al.* 1993). V79 cell lines were grown in Dulbecco's modified Eagles medium (DMEM) complemented with 10% foetal bovine serum, 100 units ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Gibco Life Technologies, Paisley, UK) in 75- cm^2 flasks (CoStar, Cambridge MA, USA) fitted with vented caps in humidified incubators at 37°C with the atmosphere maintained at 5% CO_2 . V79 heterologous-expressing human UGTs were maintained under constant selection concentrations of geneticin (G418, Gibco). Cells were rinsed twice with prechilled phosphate-buffered saline (pH 7.4), harvested by gentle scraping of the monolayer into 2.5 ml ice-cold PBS. Cells in suspension were pelleted by centrifugation at 1000g for 3 min and stored at -80°C and used within 2 months of harvesting.

UGT1A4 baculovirus was kindly provided by Robert Tukey (La Jolla, CA, USA). Briefly, SF9 cells (2×10^6) had previously been transfected with 3 μg UGT1A4pBluebac, 1 μg linear AcMNPV DNA and 20 μl cationic liposome solution in 1 ml Grace's media with no supplements for 1 h at room temperature. TNM-FH insect media + 10% foetal calf serum (Sigma Chemical Co., St Louis, MO, USA) were then added to the cells and incubated at 27°C , and the primary virus stock was collected at 48 h post-transfection. To obtain pure recombinant UGT1A4 baculovirus, several rounds of plaque purification were performed. SF9 cells in a 60-mm tissue culture dish were infected with serial dilution of primary stock virus. Virus solution was removed at 1 h post-infection, and the cells were overlaid with TNM-FH insect media + 10% foetal calf medium containing 1.5% low melting Sea Plaque agarose and 150 $\mu\text{g ml}^{-1}$ isopropyl- β -D-thiogalactopyranoside. Cells were incubated at 27°C for 4–6 days, and isolated recombinant plaques (visualized as blue) were expanded to produce a stock of UGT virus. For protein expression, a multiplicity of infection of 10 plaque-forming units was used to infect SF9 cells

Table 1. Characterization of patient samples.

Sample	Sex	Age (years)	Race	Diagnosis	Medication
HLM1	male	49	Caucasian	bowel adenocarcinoma	none
HLM2	male	49	Caucasian	colorectal carcinoma	codeine phosphate, warfarin
HKM1	male	22	Caucasian	intracranial bleed	pitressin, T3, insulin, DDAP
HKM2	male	46	Caucasian	intracranial bleed	noradrenaline, cefuroxime

(3.5×10^7) in 150 cm² flasks (CoStar) fitted with vented caps at 27°C. Cells were collected at 72 h post-infection and washed with ice-cold PBS (pH 7.4). Cells in suspension were pelleted by centrifugation at 1000g for 3 min and stored at -80°C and used within 2 months of harvesting.

Enzyme assays using [¹⁴C] UDPGA

Glucuronidation of almokalant and reference substrates, bilirubin or octyl gallate (UGT1A1), 4-methyl umbelliferone (UGT1A3), 2-aminobiphenyl (UGT1A4), 1-naphthol (UGT1A6), propofol (UGT1A9) and hydoxycholeic acid (UGT2B7) in human liver and kidney microsomes and recombinant human cell lines was performed by the method described by Ethell *et al* (1998). The protein was sonicated for four 5-s bursts (Soniprep 150), allowing 1 min on ice between bursts. Control substrates (1 mM, except bilirubin 122 µM) and almokalant (5 mM) were added to a mixture containing 350 µg of the sonicated microsomes or cells, 100 mM Tris/maleate buffer, pH 8.5, 10 mM saccharic acid 1,4-lactone and 5 mM MgCl₂. After 3-min pre-incubation at 37°C, the reaction was started by adding 2 mM (cell lines) or 5 mM (microsomes) UDPGA (0.2 µCi ¹⁴C-UDPGA per assay) in a final volume of 100 µl per assay. Incubations with the reference substrates and almokalant were for 1 and 2 h, respectively. The assay occurred at linear rates under these conditions. The incubations were terminated by the addition of 100 µl methanol prechilled to -20°C to precipitate the protein. Samples were then centrifuged at 14000g for 10 min to remove the proteins. The supernatant was transferred into a HPLC vial, and 150 µl of this volume were directly injected onto a gradient HPLC using solid scintillant radioactive detection as described by Ethell *et al*. (1998).

Kinetic determinations of UGT activities

Substrate solutions were prepared and diluted on the day of the assay. Typically, the almokalant concentrations used were 50, 100, 250, 500, 750, 1000, 1250, 1875, 2500, 3750, 5000 and 7500 µM. Apparent K_m values were determined at an optimum pH 8.5 for this substrate and at protein concentrations and reaction times yielding linear glucuronide formation. Kinetic parameters — V_{max} , K_m and intrinsic clearance (Cl_{int}) — were calculated by applying the Michaelis-Menten equation to the experimental data (duplicate incubations at 12 concentrations) by using non-linear least-squares regression method (Prism Version 2, GraphPad Software, San Diego, CA, USA). All data were quoted at an appropriate level of accuracy. Almokalant kinetic assays were incubated with human tissue microsomes and UGT1A9 and UGT2B7 recombinant cell lines for 2 and 3 h, respectively.

Identification of almokalant glucuronide produced in enzyme incubations by liquid chromatography-mass spectrometry (LC/MS-MS)

The identification of almokalant glucuronide was confirmed by LC/MS-MS using metabolite standards provided by AstraZeneca. The LC for the analysis of almokalant glucuronide was performed on a HP1100 (Agilent Technologies, Stockport, UK). The column used was 2.1 × 150 mm spherisorb ODS2 column (Waters, Watford, UK) at a flow rate of 0.3 ml min⁻¹ with a one-to-five split going to the MS interface. The mobile phase comprised of 0.1% formic acid in water (v/v) with an increasing gradient of acetonitrile (with 0.1% formic acid) from 0 to 100% over 10 min, with suitable re-equilibration.

Incubation of almokalant with human liver microsomes was performed under the same conditions as the radiolabelled assays above, except in respect of using only non-radioactive UDPGA. Detection of the almokalant glucuronide was achieved with injection of 10 µl of a 10-fold dilution of the supernatant onto the LC/MS-MS system.

The glucuronide peak from the LC column was analysed by electrospray mass spectrometry with a Quattro LC (Micromass, Manchester, UK). The spectrum of the almokalant glucuronide was analysed in positive-ion mode over a mass range of 200–600 m/z and a scan speed of 2 s per spectrum. The spectrum was generated with a cone voltage of 40 eV and a capillary voltage of 3.5 eV. The desolvation and the source block temperatures were 250 and 80°C, respectively. The nebulizing gas was applied at a flow of 100 l h⁻¹, the desolvation gas at 500 l h⁻¹.

The metabolite spectrum was confirmed by LC/MS-MS analysis of the daughter ions of the parent glucuronide mass of 529.4⁺. MS settings were as above with the collision energy at 20 eV and collision gas at a pressure of 2.5×10^{-3} mbar.

Immunoblotting of UGTs

SDS-PAGE was performed according to the methods of Laemmli (1970) using mini-gel electrophoresis equipment (Bio-Rad, Hercules, CA, USA). V79-1A9 and V79-2B7 (0–50 µg/lane) were loaded onto 9% polyacrylamide gels. The gels were subject to 50 V through the stack and 100 V through the main resolving section. The electrophoretic transfer to PVDF membrane (Millipore Corporation, Bedford, MA, USA) was carried out overnight at 27 V according to the methods of Towbin *et al*. (1979). Non-specific binding sites were blocked in 5% (w/v) milk powder in TBST buffer (10 mM Tris, 154 mM NaCl, 0.05% (w/v) Tween 20, pH 9.0) for 1 h. Detection of recombinant proteins was carried out using antibodies raised against rat liver UGT that recognized several UGT isoforms (Coughtrie

et al. 1987). This polyclonal antibody interacts with the common region of all UGTs, and therefore binding to each UGT will be similar. The membrane was then incubated with the primary antibody diluted 1:2500 in TBST/5% (w/v) milk for 3 h followed by 4×5 -min washing steps with TBST buffer alone. It was then incubated in secondary antibody (rabbit anti-sheep peroxidase conjugated IgG; Pierce Biotechnology, Rockford, IL, USA) diluted 1:20 000 in TBST/5% (w/v) milk for 1 h followed by 4×10 -min washes as above. The membrane was then covered in 1 ml chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA, USA) for 1 min and exposed to autoradiography film for 6 min. The autoradiographs were developed for 1 min, washed briefly in water, fixed for 1 min, washed in running water for 5 min and then allowed to air dry. The apparent molecular weights of any bands were determined by comparison with standard proteins of known molecular mass (Invitrogen, Paisley, UK). The intensity of the signals revealed on immunoblots was analysed by densitometry (Hewlett Packard, Stockport, UK) and quantified with dedicated software (Biosoft, Cambridge, UK). The intensity of the signal as a function of the amount of protein was linear for a concentration of range of 0–50 μ g protein loaded on the gel with this primary antibody.

Protein determinations

Protein concentrations in the microsomal preparations and recombinant cellular sonicates were determined according to Lowry *et al.* (1951). Standards were made from bovine serum albumin and samples were diluted 1:200 for the protein assay.

Results

Mass spectral analysis of almokalant glucuronide formed in UGT assays

The elution profile of the glucuronide formed by *in vitro* incubation of almokalant was chromatographically identical to the synthesized reference metabolites, M18a provided by AstraZeneca (figure 1). The fragmentation of the parent ion of the product of almokalant incubated with microsomes showed an identical mass spectrum to the authentic almokalant glucuronide standard, with fragments attributable to the parent glucuronide (figure 2). Assays incubated in the absence of UDPGA had no almokalant glucuronide peak present (data not shown).

Formation of almokalant glucuronide by human tissue microsomes

The human tissue microsomes used were characterized by assaying with reference marker substrates of known specificity to demonstrate that they were competent for glucuronidation activity (table 2). In agreement with previous reports, glucuronidation of general marker substrates is considerably lower in human kidney than in liver microsomes (table 2). UGT1A9 activity levels towards propofol are higher (about threefold) in kidney than liver and UGT1A1 activity towards bilirubin is absent from human kidney (table 2).

Screening and kinetic analysis of human liver and kidney microsomes showed that both organs were capable of glucuronidating almokalant (table 2) and had K_m values in the same range: 1.68 mM (HLM) and 1.06 mM (HKM). The difference in glucuronidation rate between liver ($676 \text{ pmol min}^{-1} \text{ mg}^{-1}$) and kidney ($268 \text{ pmol min}^{-1} \text{ mg}^{-1}$) resulted in similar intrinsic clearances for almokalant glucuronide by these organs with $0.40 \text{ } \mu\text{l min}^{-1} \text{ mg}^{-1}$ in HLM and $0.25 \text{ } \mu\text{l min}^{-1} \text{ mg}^{-1}$ in HKM.

Formation of almokalant glucuronide catalysed by expressed UGT isoforms

Screening of six recombinant V79 cell lines expressing major isoforms involved in drug glucuronidation, UGT1A1, 1A3, 1A6, 1A9, 2B7 and SF9 cells expressing UGT1A4, revealed that only UGT1A9 and 2B7 were capable of forming almokalant glucuronide (table 3).

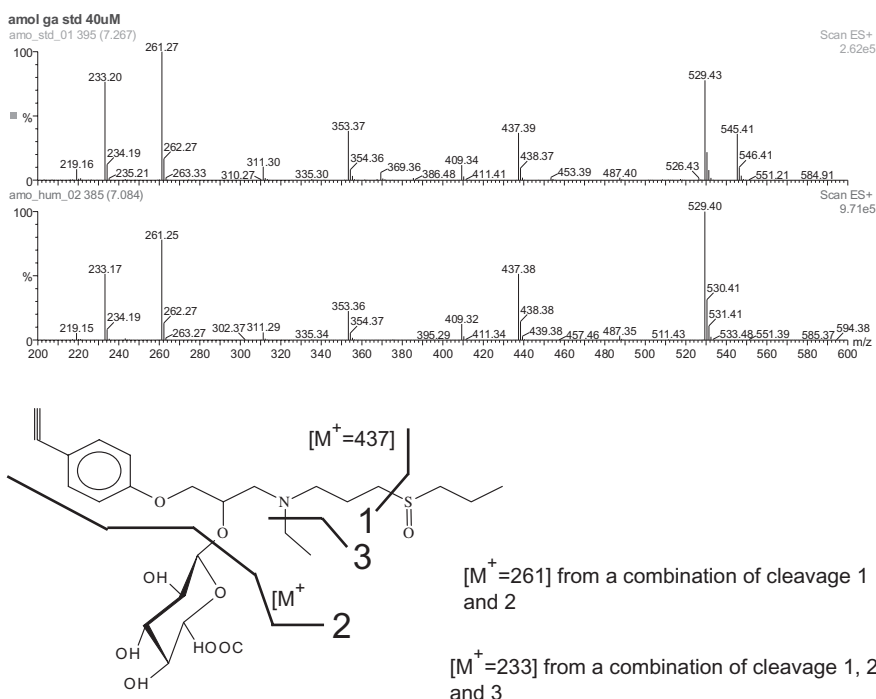


Figure 2. Mass spectra of almokalant (M18a) glucuronide standard (upper) and almokalant glucuronide produced from human liver microsomes (lower). The proposed collision-induced fragmentation of the almokalant glucuronide is also shown. The amokalant glucuronide initially fragments from the parent ion $[529^+]$ to lose propyl-sulfoxy (1) $[437^+]$ or to lose the glucuronide to leave the parent aglycone (2) $[353^+]$. These fragment then under go further fragmentation; $[437^+]$ fragment cleaves at (2) or $[353^+]$ fragment cleaves at (1) to give $[261^+]$. The nitrogen of the $[261^+]$ fragment also de-ethylates (3) to give $[233^+]$.

Table 2. Comparison of reference UGT substrates and almokalant with human liver and kidney microsomes.

Substrate	(UGT activity, nmol min ⁻¹ mg ⁻¹ protein)			
	HLM1	HLM2	HKM1	HKM2
Bilirubin	0.3	0.4	ND	ND
2-Aminobiphenyl	0.5	0.7	0.2	0.3
1-Naphthol	7.8	8.1	3.4	3.8
Propofol	0.4	0.5	1.4	1.5
Morphine	0.7	0.6	0.1	0.2
Almokalant	0.50 (0.7) ^ψ	0.68 (1.7)	0.33 (1.0)	0.27 (0.8)

Data are from the average of two determinations for screening purposes.

ND, below the level of detection for assay conditions (8 pmol min⁻¹ mg⁻¹).

UGT activity data were obtained using probe substrate (1 mM), except bilirubin (122 μM) and UDP-glucuronic acid (5 mM).

^ψ K_m values (mM) shown in parentheses were obtained using almokalant (0–7.5 mM) as described in the Materials and methods.

Kinetics analysis revealed UGT1A9 and UGT2B7 glucuronidate almokalant with similar K_m values of 1.06 mM (UGT1A9) and 0.97 mM (UGT2B7) to the human kidney microsomes (figure 3). However, the maximal rate of glucuronidation in the recombinant cells was four times higher with UGT1A9

Table 3. Glucuronidation of almokalant catalysed by cloned/expressed UGTs.

UGT isoform (substrate)	UGT activity ($\text{pmol min}^{-1} \text{mg}^{-1}$)		Almokalant
		Reference substrate	
1A1	(octylgallate)	571	ND
1A3	(4-methylumbelliferone)	57	ND
1A4 bv	(2-aminobiphenyl)	100	ND
1A6	(1-naphthol)	2413	ND
1A9	(propofol)	1066	26.8
2B7	(hyodeoxycholate)	32	13.4

ND, below the level of detection for assay conditions ($8 \text{ pmol min}^{-1} \text{mg}^{-1}$).

All UGT isoforms were expressed in V79 cells except for UGT1A4 bv, which was prepared from baculovirus-infected SF9 cells (see the Materials and methods).

Data are from the average of two determinations for screening before kinetic analysis (see figure 3).

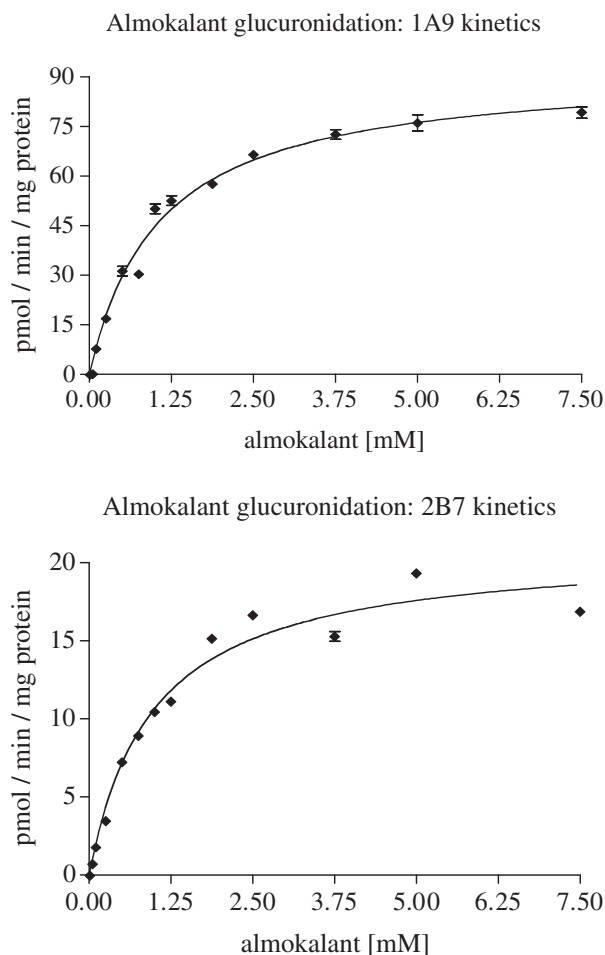


Figure 3. Kinetic analysis for the glucuronidation of almokalant by recombinant human UGT1A9 and 2B7. $K_m = 1.064 \text{ mM}$ and $V_{\max} = 92.31 \text{ pmol}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$ for UGT1A9 and $K_m =$ and 0.971 mM and $V_{\max} = 21.01 \text{ pmol}^{-1} \text{ min}^{-1} \text{ mg}$ for UGT2B7. Data are the mean of three determinations and error bars are the SEM.

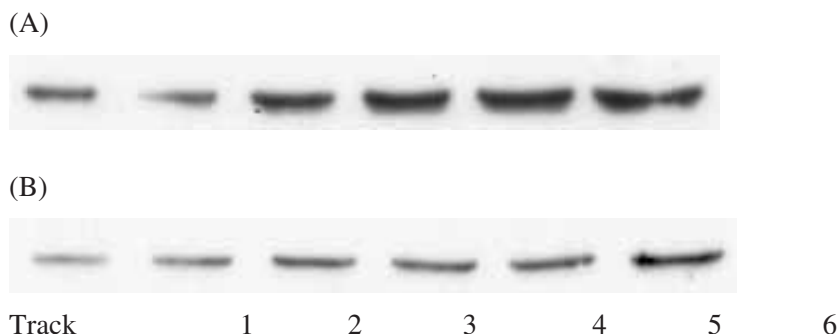


Figure 4. Quantification of (A) UGT1A9 and (B) UGT2B7 expression in V79 recombinant cell lines by immunoblotting and densitometry (see the Materials and methods). Track 1 (10 μg), 2 (20 μg), 3 (25 μg), 4 (30 μg), 5 (40 μg) and 6 (50 μg) recombinant protein. Relative expression of UGT1A9 was two fold that of UGT2B7 in V79 cell lines.

(92 $\text{pmol min}^{-1} \text{mg}^{-1}$) compared with UGT2B7 (21 $\text{pmol min}^{-1} \text{mg}^{-1}$), but was significantly lower than human tissue microsomes. Accordingly, the intrinsic clearances were 0.087 $\mu\text{l min}^{-1} \text{mg}^{-1}$ for UGT1A9 and 0.022 $\mu\text{l min}^{-1} \text{mg}^{-1}$ for UGT2B7.

Quantitation of UGT1A9 and UGT2B7 expression in V79 cells

UGT1A9 and UGT2B7 preparations used in this work were blotted to compare the relative level of expression of UGTs to the rates measured in the kinetic experiment (figure 4). Although the V_{max} for UGT1A9 was approximately fourfold higher than that measured for UGT2B7, blotting and densitometric analysis revealed that UGT1A9 was expressed at approximately a twofold higher level than the UGT2B7 in the recombinant cell lines. Therefore, the contribution of UGT2B7 would be approximately half that of UGT1A9.

Discussion

The identification of which UGT is involved in the glucuronidation of a particular xenobiotic is partially dependent on the availability of recombinant human UGT isoforms. However, the relative catalytic significance of an individual isoform is difficult to predict when multiple isoforms are involved in the glucuronidation of a compound. Comparison of simple metabolic rates is often misleading, but kinetic analysis of probe substrate glucuronidation with human recombinant enzymes in combination with human tissue microsomes provides meaningful data in estimating the contribution of each isoform.

The results of the current study show that at least two human UGT isoforms, UGT1A9 and UGT2B7, are involved in the *in vitro* glucuronidation of almokalant. The reaction with both isoforms yielded almost identical K_{m} values for UGT1A9 (1.06 mM) and UGT 2B7 (0.97 mM). Interestingly, these compare very well with the K_{m} values found for HLM (1.68 mM) and particularly HKM (1.06 mM). Propofol was used as a probe substrate for UGT1A9 in these tissues and the levels of activity towards this substrate suggest that UGT1A9 is the only isoform which has a comparable or even higher activity in human kidney than in human liver (McGurk *et al.* 1998, Soars *et al.* 2001). It is therefore likely that the

glucuronidation of almokalant in human kidney microsomes is primarily due to UGT1A9 activity. The very similar K_m values in HKM and V79-UGT1A9 cell lines support this speculation. However, the contribution of UGT2B7 to the renal glucuronidation of almokalant cannot be ruled out since it was demonstrated to be the only isoform capable of specifically glucuronidating endogenous compounds such as hyodeoxycholic acid and oestriol in human kidney (McGurk *et al.* 1998).

The difference in glucuronidation rate between liver ($676 \text{ pmol min}^{-1} \text{ mg}^{-1}$) and kidney ($268 \text{ pmol min}^{-1} \text{ mg}^{-1}$) might be attributable to the presence of another UGT isoform. The recombinant data suggest that UGT2B7 makes a significant contribution towards the glucuronidation of almokalant in human liver, but this does not rule out the possibility of other UGTs contributing to almokalant glucuronidation in liver microsomes that may not have been included in the panel of tested isoforms or may have turnovers under the detection limit of the assay ($8 \text{ pmol min}^{-1} \text{ mg}^{-1}$). However, almokalant glucuronidation by UGT1A9 and UGT2B7 obeyed Michaelis–Menten kinetics and together with the similar K_m values suggest the existence of another isoform with a very divergent K_m and a significant rate of glucuronidation is rather improbable. As the relative expression levels of UGT1A9 and UGT2B7 in liver are unknown, and the involvement of further UGT isoforms cannot be ruled out, predictions about the contribution of the two isoforms in human liver glucuronidation *in vivo* cannot be made. Soars *et al.* (2001) reported that the *in vitro* clearances were always lower than the actual *in vivo* clearances, so the intrinsic clearances of almokalant in HLM ($0.40 \mu\text{l min}^{-1} \text{ mg}^{-1}$) and HKM ($0.25 \mu\text{l min}^{-1} \text{ mg}^{-1}$) will probably be higher *in vivo*.

The maximum rate of almokalant glucuronidation in the current study was highest in HLM ($675.5 \text{ pmol min}^{-1} \text{ mg}^{-1}$). Andersson *et al.* (2001) demonstrated a similar rate of formation for the stereo-specific metabolite M18a ($658 \text{ pmol min}^{-1} \text{ mg}^{-1}$), but a significantly higher rate ($6222 \text{ pmol min}^{-1} \text{ mg}^{-1}$) for M18b. The K_m values for both metabolites, $936 \mu\text{M}$ for M18a and $1444 \mu\text{M}$ for M18b, were very close to the ones reported here. Unfortunately, the two isomeric glucuronides did not elute as two different peaks in the methods used here. Thus, further information about the kinetic parameters and also about possible stereo-specific catalysis by different UGT isoforms remains unknown and provides an opportunity for additional research.

In previous studies, a variety of substrates have been identified to be metabolized by UGT isoforms 1A9 and 2B7. UGT1A9 catalyses glucuronidation of a wide variety of bulky phenolic substrates. Those include β -adrenoceptor agonists, non-steroidal anti-inflammatory analgesics, diuretics, hydroxylated polyaromatic hydrocarbons and the anaesthetic propofol, which was used as probe substrate in this study (Bock *et al.* 1993, Ebner and Burchell 1993). The substrates for UGT2B7 include opiates, carboxylic acid drugs, hydroxylated metabolites of benzo(a)pyrene and the probe substrate hyodeoxycholic acid (Ritter *et al.* 1992, Jin *et al.* 1993). The physico-chemical structure of almokalant may predispose it to be metabolized by these isoforms.

In conclusion, of the two isoforms identified by HPLC with radiochemical detection to be involved in the metabolism of almokalant, UGT1A9 has the higher intrinsic clearance. It seems probable that the glucuronidation of this compound in human kidney is mainly catalysed by UGT1A9 due to the relative abundance of this enzyme in this organ. Although the lower relative abundance of UGT2B7 in

kidney is reflected in its contribution to renal almolokant glucuronidation, this is not the case in the liver. The percentage contribution of UGT1A9 and UGT2B7, or further isoforms to the overall glucuronidation, cannot be estimated. However, the similar K_m values and the comparatively small difference in the intrinsic clearances suggest that both isoforms do indeed play a significant role in almolokant metabolism *in vivo*.

Acknowledgements

The authors thank AstraZeneca for providing almolokant and its glucuronide standards, Robert Tukey (La Jolla, CA, USA) for providing UGT1A4 baculovirus and The Wellcome Trust for financial support.

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Transcriptional regulation of cytochrome P4503A4 gene expression: effects of inherited mutations in the 5'-flanking region

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Received 1 June 2003

1. Understanding the genetic basis of interindividual variability in drug disposition and response is a fundamental focus for rational and individualized drug treatment. Cytochrome P4503A4 (CYP3A4) has a central role in human drug metabolism and polymorphic variation has been reported in this gene.

2. This study reports the *in vitro* functional analysis of inherited mutations in the 5' flanking region of the *CYP3A4* gene using reporter constructs in which the 1141 bp proximal promoter region from the mutant alleles was inserted between a single copy of the CYP3A4 300 bp core distal enhancer (XREM) sequence and the cDNA for human secretory alkaline phosphatase.

3. Reporter constructs were co-transfected with an hPXR expression vector into human liver and intestinal cells in culture and xenobiotic modulation of *CYP3A4* promoter activity determined by chemiluminescent secretory alkaline phosphatase assay. DNA–protein interactions were next examined using electrophoretic mobility shift assays.

4. The results demonstrated that inherited mutations in the *CYP3A4* gene proximal promoter region could cause significant up-regulation of *in vitro* transcriptional activation by *CYP3A4* xenobiotic inducers. In addition, the magnitude of the effect appeared to be dependent on the cell type used in the functional assays, possibly due to the differing availability of specific intracellular transcription factors or their activating ligands.

Introduction

Cytochrome P4503A4 (CYP3A4) is the major cytochrome P450 enzyme in adult human liver and is known to metabolize many xenobiotic compounds (Watkins 1994). Its enzyme activity in liver biopsies varies by up to 40-fold in the population (Daly *et al.* 1993, Shimada *et al.* 1994, Westlind *et al.* 1999). This *in vivo* variability is generally assumed to contribute to both the low therapeutic success of some CYP3A4 drug substrates and the harmful drug interactions frequently encountered in the development and use of such compounds. In addition to modulation of drug metabolism, interindividual variation in CYP3A4 enzyme activity may also contribute to individual predispositions to some common cancers. For example, CYP3A4 metabolizes aflatoxin B₁ (Hashimoto *et al.* 1995), which is strongly implicated in the aetiology of liver

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cancer, and probably a major cause of premature death in developing countries (Henry *et al.* 1999).

The discovery of variant alleles for the drug-metabolizing enzymes CYP2A6, CYP2D6, CYP2C9 and CYP2C19, which markedly influence their activity or expression (Daly *et al.* 1993), has added impetus to the search for inherited variation in the *CYP3A4* gene, which may affect individual hepatic CYP3A4 phenotypes. Indeed, it has been proposed that most of the substantial interindividual variability in CYP3A4 activity may be attributable to genetic factors (Ozdemir *et al.* 2000). However, *CYP3A4* polymorphisms that result in functionally altered protein variants are rare (Rebbick *et al.* 1998, Dai *et al.* 2001, Hseih *et al.* 2001, Lamba *et al.* 2002, Sata *et al.* 2000), usually heterozygous and cannot alone be responsible for the high rate of interindividual variation in CYP3A4 enzyme levels.

Since hepatic CYP3A4 enzyme activity is highly correlated with CYP3A4 mRNA abundance (Kuehl *et al.* 2001), indicating that transcriptional control by both endogenous and exogenous effectors is the primary mechanism of *in vivo* regulation, functionally relevant genetic variance in the 5' regulatory region of the *CYP3A4* gene has also been considered as a possible cause of interindividual phenotypic variation (Rebbick *et al.* 1998, Westlind *et al.* 1999, Kuehl *et al.* 2001, Hamzeiy *et al.* 2002). A distal regulatory region (termed XREM) that, in conjunction with elements in the proximal promoter region, mediates the *in vitro* transcriptional activation of the *CYP3A4* gene promoter via the pregnane-X-receptor (hPXR) has been identified (Goodwin *et al.* 1999). This region, located approximately 8 kb upstream of the *CYP3A4* gene, is a complex array of transcription factor binding sites and includes at least two elements (designated dNR1 and dNR2) capable of binding hPXR/RXR α heterodimers. Site-directed mutagenesis of these elements substantially reduces *CYP3A4* xenobiotic responsiveness *in vitro*, suggesting that the XREM may be central to the regulation of *CYP3A4* gene expression *in vivo*. However, in a recent population analysis of the XREM region in over 100 subjects (Hamzeiy *et al.* 2002), no inherited DNA sequences variation was found, suggesting extensive conservation of this essential regulatory site. Thus, genetic variation in the *CYP3A4* distal XREM is not likely to be the main source of interindividual variation of *CYP3A4* expression *in vivo*.

Rebbeck *et al.* (1998) identified a relatively frequent polymorphism (about 10% in Caucasian population) in the *CYP3A4* gene proximal regulatory region, (*CYP3A4*1B*, -392 A $\rightarrow$ G) and suggested an association with increased risk of higher grade prostate cancer. In a later study, however, the *CYP3A4*1B* allele had no effect on hepatic CYP3A4 levels *in vivo* and the proposed cancer association could not be confirmed (Westlind *et al.* 1999). More recently, we have identified three novel, heterozygous proximal promoter region mutant alleles, *CYP3A4*1E* (-369 T $\rightarrow$ A), *CYP3A4*1F* (-747 C $\rightarrow$ G) and *CYP3A4*15B* (9nt insertion -845 $\rightarrow$ -844, -392 A $\rightarrow$ G and exon 6 G $\rightarrow$ A) (Hamzeiy *et al.* 2002). Both the *CYP3A4*1B* and *CYP3A4*1E* alleles have a relatively low frequency in Caucasian and Iranian populations (allele frequency 1%), whereas the *CYP3A4*1F* allele is present at a much higher frequency (20 and 18% in Caucasian and Iranian populations, respectively). We have now examined the effect of these new mutant alleles and *CYP3A4*1B* on transcriptional activation of the *CYP3A4* promoter using *in vitro* reporter gene transfection and electrophoretic mobility shift assays.

Materials and methods

Chemicals

All chemicals and reagents used in the experimental work were of molecular biology grade purchased from Sigma-Aldrich (Dorset, UK) unless specified. FuGENE 6 transfection reagent was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). All restriction enzymes and T4 DNA ligase were supplied by Promega Corporation (Madison, WI, USA).

Recombinant DNA constructs

A total of 300 bp of the *CYP3A4* XREM region (−7972/−7673) were amplified from human genomic DNA using forward (5′-cgg**acgcgt**-ACTTCATGCAAAAATGCTGG-3′) and reverse (5′-ccg**ctcagag**-GTTCTTGTGTCAGAAGTTCAGC-3′) primers that created *Mlu*I and *Xho*I restriction enzyme digestion sites, respectively (designated in **bold**) in the PCR products. (The standard numbering system designates the first nucleotide of the ATG translation start site as position +1 and the nucleotide before it as position −1. The main *CYP3A4* transcription start site is at −104.) The digested and purified PCR products were then ligated into *Mlu*I plus *Xho*I digested and purified plasmid DNA from a human secretory alkaline phosphatase cDNA-containing reporter vector (pSEAP2-1; Basic, BD Bioscience, Oxford, UK). The recombinant XREM-SEAP plasmid (designated pX-SEAP2) was transfected into *E. coli* TOP10F′ (Invitrogen, Paisley, UK) cells and transformed colonies isolated. A total of 1143 bp of the *CYP3A4* proximal promoter region (−1203/−61) was amplified from the genomic DNA of wild-type (*CYP3A4*1A*) and previously described (Hamzeiy *et al.* 2002) subjects carrying variant alleles using forward (5′-ccc**aagctt**-GACCATGCCCCATCATTTGC-3′) and reverse (5′-ccg**gaattc**-TGCTGGGCTATGTGCATGGAGC-3′) primers, creating *Hind*III and *Eco*RI restriction enzyme digestion sites, respectively in the PCR products. The digested and purified PCR products were then ligated into *Hind*III plus *Eco*RI digested pX-SEAP2 plasmid DNA, creating pXP-SEAP2 plasmids which were transfected into *E. coli* TOP10F′ cells. As the *CYP3A4* proximal promoter PCR products were amplified from genomic DNA samples in which the mutant *CYP3A4* alleles were heterozygous, DNA sequencing of inserted PCR products from individual pXP-SEAP2 plasmid clones was used in each case to identify and authenticate reporter constructs containing the mutant alleles. The human pregnane X receptor expression plasmid (pSG5-hPXRΔATG) was a gift from Dr Steven Kliewer (GlaxoSmithKline Ltd). This expression vector was generated by PCR amplification and subcloning of 1.3 kb of hPXR cDNA, modified at the first codon (CTG → ATG) for improved protein expression, into the expression vector pSG5 (Lehmann *et al.* 1998). The positive control alkaline phosphatase expression vector (pCMV-cSPAP) was a gift from Dr Steve Hood and colleagues (GlaxoSmithKline).

Cell culture and DNA transfection

Cells were routinely grown in minimal essential medium with Earl's salts supplemented with 10% foetal calf serum, glutamine, genatamycin and non-essential amino acids (Invitrogen, Paisley, UK). HepG2 (a human hepatocyte carcinoma cell line) and Caco-2 (a human colon adenocarcinoma cell line) were obtained as low passage frozen cultures from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The HuH7 human hepatocellular carcinoma cell line (Nakabayashi *et al.* 1982) was also a gift from Dr Steve Hood. Following trypsinization, cells were diluted with growth medium to a concentration of 2×10^5 cell ml^{−1} and 120 µl of cell suspension were seeded into each well of a 96-well plate (Nunc International, Leicester, UK). Plates were then incubated at 37°C for 48 h in a humidified container. FuGENE 6-mediated DNA co-transfections, using individual pXP-SEAP2 plasmid DNAs containing wild-type or mutant *CYP3A4* promoters and pSG5-hPXRΔATG plasmid DNA, were performed as described by Goodwin *et al.* (1999). Transfections were allowed to proceed for 5 h in medium from which the serum had been omitted. Cells were then cultured for an additional 60 h in fresh serum-containing medium in the presence or absence of classical CYP3A4 inducers (Goodwin *et al.* 1999).

Determination of secreted alkaline phosphatase activity

A total of 15 µl samples of culture medium from transfected and control wells were transferred into an Optiplate polystyrene 96-well plate (Packard, Pangbourne, UK) and SEAP activity measured using the AuroraTM AP Chemiluminescent Reporter Gene assay system (ICN Biochemicals, Inc., Costa Mesa, USA). An automated LumiCountTM (Packard) plate reader was used to analyse the chemiluminescence output.

Data analysis

The change in SEAP activity measured before and 60 h after addition of xenobiotic was calculated for each reporter construct to allow for variation in cell seeding, transfection efficiency and cytotoxicity or cell proliferative effects of xenobiotics that might otherwise cause anomalous results. Activation

(induction) relative to the wild-type was calculated using these values and the statistical significance relative to the DMSO control was tested as described (Plant *et al.* 2000). In the dose-response experiments, $I_{\max}$ (representing the extrapolated maximal induction, encompassing receptor-ligand, receptor-receptor, receptor translocation and receptor-DNA interactions caused by the inducer) and EC_{50} (drug concentration required to achieve half maximum induction) were calculated using non-linear regression analysis (GraphPad Prism v2.0). An indication of the overall effect of the xenobiotic (the overall inductive ability, IA) was calculated by dividing $I_{\max}$ by EC_{50} (El-Sankary *et al.* 2001).

Preparation of nuclear extracts

Nuclear protein extracts were isolated according to the protocol of Dignam *et al.* (1983). Briefly, HepG2 cells were grown to approximately 90% confluence and then collected by trypsinization. Cells were pelleted by centrifugation (1300g for 5 min) and washed twice with PBS. After the second wash, cells were resuspended in $5 \times$ packed cell volume of ice-cold PBS. Cells were pelleted, resuspended in $2 \times$ packed cell volume of buffer A (10 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and allowed to swell on ice for 10 min before disruption using a Dounce homogenizer. Nuclei were pelleted (2000g for 15 min) and resuspended $0.5 \times$ packed nuclear volume (homogenate volume-supernatant volume) of buffer C (25% glycerol, 20 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF). A total of $0.5 \times$ packed nuclear volume of high salt buffer (buffer C containing 1.2 M NaCl) was then added drop wise with swirling, and the suspension homogenized with a Dounce homogenizer. The resulting homogenate was centrifuged at 16 000g for 30 min and supernatant (nuclear protein) aliquots stored at $-80^\circ C$. Protein concentration was determined by a modification of the method of Stoscheck (1990) and integrity assessed by SDS-PAGE. Each aliquot was taken through only three freeze/thaw cycles to maintain protein integrity.

Electrophoretic mobility shift assays

Double-stranded DNA probes representing short mutation-containing DNA sequence from the variant *CYP3A4* alleles and their corresponding wild-type sequences were generated by annealing complimentary sense and anti-sense synthetic oligonucleotides (MWG-Biotech, Milton Keynes, UK). The fragments used (sense strand only shown) were as follows:

*CYP3A4*1B* (−392 A → G transition) 5'-GAGACAAGGGCAGGAGAGAGGCGAT-3' and its corresponding wild-type sequence 5'-GAGACAAGGGCAAGAGAGAGGCGAT-3'
*CYP3A4*1E* (−369 T → A transversion) 5'-TTAATAGATTATTATGCCAATG-3' and its corresponding wild-type sequence 5'-TTAATAGATTATTTTGCCAATG-3'
*CYP3A4*1F* (−747 C → G transition) 5'-GTGTACAGCACGCTGGTAGGGAC-3' and its corresponding wild-type sequence 5'-GTGTACAGCACCTGGTAGGGAC-3'.

Allele *CYP3A4*15B* was not examined owing to the nature of the mutation (9 bp insert at −844). Whereas it would be possible to detect if proteins bound to this region, the lack of an appropriate control (i.e. oligomer probe of the same length but without the insertion) would make interpretation of such data speculative at best. The protein binding reactions with ^{32}P -end-labelled wild-type DNA probes and competing unlabelled wild-type or mutant fragments were performed as described (El-Sankary *et al.* 2002). After incubation, the reaction mixtures were electrophoresed in 4% non-denaturing polyacrylamide gels. The gels were exposed overnight to Kodak X-Omat film and band intensity quantified by densitometry. Control experiments, in which nuclear protein extracts were pretreated with proteinase K or heat denatured before addition of probes, were also performed to ensure that the observed retarded bands were due to DNA-protein interactions.

Results

Transcriptional activation of reporter gene constructs

As shown in figure 1, in HepG2 liver cells the *CYP3A4*1B*, *CYP3A4*1F* and *CYP3A4*15B* promoters all produced significantly greater transcriptional activation in response to the xenobiotics used (except *CYP3A4*15B* with phenobarbital) compared with the wild-type promoter, whereas the *CYP3A4*1E* promoter showed an increase only with dexamethasone. By contrast, in HuH7 cells all of the mutant promoters (apart from *CYP3A4*15B* with phenobarbital) produced a significant increase in transcriptional activation with all xenobiotics when compared with the wild-type promoter. However, in the intestinal CaCo-2 cells, the only promoter consistently to produce greater transcriptional activation with all

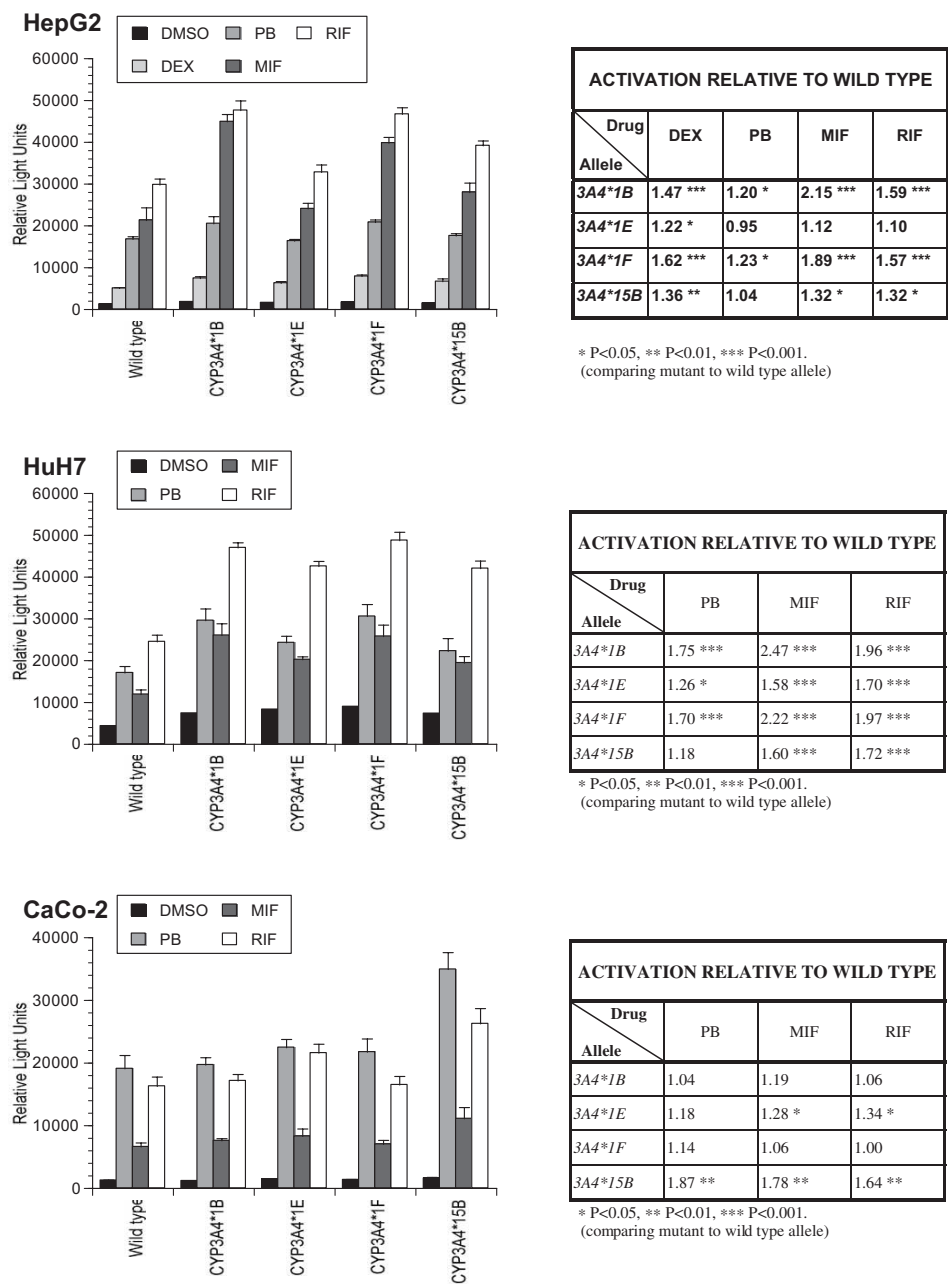
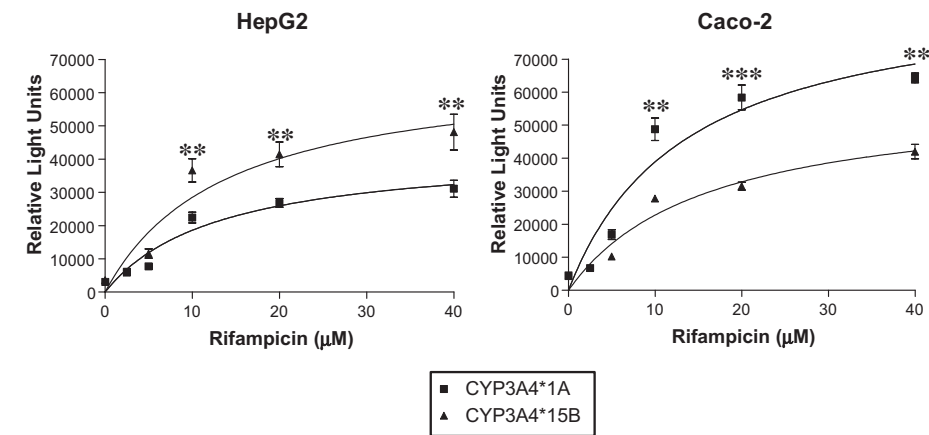


Figure 1. Activation profile of wild-type and mutant reporter gene constructs in HepG2, HuH7 and CaCo-2 cells. Cells were transfected with the wild-type (*CYP3A4*1A*) or mutation-containing pXP-SEAP2 reporter plasmid DNA in the presence of pSG5-hPXRΔATG. After DNA transfection, cells were exposed to CYP3A4 inducers at 10 μM (phenobarbital, 1 mM) and incubated for a further 60h before chemiluminescent SEAP assay. DEX, dexamethasone; PB, phenobarbitone; MIF, mifipristone; RIF, rifampicin. The results were derived from four experiments in which each transfection and exposure was performed six times ($n = 24$). Relative inductions of mutation-containing plasmid constructs compared with the wild-type are presented beside each plot. Differences were analysed by a two-sided t -test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing mutant with the wild-type.

the xenobiotics was *CYP3A4*15B*. In fact, promoter *CYP3A4*1F*, which gave the greatest increase in transcriptional activation in both of the liver cell lines, produced no significant increase in CaCo-2 cells compared with the wild-type promoter. Thus, it would appear that in this *in vitro* transfection-based assay, the recipient cell type is of critical importance for demonstrating the different responses of mutant promoters to the different CYP3A4 inducers.

As the *CYP3A4*15B* promoter consistently showed greater transcriptional activation with rifampicin compared with the wild-type in both liver and intestinal cells, it was used to determine whether this difference was present throughout the dose response curve. As shown in figure 2, significant differences between mutant and wild-type promoters were seen in both cell lines at 10 µM rifampicin and above, with the *CYP3A4*15B* activation relative to the wild-type being greater in both HepG2 and CaCo-2 cells. Subsequent analysis of the dose–response curves indicated that whereas there was a significant increase in $I_{\max}$ for the mutant promoter compared with the wild-type, there was no significant difference in EC_{50} values. The lack of change in EC_{50} values obtained for rifampicin activation of the *CYP3A4*15B* promoter compared with the wild-type would suggest that binding of activated hPXR-RXR α heterodimers to the PXREs in the two reporter constructs occurred in an unchanged manner. As the DNA sequence alterations in this allele do not modify the proximal PXRE, this is not surprising. The increase in $I_{\max}$ suggests an alteration in the *capacity* for activation (induction)



Cell line	Compound	$I_{\max}$		EC_{50}		Intrinsic Activation	
		wt	<i>3A4*15B</i>	wt	<i>3A4*15B</i>	wt	<i>3A4*15B</i>
HepG2	Rifampicin	4296 ± 780	6808 ± 1502	13.1 ± 5.7	13.9 ± 7.2	327.7	489.8
CaCo-2	Rifampicin	5903 ± 1097	9190 ± 1989	15.9 ± 6.7	13.6 ± 7.0	370.1	673.3

Figure 2. Concentration-dependent activation of the wild-type and *CYP3A4*15B* reporter constructs in different cell types. HepG2 and CaCo-2 cells were transfected with wild-type (*CYP3A4*1A*) or *CYP3A4*15B* pXP-SEAP2 plasmid DNAs in the presence of pSG5-hPXR Δ ATG. (*CYP3A4*15B* was the only mutant promoter to show transcriptional activation with the classical CYP3A4 inducers in both liver and intestinal cells.) Transfected cells were then exposed to varying concentrations of rifampicin. The results were derived from a representative experiment in which each datum point was the mean ($\pm$ SEM) of six replicate transfections. Differences were analysed by a two-sided *t*-test. * p < 0.05, ** p < 0.01, *** p < 0.001 comparing mutant with wild-type.

perhaps due to indirect modulation of *CYP3A4*15B* proximal promoter responsiveness to hPXR by the 9 bp insert at -844 in this allele.

Electrophoretic mobility shift assay

As shown in figure 3, electrophoretic mobility shifts due to protein binding were observed for wild-type ³²P-labelled synthetic double-stranded DNA fragments from the three regions of the *CYP3A4* promoter examined. The binding was specific since it could be competed out by unlabelled DNA fragments derived from the same wild-type sequences. This competition by wild-type sequences could also be reproduced using sequences derived from the corresponding regions of the *CYP3A4*1B* and *CYP3A4*1E* promoters indicating that the same proteins could still bind to the mutation-containing sequences, although perhaps with slightly altered binding affinity. In the case of DNA fragments derived from the *CYP3A4*1F* region, the wild-type sequence appeared to displace bound proteins with greater efficiency compared with the other alleles. However, with the corresponding *CYP3A4*1F* mutation-containing sequence, relatively little competition for protein(s) specifically bound to the wild-type sequence was observed, indicating that specific protein binding at the mutation site in this allele had been substantially reduced.

Discussion

To identify possible transcriptional binding motifs that might co-locate with the mutation sites in the variant *CYP3A4* alleles, context DNA sequences from wild-type and mutant promoters were analysed for protein-binding regulatory elements by screening against the TRANSFAC database using the AliBaba and MatInspector search engines. As shown in figure 4, the *CYP3A4*1B* A → G transversion is situated in the so-called NFSE element (Hashimoto *et al.* 1993) between two potential Sp-1 binding sites and thus may have relatively little effect on protein binding at these sites. However, changes in putative transcription factor binding motifs were indicated for both the *CYP3A4*1E* and *CYP3A4*1F* mutations. In the case of the *CYP3A4*1E* allele, the T → A transversion could result in altered NF-1 (nuclear factor 1) and Oct-1 (octamer 1) binding affinities at this location but the change might be small since the binding sites are modified but not eliminated. On the other hand, the bioinformatic analysis of the *CYP3A4*1F* allele, suggests that the C → G transversion could eliminate a potential Sp-1 binding site and thus cause a more dramatic change in protein binding at this location.

This analysis is in agreement with data from both the electrophoretic mobility shift and the transcriptional activation assays. In the former, protein binding and hence ability to compete with wild-type sequences was reduced substantially only in experiments with the *CYP3A4*1F* allele. In the case of the *CYP3A4*1B* and *CYP3A4*1E* alleles, little difference from wild-type was observed. The differences in protein binding ability between these three mutant promoters as indicated by EMSA are reflected by significant differences in their transcriptional activation responses to xenobiotics. However, the changes are small and hence may not have a major impact *in vivo*. Indeed, in the case of *CYP3A4*1B*, this is in agreement with the *in vivo* data (Westlind *et al.* 1999). However, all of the mutant promoters examined here appear to give greater xenobiotic activation *in vitro* than the

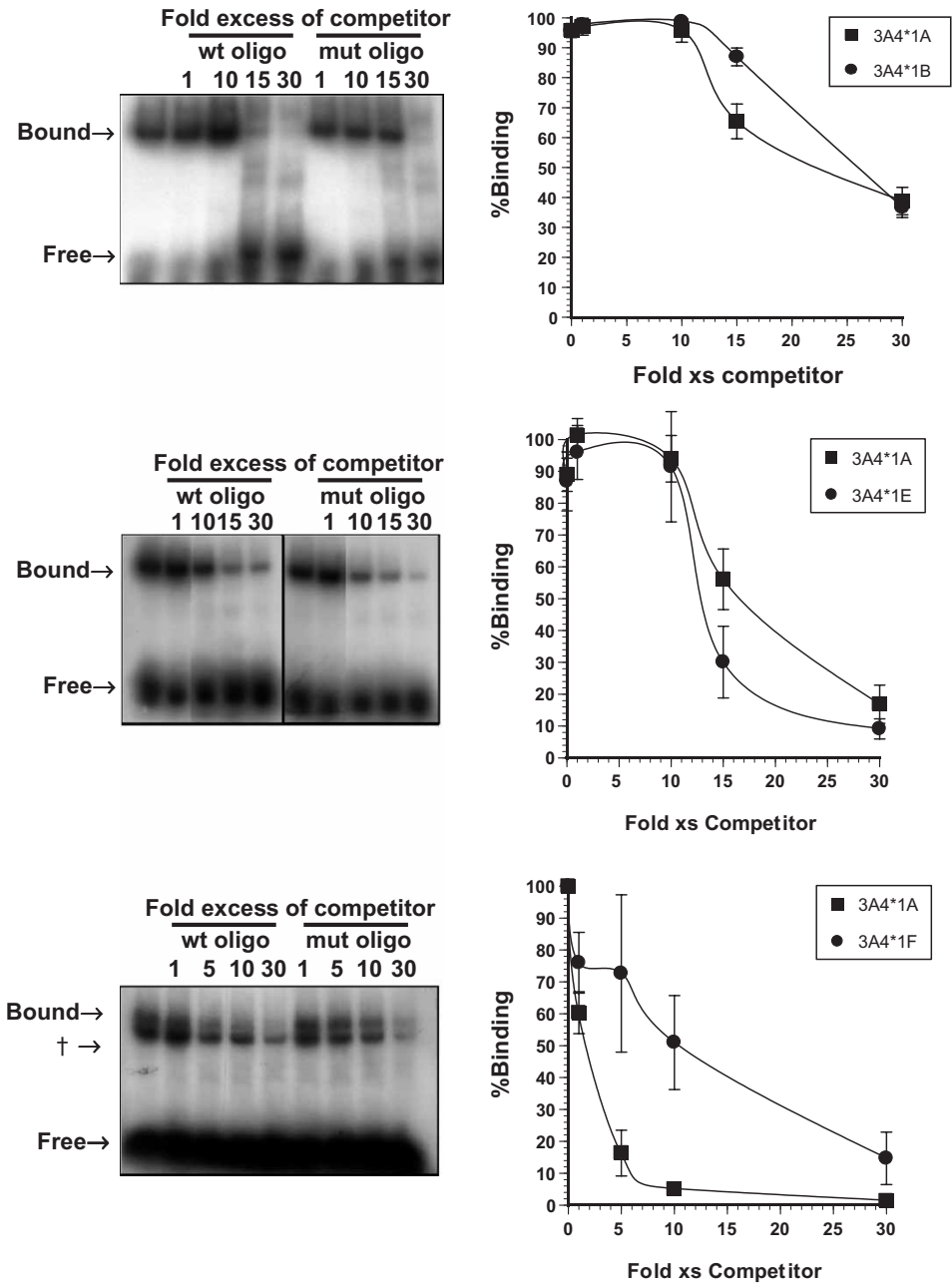


Figure 3. Influence of mutations in the *CYP3A4* promoter on DNA-protein interactions. Synthetic double-stranded DNA fragments covering the relevant sections of the wild-type (*CYP3A4*1A*) 5' flanking region were end-labelled and added to HepG2 cell nuclear protein extracts as described in the Materials and methods. Increasing amounts of unlabelled wild-type or mutant competitor DNA fragments were added to the binding reactions. Reactions products were electrophoresed in 4% non-denaturing polyacrylamide gels and autoradiography performed for 16 h. Band intensity was calculated using video-based densitometry: All exposures were within the linear range of the film. Error bars indicate standard error of the mean ($n=6$); †non-competable, non-specific binding.

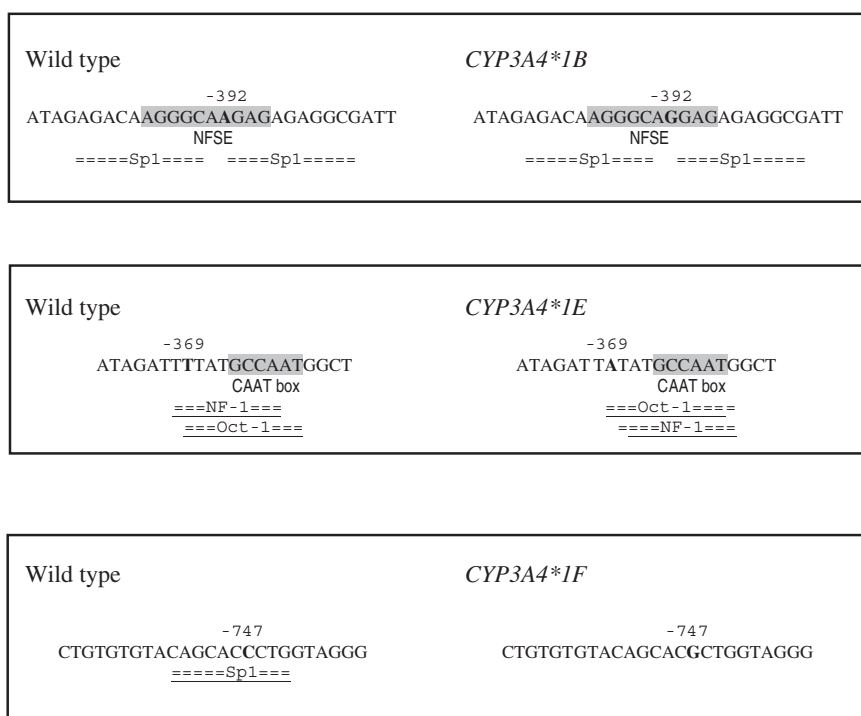


Figure 4. Predicted protein-binding sites in the sequence contexts of the *CYP3A4* promoter mutations. The *CYP3A4* wild-type and mutant promoter sequences at the various mutation sites were analysed for potential transcription factor binding sites using the AliBaba search engine to interrogate the TRANSFAC database. The mutation sites are shown in bold and numbered to aid comparison. Shaded boxes identify landmark features in the *CYP3A4* promoter (Hashimoto *et al.* 1993); dotted lines indicate the extent of the predicted protein binding motifs.

wild-type promoter, with the exact level of response being dependent on the xenobiotic used and the recipient cell line. The reason for this is not clear. Whereas it could be argued that the transfections were performed in the presence of abnormally high intracellular levels of hPXR (due to co-transfection with pSG5-hPXRΔATG) thus maximizing responsiveness to hPXR ligands such as rifampicin, other xenobiotics also gave elevated levels of activation compared with the wild-type. If the basis of transcriptional activation of *CYP3A4* by xenobiotics resides mainly in the binding of activated xenobiotic-responsive nuclear receptors (e.g. hPXR, hCAR) to their specific response elements in the promoter region, then modification or abolition of the binding sites for other transcription factors such as NF-1, Oct-1 or Sp-1 which are not activated by such compounds, might not be expected to result in substantial change (Gibson *et al.* 2002). Indeed, *in vitro* mutation of specific hPXR binding sites in the *CYP3A4* proximal promoter or XREM only produces a 50% reduction in xenobiotic transcriptional activation (Goodwin *et al.* 1999). This would support the hypothesis that the responsiveness of the *CYP3A4* gene to both exogenous and endogenous factors represents the summation of modulatory effects from all of the proteins binding to the promoter region (Quattrochi and Guzelian 2001, Saito *et al.* 2001, El-Sankary *et al.* 2002). This does not explain why for the *CYP3A4* alleles examined here, the effect of the mutations is to increase rather than decrease the *in vitro* response to xenobiotics,

although a possible scenario may be envisaged. The *CYP3A4* promoter may be under very tight control with multiple transcription factor interactions required for its regulated activation. PXR probably exists associated with DNA at the PXRE, possibly in an unliganded state, until activated by xenobiotics, whereupon transcriptional activation occurs. However, this activation may be regulated by the association of other transcription factors with the surrounding DNA, potentially through the modulation of chromatin structure. In these mutant alleles, the disruption of protein:DNA interactions other than at the PXRE may result in the alteration of repressive chromatin status, causing an increased ability of xenobiotics to activate *CYP3A4* transcription via PXR activation.

Whether, the *in vitro* response of the mutant promoters to xenobiotics is a true representation of the situation *in vivo* remains to be determined. However, interindividual variation in the metabolism of *CYP3A4* substrates is unlikely to be a result just of variation in the *CYP3A4* genotype, but may depend on a haplotype characterized by SNPs in other genes in the *CYP3A* locus (Kuehl *et al.* 2001) and/or in genes coding for the receptors and other DNA-binding proteins which control their transcription (Hustert *et al.* 2001, Zhang *et al.* 2001).

In summary, it has been shown that inherited mutations in the human *CYP3A4* gene 5' regulatory region can significantly affect transcriptional activation by xenobiotic inducers *in vitro*. This response also appears to be modulated by cell type and possibly the presence of endogenous activating ligands. Owing to their location and heterozygous nature, these mutations in the *CYP3A4* 5' regulatory region may have a limited effect on hepatic *CYP3A4* expression *in vivo*.

Acknowledgements

The authors thank Nasser Vahdati-Mashhadian and Karen Swales for helpful discussions. H. H. was supported by a studentship from the Iranian Ministry of Health. V. B. was supported by a Research Committee studentship from the University of Surrey.

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Pharmacokinetic analysis of factors determining elimination pathways for sulfate and glucuronide metabolites of xenobiotics II: Studies with isolated perfused rat liver

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Received 2 May 2003

1. To elucidate the determining factors for elimination pathways of sulfate and glucuronide metabolites of xenobiotics, a single-pass perfusion of 4-methylumbelliferone (4MU) or *p*-nitrophenol (pNP) was performed with an isolated rat liver preparation.

2. Without bovine serum albumin in the perfusion system, clearance calculated based on the unbound concentration in the liver clearly showed that the net efflux clearances (CL_{eff}) of sulfates from the sinusoidal membrane were much higher than those of glucuronides and that the biliary excretion clearances (CL_b) of glucuronides were approximately two times larger than those of sulfates.

3. The ratios of CL_{eff} to CL_b were much higher for sulfates than those for glucuronides. The bile-oriented elimination of glucuronides or sinusoidal efflux-oriented elimination of sulfates was observed even using the perfusate including 3% bovine serum albumin, but the sinusoidal efflux of sulfates was extensively enhanced by bovine serum albumin in the perfusate. The mechanisms behind this stimulatory effect remain to be elucidated.

4. For both compounds, CL_b of glucuronide was comparable with CL_b of sulfate, meaning that CL_b is not responsible for the biliary excretion of glucuronides at extensively higher rate than sulfates.

5. Higher concentration of glucuronides in the liver, partly caused by much lower CL_{eff} of glucuronides than that of sulfates, is likely responsible for the bile-oriented excretion of glucuronides. The extensive sinusoidal efflux of sulfates, leading to the urine-oriented excretion, is attributed to the substantially higher CL_{eff} than CL_b .

6. In conclusion, the sinusoidal efflux is an important factor for determining elimination pathways of both sulfates and glucuronides, although further studies are needed to clarify the mechanisms of the sinusoidal efflux.

Introduction

Xenobiotics are usually excreted into urine or bile unchanged or as metabolites. Phase II metabolism, conjugation reactions, often after phase I metabolism is one of the most important processes for the detoxification of xenobiotics. In conjugation reactions, the metabolic product or the parent drug, if it has a polar group that allows conjugation, reacts with an endogenous compound such as glucuronic acid, sulfate, glutathione and glycine to yield water-soluble conjugated metabolites that are easily excreted into urine or bile (Moller and Sheikh 1983, Klaassen and Watkins III 1984). Biliary excretion

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and urinary excretion are subsequently the major ways of clearing the internal milieu from potentially dangerous xenobiotics and their conjugated metabolites. Most xenobiotics and/or their metabolites are eliminated from the body via both pathways (Fleck and Braunlich 1990) except for a few compounds such as *p*-aminohippuric acid (Fleck and Braunlich 1986) and indocyanine green (Guarino and Anderson 1976, Fleck and Braunlich 1986), which are excreted via almost entirely by one of these two pathways into urine and bile, respectively. When one excretion process is damaged, the other can compensate in terms of the excretion of a given xenobiotic (Siegers and Klaassen 1984, Besseghir and Roch-Ramel 1987, Kimura *et al.* 1988). On the other hand, it has been reported that both glucuronides and sulfates can be handled by both the liver and kidney (Fleck and Braunlich 1990). However, glucuronides are usually excreted into both urine (Moller and Sheikh 1983) and bile (Klaassen and Watkins III 1984), whereas sulfates are extensively excreted into urine (De Vries *et al.* 1985, Mulder *et al.* 1985). Although there is an empirical rule stating that lipophilic compounds of molecular weight higher than 300–500 are preferentially excreted into bile after metabolism, whereas the kidney would preferentially eliminate hydrophilic substances of molecular weight smaller than 500 (Besseghir and Roch-Ramel 1987), the specific determinants of the preferential excretion pathway of xenobiotics including glucuronides and sulfates have not been fully clarified to date. Transporters that may be involved in the biliary (Kusuhara *et al.* 1998) and/or renal (Burckhardt and Wolff 2000) excretion of xenobiotics have been reported and are very important. However, pharmacokinetic analysis and estimation are also very important to elucidate quantitatively the processes determining their preferential excretion.

We have already evaluated the hepatic and renal handling of three phenolic compounds, 4-methylumbelliferone (4MU), *p*-nitrophenol (pNP) and acetaminophen, and of their glucuronides and sulfates in an *in-vivo* constant infusion study (Ishii *et al.* 2002). The excretion of their glucuronides was more balanced in urine and bile and their sulfates were primarily excreted into urine. However, the biliary excretion clearances (CL_b) of glucuronides are much larger than their renal excretion clearances (CL_r), and there was not any large difference between CL_r and CL_b in the case of sulfates. On the other hand, the tissue-to-plasma concentration ratio (K_p) of both glucuronides and sulfates for every phenolic compound was much higher in the kidney than that in the liver. These results suggest that one of the most important determinants for the preferential excretion of these conjugates into bile or urine is the extent of disposition of each compound to the liver or kidney and that the clearances such as CL_b and CL_r do not necessarily reflect the actual excretion rate of conjugates. In addition, K_p values of both glucuronides and sulfates in the liver, where these conjugates are mainly formed, were found small. The K_p values of sulfates were quite low, suggesting that sulfates generated in the liver are subject to an extensive sinusoidal efflux.

The present study focused on the hepatic handling of glucuronides and sulfates for 4MU and pNP. Using an isolated liver perfusion technique, the biliary excretion and the efflux from the sinusoidal membrane of the liver were estimated to elucidate the determining process for the elimination pathway. Furthermore, we investigated the effect of protein binding on the hepatic handling of these conjugates by using bovine serum albumin (BSA).

Materials and methods

Materials

4MU, 4MU glucuronide (4MUG), 4MU sulfate (4MUS), pNP glucuronide (pNPG) and pNP sulfate (pNPS), of which the chemical purities are over 98%, were obtained from Sigma Chemical Co. (St Louis, MO, USA). pNP, of which the chemical purity is above 98.5%, was purchased from Ishizu Pharmaceutical Co. (Osaka, Japan). As the internal standard for the determination of 4MU and pNP including their conjugates, umbelliferone (Tokyo Chemical Co., Tokyo, Japan) and phenol (Sigma), respectively, were used. All other reagents were of the highest grade available.

Animals

Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25°C and 55% humidity, were allowed free access to standard laboratory chow (Clea Japan, Tokyo) and water before the experiments. Rats weighing 240–290 g were randomly assigned to each experimental group. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with Principles of Laboratory Animal Care (NIH Publication #85–23).

Isolated liver perfusion study

The isolated liver perfusion study was performed according to Mortimore's method (Mortimore 1961) as described (Higaki *et al.* 1992). Briefly, after the animals had been anaesthetized with an intraperitoneal injection of pentobarbital (30 mg kg⁻¹), the bile duct was first cannulated with polyethylene tubing (PE 50, 0.023 inch i.d. × 0.038 inch o.d., Division of Becton Dickinson, Parsippany, NJ, USA) followed by cannulation of the portal vein and the inferior vena cava with polyethylene tubing PE 160 (0.045 inch i.d. × 0.062 inch o.d., Division of Becton Dickinson) and PE 240 (0.066 inch i.d. × 0.095 inch o.d., Division of Becton Dickinson), respectively. The perfusate consisted of about 20% washed human erythrocytes, kindly supplied by the Japanese Red Cross Society (Okayama, Japan), and 0.1% D-glucose in Krebs–Ringer bicarbonate buffer with or without 3% BSA (Fraction V, Sigma) was delivered at 13.5 ml min⁻¹.

Estimation of unbound fraction

Unbound fraction of compounds in perfusate (f_p) or in 16.7% liver homogenate (f_T) was determined by an ultrafiltration method (1300 g for 20–30 min) using MPS-1 (Amicon Co., Tokyo, Japan). Liver homogenate (16.7%) was prepared by homogenizing liver with its fivefold volume of an ice-cold isotonic phosphate buffer (pH 7.4).

Determination of drug concentrations

Drug concentrations in perfusate, bile and liver were determined by HPLC. 4MU and pNP and their conjugates were assayed according to the methods reported by Zimmerman *et al.* (1991) and Klaassen and Watkins III (1984), respectively, with minor modifications. Briefly, the perfusate obtained after centrifugation was deproteinized with methanol after the addition of an internal standard. Bile was diluted with the mobile phase used in the HPLC analysis after the addition of an internal standard. The isolated liver was homogenized in isotonic phosphate buffer (pH 7.4), and the resultant homogenate was deproteinized with methanol after the addition of an internal standard. An aliquot of each supernatant was introduced onto HPLC system, consisting of a model LC-6A HPLC pump (Shimadzu, Kyoto, Japan), a model SIL-9A system controller (Shimadzu), and a model SPD-2A UV detector (Shimadzu) set at 313 and 280 nm for the cases of 4MU and pNP, respectively. A ChemcoPak Nucleosil column (150 × 4.6 mm i.d.; pore size, 120 Å; particle size, 5 µm; Macherey-Nagel, Inc., Easton, PA, USA) was used at room temperature. The mobile phase for determination of 4MU and its conjugated metabolites consisted of 25% methanol in 0.05 M KH₂PO₄, and that for pNP and their conjugates consisted of acetic acid–methanol–0.05 M KH₂PO₄, 1:10:10 or 7.5:15:1000 (v/v), respectively. Each of the mobile phases was delivered at 1.0 ml min⁻¹. The coefficient of variation (CV) for each standard curve ranged from 0.3 to 5% and the squared correlation coefficient was over 0.998.

Pharmacokinetic estimation of hepatic handling of conjugates

CL_b and CL_{eff}, the net sinusoidal efflux clearance, were calculated based on the unbound concentration of conjugates in the liver using the unbound fraction obtained in 16.7% liver homogenate as follows:

$$CL_b = \frac{\text{Biliary excretion rate at steady state}}{f_T \cdot C_{\text{liver,ss}}} \quad (1)$$

$$CL_{\text{eff}} = \frac{\text{Net efflux rate at steady state}}{f_T \cdot C_{\text{liver,ss}}} \quad (2)$$

where f_T and $C_{\text{liver,ss}}$ are the unbound fraction of conjugates in 16.7% liver homogenate and the concentration of conjugates in the liver at steady state, respectively. A net efflux rate was calculated by multiplying the concentration in the outflow perfusate by the perfusion rate.

Statistical analysis

Results are expressed as the mean $\pm$ SD of three or more experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance in the differences of the means was determined by Dunnett's method or Student's *t*-test.

Results and discussion

To estimate the hepatic handling of 4MUG and 4MUS at steady state, 4MU was constantly infused through the isolated liver preparation at $20 \mu\text{g ml}^{-1}$ without BSA (figure 1, A) and with 3% BSA (figure 1, B). Based on our preliminary studies, it was found that the perfusion of $20 \mu\text{g ml}^{-1}$ of 4MU gave 4MUG and 4MUS of the similar concentration to those obtained in *in-vivo* experiments performed in the previous study (Ishii *et al.* 2002). The outflow concentration–time profiles of 4MU, 4MUG and 4MUS and their biliary excretion rate–time profiles are shown in the upper and lower panels, respectively. Both outflow concentration and biliary excretion rate reached steady state in 25–35 min for all the chemicals regardless of the presence of BSA. Pharmacokinetic parameters at steady state are summarized in table 1. In the present study, as 4MU instead of conjugates was constantly perfused, the conjugates found in the outflow and bile are all generated in the liver where 4MU is metabolized. The biliary excretion rate of 4MUG was substantially higher than that of 4MUS in both 0% BSA and 3% BSA perfusion systems. The sum of biliary excretion rates and net efflux rates also gave much higher values for glucuronide than those for sulfate in both perfusion systems. These results clearly support the relatively higher capacity of the glucuronosyltransferases than that of

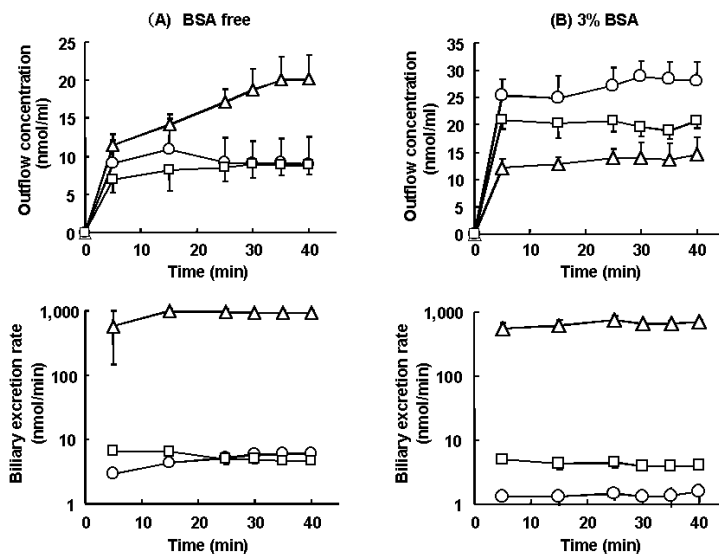


Figure 1. Appearance of 4MUG and 4MUS in outflow perfusate and bile during single-pass perfusion of 4MU through isolated liver preparations. Perfusion of $20 \mu\text{g ml}^{-1}$ 4MU ($113.5 \text{ nmol ml}^{-1}$) was performed at 13.5 ml min^{-1} of perfusion rate, using KRB buffer without BSA (A) and with 3% BSA (B). Results are a mean with a vertical bar showing SD of three experiments. ○, 4MU; △, 4MUG; □, 4MUS.

Table 1. Pharmacokinetic parameters describing hepatic handling of 4MU and its conjugates in the isolated perfused liver with or without BSA.

BSA (%)	Chemicals	Pharmacokinetic parameters							
		$C_{p,ss}$ (nmol ml ⁻¹)	f_p	Net efflux rate (nmol min ⁻¹)	Biliary excretion rate (nmol min ⁻¹)	$C_{liver, ss}$ (nmol g ⁻¹)	CL _b (ml min ⁻¹)	CL _{eff} (ml min ⁻¹)	CL _{eff} /CL _b
0	4MU	8.97 ± 3.56	1	–	6.04 ± 1.11	109.3 ± 10.5	n.c.	n.c.	n.c.
	4MUG	20.1 ± 3.3	1	271.4 ± 44.6	648.4 ± 43.2	223.2 ± 18.9	6.53 ± 0.43	1.86 ± 0.24	0.29 ± 0.03
	4MUS	**8.81 ± 1.20	1	**118.8 ± 16.2	**4.63 ± 0.20	**6.88 ± 2.30	**3.19 ± 0.98	*79.9 ± 16.2	**25.7 ± 3.6
	4MU	28.0 ± 6.3	0.08 ± 0.01	–	1.60 ± 0.46	56.5 ± 10.7	n.c.	n.c.	n.c.
3	4MUG	14.6 ± 0.5	0.59 ± 0.03	197.1 ± 6.8	693.9 ± 55.2	115.1 ± 27.5	9.07 ± 1.76	2.59 ± 0.62	0.28 ± 0.02
	4MUS	*20.6 ± 1.9	**0.03 ± 0.00	*278.1 ± 5.7	**4.04 ± 0.49	**3.68 ± 0.70	**4.73 ± 0.82	**312.8 ± 45.0	**66.9 ± 10.5

Single-pass perfusion of 20 µg ml⁻¹ 4MU (113.5 nmol ml⁻¹) was performed at 13.5 ml min⁻¹ of perfusion rate, using KRB buffer with or without BSA. All parameters were calculated at steady-state. CL_b and CL_{eff} mean the biliary excretion clearance and the net sinusoidal efflux clearance, respectively. Both clearances were calculated based on the unbound concentration of each substrate in the liver, using the unbound fraction in 16.7% liver homogenate as follows: $f_T^{4MUG} = 0.653 \pm 0.086$ ($n = 11$), $f_T^{4MUS} = 0.226 \pm 0.033$ ($n = 11$, $p < 0.01$ compared with f_T^{4MUG}). Results are the mean ± SD of three experiments. ** $p < 0.01$; * $p < 0.05$ compared with 4MUG in each BSA concentration. n.c., Not calculated.

the sulfotransferases (Benet *et al.* 1995). However, the CL_b of 4MUG was only two times larger than that of 4MUS, whereas its biliary excretion rate was 140 or 172 times larger than that of 4MUS. The substantial difference in the ratio of glucuronide to sulfate between CL_b and actual biliary excretion rate obviously reveals that a determining factor for the biliary excretion of 4MUG is not CL_b , but hepatic concentration of 4MUG. This result supports the prior conclusion that the clearances do not necessarily reflect the actual excretion rates as suggested previously based on an *in-vivo* study (Ishii *et al.* 2002). The difference in the net efflux rate between 4MUG and 4MUS is much smaller than that in the biliary excretion rate in both perfusion systems. However, CL_{eff} of 4MUS was about 40 or 120 times larger than that of 4MUG due to much lower hepatic concentration of 4MUS. In the present study, CL_{eff} was calculated using the net efflux rates of conjugates, but their influx permeabilities are very small (Miyachi *et al.* 1988) such that CL_{eff} estimated here almost reflects the efflux of the conjugates generated in the liver. Therefore, the efflux of sulfates is determined by the large value of CL_{eff} . Higher concentrations of 4MUG are certainly generated by higher capacity of glucuronidation (Benet *et al.* 1995) and probably by much lower CL_{eff} of 4MUG. On the other hand, the efflux from the sinusoidal membrane, leading to the urinary excretion *in vivo*, is dependent upon its very high value of CL_{eff} . The lower concentration of sulfate in the liver is also certainly ascribed to the efflux at higher rate regardless of its relatively lower f_T . The ratio of CL_{eff} to CL_b was extensively higher for 4MUS than that for 4MUG, which also reveals a distinct difference in the hepatic handling of 4MUG and 4MUS. The effect of BSA on the hepatic handling will be discussed below along with the results for pNP.

Figure 2 and table 2 show the results of the liver perfusion studies employing pNP as a substrate. The hepatic handling of glucuronide and sulfate of pNP is very similar to that of 4MU conjugates regardless of the presence of BSA. Although net

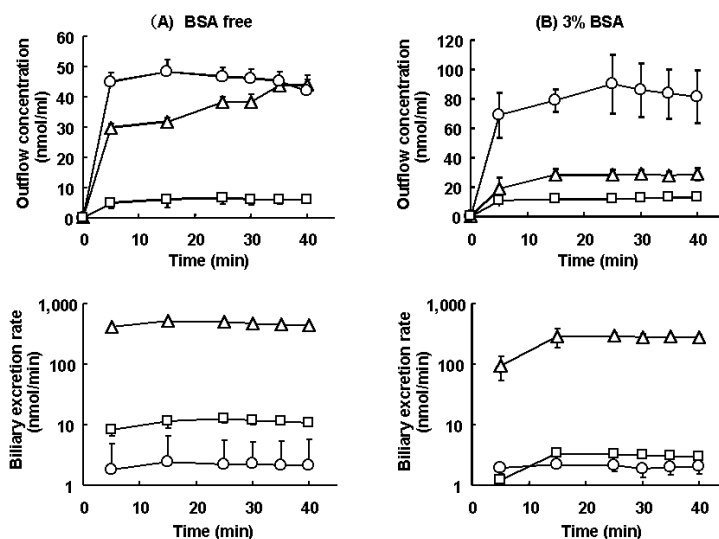


Figure 2. Appearance of pNPG and pNPS in outflow perfusate and bile during single-pass perfusion of pNP through isolated liver preparations. Perfusion of $20 \mu\text{g ml}^{-1}$ pNP ($143.8 \text{ nmol ml}^{-1}$) was performed at 13.5 ml min^{-1} of perfusion rate, using KRB buffer without BSA (A) and with 3% BSA (B). Results are expressed as a mean with a vertical bar showing SD of four experiments. ○, pNP; △, pNPG; □, pNPS.

Table 2. Pharmacokinetic parameters describing hepatic handling of pNP and its conjugates in the isolated perfused liver with or without BSA.

BSA (%)	Chemicals	Pharmacokinetic parameters							
		$C_{p,ss}$ (nmol ml ⁻¹)	f_p	Net efflux rate (nmol min ⁻¹)	Biliary excretion rate (nmol min ⁻¹)	$C_{liver, ss}$ (nmol g ⁻¹)	CL_b (ml min ⁻¹)	CL_{eff} (ml min ⁻¹)	CL_{eff}/CL_b
0	pNP	42.1 ± 12.7	1	–	2.16 ± 0.27	70.4 ± 9.4	n.c.	n.c.	n.c.
	pNPG	44.0 ± 7.0	1	593.4 ± 95.1	434.2 ± 61.7	173.4 ± 24.6	3.67 ± 0.33	5.12 ± 1.20	1.39 ± 0.32
	pNPS	**6.02 ± 0.77	1	**81.2 ± 10.4	**10.6 ± 2.0	**3.43 ± 0.89	5.95 ± 1.98	**44.3 ± 5.4	**7.87 ± 1.74
3	pNP	81.4 ± 17.7	0.16 ± 0.02	–	2.06 ± 0.52	24.3 ± 6.4	n.c.	n.c.	n.c.
	pNPG	28.7 ± 4.1	0.87 ± 0.06	387.5 ± 55.4	275.4 ± 33.6	128.3 ± 10.6	3.17 ± 0.63	4.43 ± 0.56	1.42 ± 0.19
	pNPS	**13.2 ± 1.7	**0.06 ± 0.02	**178.2 ± 23.0	**2.92 ± 0.25	**2.12 ± 0.25	2.56 ± 0.48	**154.3 ± 15.0	**62.0 ± 13.0

Single-pass perfusion of 20 µg ml⁻¹ pNP (143.8 nmol ml⁻¹) was performed at 13.5 ml min⁻¹ of perfusion rate, using KRB buffer with or without BSA. All parameters were calculated at steady-state. CL_b and CL_{eff} mean the biliary excretion clearance and the net sinusoidal efflux clearance, respectively. Both clearances were calculated based on the free concentration of each substrate in the liver, using the unbound fraction in 16.7% liver homogenate as follows: $f_T^{pNPG} = 0.684 \pm 0.177$ ($n=8$), $f_T^{pNPS} = 0.547 \pm 0.173$ ($n=8$). Results are the mean ± SD of four experiments. ** $p < 0.01$ compared with pNPG in each BSA concentration. n.c., Not calculated.

efflux rates of pNPG were larger than its biliary excretion rates, biliary excretion rates of pNPG were still much higher than those of pNPS. Furthermore, the difference in CL_b between pNPG and pNPS was very small, which is similar to that between 4MUG and 4MUS. What is different for the case of 4MU is that CL_{eff} of pNPG is close to CL_b of pNPG, which is probably responsible for its more balanced excretion into both bile and urine (Ishii *et al.* 2002).

To estimate the effect of BSA on the hepatic handling of conjugates of the two xenobiotics, the ratio of 3% BSA to 0% BSA was calculated in terms of CL_b , CL_{eff} and CL_{eff}/CL_b (figure 3). For both 4MU and pNP, the presence of BSA in the perfusate enhanced the sinusoidal efflux of sulfates extensively, leading to the more efflux-oriented excretion of sulfates in the perfusion system with 3% BSA (tables 1 and 2). The sinusoidal efflux of dibromosulphophthalein (DBSP) is also enhanced by BSA in the perfusate (Meijer *et al.* 1984, Nijssen *et al.* 1994), but other proteins such as fatty acid binding protein and glutathione *S*-transferase (ligandin) also have the same effect on DBSP efflux (Nijssen *et al.* 1994), meaning that the stimulatory effect is not albumin-specific. The decrease in the reuptake of DBSP secreted into the perfusate possibly results in the enhancement of the net efflux (Nijssen *et al.* 1994) such that the inhibition of the reuptake by BSA due to the protein binding might be partly responsible for the stimulated efflux of the sulfate conjugates in the present study. However, DBSP is a typical substrate as excreted into bile (Klaassen and Plaa 1968) and is extensively taken up by the liver (Blom *et al.* 1981) probably via *oatp1* and *oatp2* (Suzuki and Sugiyama 2000), although the sinusoidal efflux of DBSP is suggested to be via some transporter (Nijssen *et al.* 1991). On the other hand, the hepatic uptake and extraction of 4MUS and 4MUG is so low (Miyachi *et al.* 1988) that the contribution of the reuptake inhibition to the stimulated efflux of 4MUS and pNPS is probably very small. The very low unbound fraction of sulfate in the perfusate, which is much lower than that in the liver (16.7% homogenate) (tables 1 and 2), might be important, because the sinusoidal efflux was not stimulated for either 4MUG or pNPG, of which the unbound fraction is almost equivalent to or larger than that in the liver (tables 1

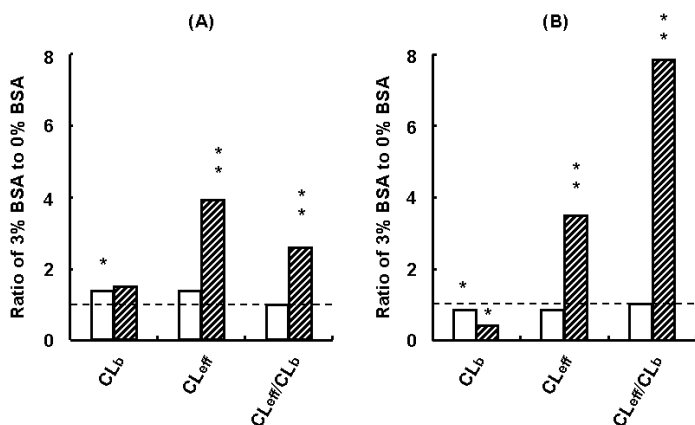


Figure 3. Effect of BSA in perfusate on CL_{eff} and CL_b for glucuronides and sulfates of 4MU (A) and pNP (B). Ratio of 3% BSA to 0% BSA was calculated for 4MU or pNP using the data in tables 1 or 2, respectively. CL_b and CL_{eff} are the biliary excretion clearance and the efflux clearance, respectively. Both clearances were calculated based on the free concentration of each substrate in the liver. Statistically significant differences between 0% BSA and 3% BSA are indicated as follows: ** $p < 0.01$; * $p < 0.05$. (A) □, 4MUG; ▨, 4MUS. (B) □, pNPG; ▨, pNPS.

and 2). This condition can facilitate the passive diffusion of solutes from the liver to the perfusate across the sinusoidal membrane, but further studies are needed to refer to the similar effect on the transport of xenobiotics via some transporter. Furthermore, the mechanisms behind the increasing tendency or the decreasing tendency in CL_b for conjugates of 4MU or those of pNP (figure 3) remain to be clarified.

Figure 4 shows the concentration dependency in terms of CL_b , CL_{eff} and the ratio of CL_{eff} to CL_b in the case of 4MU conjugates. For 4MUG (figure 4, A), saturation was observed for CL_b , but not for CL_{eff} over the concentration range examined. Therefore, the ratio of CL_{eff} to CL_b significantly increased from 0.29 to 0.44. As the hepatic concentration of 4MUG increased from 149.4 ± 35.3 ($n=4$) to 645.7 ± 31.0 nmol g⁻¹ liver ($n=4$) without any change in f_T , the biliary excretion process might be saturated, suggesting that the capacity of the biliary excretion system is relatively lower than that of the sinusoidal efflux system for 4MUG. On the other hand, there is a saturable tendency in both CL_b and CL_{eff} for 4MUS (figure 4, B). The hepatic concentration of 4MUS increased from 3.1 ± 0.3 ($n=4$) to 10.4 ± 2.4 nmol g⁻¹ liver ($n=4$), although no change in f_T was observed. 4MUS transport might be saturated in both processes like the biliary excretion of 4MUG. Consequently, the sinusoidal efflux-oriented excretion of 4MUS was probably enhanced as the infusion concentration increased. The contribution of the urinary excretion increased as the dose was increased in our previous *in-vivo* study (Ishii *et al.* 2002), suggesting that the hepatic handling of conjugates basically regulates their excretion pathways. As for pNP, the hepatic perfusion of pNP at 10 µg ml⁻¹ was performed and no significant difference was found in any kinetic parameters, meaning the linear kinetics for biliary excretion and sinusoidal efflux of both conjugates in the range of 10–20 µg ml⁻¹ (data not shown). Because of toxicity, the perfusion of pNP at more than 20 µg ml⁻¹ was not acceptable.

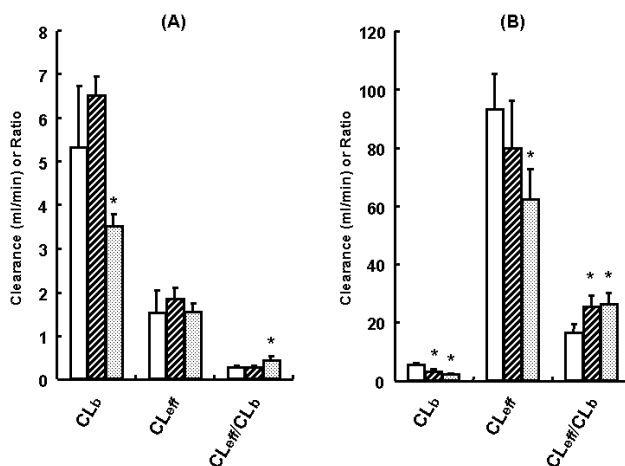


Figure 4. Concentration dependency of CL_{eff} and CL_b for 4MUG (A) and 4MUS (B). Single-pass perfusion of 4MU at 10 µg ml⁻¹ (□), 20 µg ml⁻¹ (▨) or 100 µg ml⁻¹ (■) was performed using KRB without BSA. Both CL_b and CL_{eff} were calculated based on the free concentration of each substrate in the liver. Results are expressed as a mean with a vertical bar showing SD of three or four experiments. Statistically significant difference from the values at 10 µg ml⁻¹ is indicated as follows: * $p < 0.05$.

Biliary excretion of both conjugates is probably mediated by mrp2, multidrug resistance-associate protein 2, located in the canalicular membrane of the liver. It has already been reported that many anionic xenobiotics and conjugates are transported via mrp2 into bile (Suzuki and Sugiyama 1999).

Extrahepatic conjugation reactions including sulfation take place for several phenolic compounds including 4MU and pNP (Mulder *et al.* 1984). However, the liver is certainly a main organ where phase II metabolism takes place, meaning that sulfates generated in the liver have to be subject to extensive efflux from the liver. It has been reported that harmol sulfate is transported back to the blood via a carrier-mediated mechanism (De Vries *et al.* 1985). Even indocyanine green, sulfobromophthalein (Scharschmidt *et al.* 1975) and DBSP (Nijssen *et al.* 1994), which are well known to be extensively excreted into bile, are subject to a sinusoidal efflux to the blood. However, details about the efflux of these conjugates remain to be clarified, although they are transported back to the blood probably via some carrier (Scharschmidt *et al.* 1975, De Vries *et al.* 1985, Nijssen *et al.* 1991).

Several transporters that can be responsible for the sinusoidal efflux of organic anions have been suggested such as Npt1, cloned as renal inorganic phosphate transporter (Yabuuchi *et al.* 1998), sGshT, glutathione transporter (Yi *et al.* 1995), Oatps, organic anion transporting polypeptide (Suzuki and Sugiyama 2000) and OAT/OCTs, organic anion/cation transporter (Sekine *et al.* 2000). Some of these transporters are thought to support the efflux as well as the uptake of anions across the sinusoidal membrane (Tsuji and Tamai 1999), whereas an independent system has also been postulated for each process (Nijssen *et al.* 1991). Although mrp3, multidrug resistance-associated protein 3, has also been demonstrated to mediate the sinusoidal efflux of anions (Xiong *et al.* 2002), its constitutive expression level is quite low so that this transporter might not be responsible for the extensive efflux of sulfates under the physiological conditions. Further investigations to clarify the mechanisms behind the extensive sinusoidal efflux of sulfates are currently in progress.

In conclusion, the bile-oriented excretion of glucuronides is ascribed not to their CL_b which are comparable with CL_b of sulfates, but to high concentrations of glucuronides in the liver that is partly caused by their CL_{eff} values being much lower than the CL_{eff} of sulfates. On the other hand, the sinusoidal efflux of sulfates, leading to the urine-oriented excretion, is dependent on higher CL_{eff} than CL_b . The sinusoidal efflux must be one of the most important factors for determining the elimination pathways of both conjugates, although further studies are needed to determine the mechanisms and transporters involved in the extensive efflux of sulfates.

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Application of structure–metabolism relationships in the identification of a selective endothelin A antagonist, BMS-193884, with favourable pharmacokinetic properties

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Received 11 April 2003

1. Based on binding affinity, 2'-amino-N-(3,4-dimethyl-5-isoxazolyl)-4'-(2-methylpropyl)[1,1'-biphenyl]-2-sulfonamide (**2**) was identified as an initial lead in a programme to identify selective endothelin (ET) receptor antagonists. However, the compound was extensively metabolized in preclinical animal species and human *in vitro* systems due to oxidative biotransformation.

2. To optimize this structural class, the site of metabolism of **2** was determined. This allowed for focussed structure–activity and structure–metabolism studies aimed at finding more metabolically stable analogues that maintained potency. New analogues were screened for their ET binding characteristics and their stability in rat and human liver microsomes.

3. The use of the microsomal stability screen was tested by the determination of the pharmacokinetic parameters of select analogues. A good correlation was found between reduced rates of rat microsomal metabolism and reduced clearance in the rat.

4. N-(3,4-dimethyl-5-isoxazolyl)-4'-(2-oxazolyl)[1,1'-biphenyl]-2-sulfonamide (**3**) was identified as an analogue with improved *in vitro* properties and further studies revealed that the compound had improved pharmacokinetic properties.

5. N-[[2'-[[[3,4-dimethyl-5-isoxazolyl]amino]sulfonyl]-4-(2-oxazolyl)[1,1'-biphenyl]-2-yl]methyl]acetamide (**4**) was subsequently identified as a compound with superior *in vitro* properties compared with compound **3**, but when tested *in vivo* it had a substantially increased rate of clearance. Further studies demonstrated that the clearance of this closely related structural analogue was not dictated by metabolic processes, but was mediated by transport-mediated direct biliary excretion.

6. The utility of screening for *in vitro* liver microsomal stability as part of the lead optimization process for compounds with metabolic liabilities was shown. It was also shown that relatively small molecular changes can dramatically change the disposition of closely related analogues and care must be used when screening for a single property.

Introduction

The endothelins are a closely related family of peptides with potent vasoactive and mitogenic activity. They cause numerous biological effects and have been the subject of several reviews (Rubanyi and Polokoff 1994, Ohlstein *et al.* 1996, Webb and Meek 1997, Kaur *et al.* 2001). The endothelins have been implicated in a variety of disease states including hypertension (Brunner 1998), congestive

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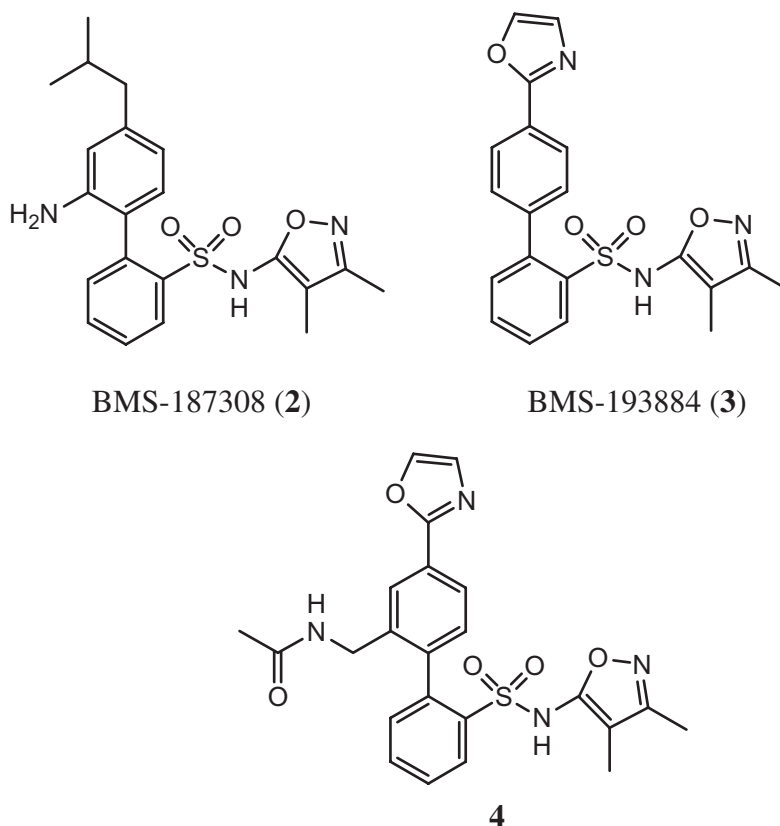


Figure 1. Structures of key endothelin A antagonist compounds.

heart failure (Love and McMurray 1996, Pousset *et al.* 1997, Nguyen and Johnson 1998), renal failure (Takahashi *et al.* 1994, Rohmeiss *et al.* 1999), pulmonary hypertension (Underwood *et al.* 1997), and metastatic prostate cancer (Nelson *et al.* 1995). The endothelins exert their activity through two receptors, ET_A and ET_B (Arai *et al.* 1990, Sakurai *et al.* 1990). For this reason, an intense effort has been mounted recently to prepare non-peptide antagonists of these receptors.

Several potent, ET_A-selective antagonists have been discovered at Bristol-Myers Squibb (Stein *et al.* 1994, 1995, Murugesan *et al.* 1998, 2000). Owing to superior potency, 2'-amino-N-(3,4-dimethyl-5-isoxazolyl)-4'-(2-methylpropyl) [1,1'-biphenyl]-2-sulfonamide (**2**, BMS-187308, figure 1) was selected as the subject of an in-depth structure-activity relationship (SAR) programme. The major goal of the SAR programme was to maintain potency while improving the pharmacokinetic behaviour and duration of action of the compound. This was accomplished through the initial identification of the primary metabolic pathways of **2** followed by the synthesis of a number of analogues at that site. These analogues were subjected to a microsomal metabolism screen in tandem to the *in vitro* potency screens. The most potent and metabolically stable compounds were investigated *in vivo*. This process led to the identification of N-(3,4-dimethyl-5-isoxazolyl)-4'-(2-oxazolyl)[1,1'-biphenyl]-2-sulfonamide (**3**, BMS-193884, figure 1) as a potent selective antagonist of ET_A that had improved potency and pharmacokinetics over **2**.

Materials and methods

Materials

All experimental compounds were synthesized at the Bristol-Myers Squibb Pharmaceutical Research Institute and were at least 97% pure. NADPH was obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were reagent grade or better.

Studies in rats

All procedures were approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee. The rats were housed under standard conditions and had free access to water and standard rodent laboratory diet.

Pharmacokinetic and bioavailability studies in rats. Adult male Sprague-Dawley rats (250–300 g, Harlan Sprague-Dawley, Indianapolis, IN, USA) were surgically prepared with indwelling jugular vein cannulae 1 day before drug administration. Rats were fasted overnight before dosing and were fed 8 h after dosing. The animals had free access to water and were conscious and unrestrained throughout the study. Each rat was given either a single intravenous or oral dose ($10\text{--}100\text{ }\mu\text{mol kg}^{-1}$, $n = 3$, both routes). The intravenous (i.v.) doses were administered as a bolus through the jugular vein cannula and the oral doses were by gavage. The compounds were administered as a solution in either propylene glycol (for i.v. studies) or 5% sodium bicarbonate (for oral studies). Blood samples (250 μl) were collected at serial time points for 12 h after dose into heparin containing tubes. Plasma was prepared immediately, frozen and stored at -20°C before analysis.

Metabolism studies with bile duct cannulated rats. Adult male Sprague-Dawley rats (300–350 g) were surgically prepared with an indwelling jugular vein, bile duct and duodenal cannulae 2 days before drug administration. The bile duct and duodenal cannulae were connected to allow normal bile flow during recovery. After dosing, the bile was diverted and control bile infused into the duodenal cannula at 1 ml h^{-1} . The rats were fasted overnight before dosing. The animals had free access to water and were conscious and unrestrained throughout the study. Each rat was given a single i.v. dose ($100\text{ }\mu\text{mol kg}^{-1}$, $n = 3$). The compounds were administered as solutions in propylene glycol. Bile and urine were collected for 12 h.

Plasma sample analysis

Plasma proteins were precipitated with 1 vol. acetonitrile and the supernatants evaporated to dryness under a stream of nitrogen. The samples were reconstituted in mobile phase and the compound of interest was analysed by ion-spray LC/MS. BMS-182874 [5-(dimethylamino)-N-(3,4-dimethylisoxazoyl)-1-naphthalene sulfonamide], a related ET receptor antagonist, was used as the internal standard. The LC/MS system consisted of a Hewlett Packard 1090 HPLC (Agilent Technologies, Wilmington, DE, USA) equipped with a Keystone Deltabond column (Keystone Scientific, Bellefonte, PA, USA, C8, $2 \times 100\text{ mm}$, held at 60°C) and a Sciex API I mass spectrometer (PE Sciex, Thornhill, Ont., Canada) with an ion-spray source. The mobile phase was water:methanol:ammonium acetate at a flow rate of 0.3 ml min^{-1} (split 1/6 before the source) eluted with a linear gradient. Protonated molecular ions were measured by single-ion monitoring. Sample quantitation was completed by comparison with a standard curve prepared in plasma and quality control samples were run to ensure assay performance. The quantifiable limit of the assay was 5 nM for all analytes. AUCs were calculated by the trapezoidal rule (oral) or by Lagrange integration (i.v.). The AUC_∞ after oral doses was calculated by extrapolating to infinity from the last measured concentration after oral administration with the half-life calculated from the i.v. data. In these experiments, this extrapolation added less than 10% to the total AUC for all compounds examined. Oral bioavailability was estimated by dividing the average AUC calculated for the oral route by the average AUC calculated for the i.v. route.

Bile and urine sample analysis

Bile and urine samples were analysed by HPLC-UV. Urine samples were analysed directly and bile samples mixed with 1 vol. acetonitrile and the supernatants analysed. The LC/MS system consisted of a Waters Millennium HPLC system (Millipore Corp., Bedford, MA, USA) equipped with a YMC-ODS AQ column (C18, $4.6 \times 150\text{ mm}$, 3 μ). The mobile phase was water:acetonitrile:ammonium acetate at a flow rate of 1.0 ml min^{-1} eluted with a linear gradient. Parent compound was quantified by comparison with limited standard curves (typically three-point curves prepared to bracket the observed experimental samples) of authentic material spiked into bile.

Microsomal metabolism

Liver microsomes were prepared from male Sprague-Dawley rats (250–300 g) by standard differential centrifugation methods (Raucy and Lasker 1991). Protein concentrations were determined by the Lowry method (Lowry *et al.* 1951). Incubations contained 56 mM potassium phosphate, about 5 mg ml^{-1} microsomal protein, 10 mM NADPH and 400 μM BMS compound (from a 10 mM stock in 5% sodium bicarbonate).

For the screening assays, the final incubation volume was 1.0 ml. Reactions were initiated by the addition of substrate and carried out in a shaking water bath at 37°C. Incubations were terminated by the addition of an equal volume of acetonitrile and centrifugation. The supernatants were analysed by HPLC with no additional processing. The LC/MS system consisted of a Waters Millennium HPLC system (Millipore) equipped with a YMC-ODS AQ column (C18, 4.6 × 150 mm, 3 µ). The mobile phase was water:acetonitrile: ammonium acetate at a flow rate of 1.0 ml min⁻¹ eluted with a linear gradient. Conditions were adjusted such that there was never more than 20% of the substrate consumed during the incubation. Rates of metabolism were calculated by measuring peak areas of metabolite(s) and parent and determining per cent metabolism by dividing the total metabolite peak area by the total drug related peak area. This method assumes that the molar extinction coefficient of the parent is the same as the metabolite. The per cent of metabolism is then multiplied by the total drug in the incubation to determine the moles of compound metabolized.

For the incubations done with compound **2** in order to purify metabolites for structure elucidation the final incubation volume was 10 ml. The incubation was quenched at 60 min. The metabolites that had the retention times of the major monohydroxylated compounds seen in bile were isolated. Isolation was accomplished by two steps of HPLC. The first step was with a Novapak column (octadecylsilyl, 3.9 × 150 mm, Waters) eluted with a linear gradient of 70%A:30%B to 20%A:80%B over 30 min (where A = 50 mM ammonium acetate (pH 5.0) and B = CH₃OH). The second step was with the same column with a linear gradient of 70%A:30%B to 20%A:80%B over 30 min (where A = 0.1% aqueous TFA and B = acetonitrile).

Structural elucidation of metabolites of 2

The major metabolites from a rat microsomal incubation were isolated as described above and characterized by LC-MS/MS and NMR. LC-MS/MS was carried out with a TSQ700 mass spectrometer (Finnegan MAT, San Jose, CA, USA) operated in the positive-ion electrospray mode. The LC conditions were as follows: a Novapak column (octadecylsilyl, 3.9 × 150 mm, Waters) was eluted at 1.0 ml min⁻¹ with a linear gradient from 70%A:30%B to 20%A:80%B over 30 min (where A = 50 mM ammonium acetate (pH 5.0) and B = CH₃OH). The retention times for the parent and two metabolites in this system were 14.6 min for **1**, 15.8 for **2** and 23.6 for parent. NMR spectra were recorded on a Bruker 500 MHz spectrometer (GSK-500, Bruker Instruments, Billerica, MA, USA). Compound **2**: LC-MS; *m/z* (relative intensity), 400 (M + H⁺, 100) 288 (8); ¹H-NMR (all spectra recorded in CD₂Cl₂); δ 0.86 (d, 6H, CH₂CH(CH₃)₂), 1.81 (s, 3H), 1.83 (m, 1H, CH₂CH(CH₃)₂), 2.07 (s, 3H), 2.39 (d, 2H, CH₂CH(CH₃)₂), 6.58–7.76 (m, 7H). Metabolite **1**: LC-MS; *m/z* (relative intensity), 416 (M + H⁺, 76), 398 (100), 286 (10); ¹H-NMR; δ 0.95 (d, 3H, CH₂CH(CH₃)CH₂OH), 1.90 (s, 3H), 1.96 (m, 1H, CH₂CH(CH₃)CH₂OH), 2.15 (s, 3H), 2.42 (m, 1H, CH₂CH(CH₃)CH₂OH), 2.73 (m, 1H, CH₂CH(CH₃)CH₂OH), 3.50 (m, 2H, CH₂CH(CH₃)CH₂OH), 6.70–7.85 (m, 7H). Metabolite **2**: LC-MS; *m/z* (relative intensity), 416 (M + H⁺, 100), 286 (6); ¹H-NMR; δ 1.25 (s, 6H, CH₂C(OH)(CH₃)₂), 1.90 (s, 3H), 2.15 (s, 3H), 2.74 (s, 2H, CH₂C(OH)(CH₃)₂), 6.75–7.84 (m, 7H).

Results

Pharmacokinetics of 2 in rats

The pharmacokinetic parameters for compound **2** after i.v. and oral administration to rats are shown in table 1. The compound had an intermediate rate of clearance and although there was a long elimination phase, this phase did not begin until plasma concentrations fell below 0.2 µM.

Biotransformation of 2 in rats

The biotransformation and excretion pathways of compound **2** were monitored after i.v. administration to bile duct cannulated rats. There was < 1% of the dose recovered as intact compound in the bile and urine over a 12-h post-dose collection (table 2). Metabolites were identified in the bile and urine by LC/MS-MS. The biotransformation profile included a variety of oxidation products. These included compounds with molecular weights of 415 (M + 16, monohydroxylation), 431 (M + 32, dihydroxylation), 413 (M + 14), 429 (M + 30) and 320 (M + 16 and -aminodimethylisoxazole) (data not shown). The major metabolites (as judged

Table 1. Pharmacokinetic parameters of compound **2** in rats.

Parameter	Units	Route of administration	
		i.v.	Oral
Dose	$\mu\text{mol kg}^{-1}$	100	100
AUC_{∞}	$\mu\text{M} \times \text{h}$	343 ± 33	166 ± 30
Cl	$\text{ml min}^{-1} \text{kg}^{-1}$	4.9 ± 0.5	
V_{ss}	l kg^{-1}	0.2 ± 0.1	
$t_{1/2}$	h	> 10	
MRT	h	0.7	
$T_{\max}$	h		0.4 ± 0.1
$C_{\max}$	μM		161 ± 19
Bioavailability	%		48

Rats were administered either a single i.v. or oral dose of **2** and blood samples collected for 12 h. Results are presented as mean $\pm$ SD ($n = 3$ for all routes).

Table 2. Biliary and urinary excretion of intact compound after intravenous administration to bile duct cannulated rats.

Compound	Percentage of dose excreted as intact parent	
	Bile	Urine
2	< 1	< 1
3	< 1	< 1
4	62	2.5

Bile duct cannulated rats were administered an i.v. bolus injection of **2–4** and bile and urine samples collected for 12 h. Data are derived from experiments with $n = 2$ (compound **4**) or $n = 3$ (compounds **2** and **3**) animals.

by UV absorbance) were two monohydroxylated metabolites present in high concentrations in bile.

Metabolism in rat and human liver microsomes

Compound **2** was rapidly metabolized in rat and human liver microsomes. Incubation of **2** with rat microsomes produced several metabolites, the major metabolites matched those found *in vivo* in the bile duct cannulated rat studies as judged by LC and LC-MS. These products were purified and characterized by LC-MS as monohydroxylated species, but the site of -OH addition could not be determined. Quantities sufficient to do $^1\text{H-NMR}$ were obtained and the spectra collected. From the assignment of these spectra, it was obvious that both of the sites of addition of -OH were on the 2-methylpropyl side chain. The spectrum of **1** was missing the typical 2-methylpropyl pattern of the parent with new signals at δ 0.95, 1.96, 2.42 and 3.50 ppm (figure 2, integration ratio of 3:1:2:2). The chemical shift, splitting and integration were all consistent with a -OH substitution on one of the terminal methyl groups. The spectrum of **2** was also missing the typical 2-methylpropyl pattern. The new signals observed for this metabolite were at δ 1.25 and 2.74 ppm (integration ratio of 6:2) consistent with a structure with an -OH substitution on the methine carbon of the parent. The metabolite structures are shown in figure 2.

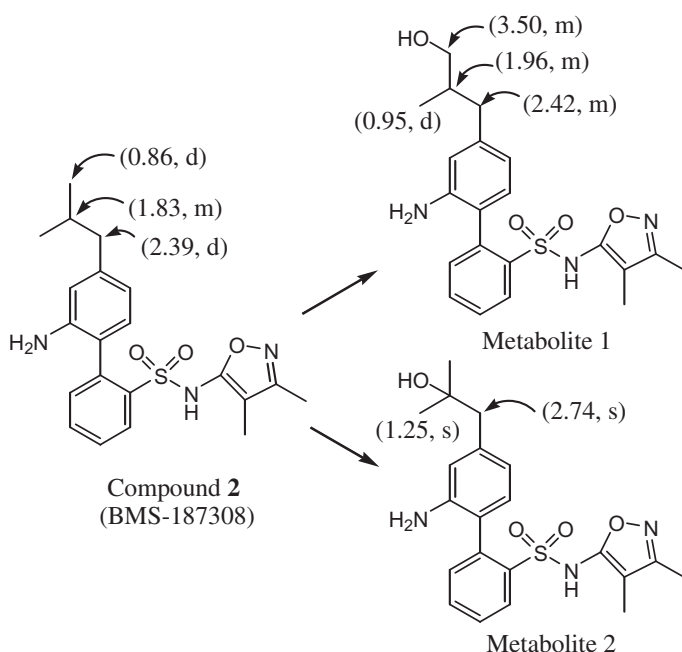


Figure 2. Metabolites of compound **2**. Assignment of the ¹H-NMR signals (chemical shift in ppm, multiplicity) for the 2-methyl propyl portion of the molecules is shown.

Microsomal stability

A series of analogues was synthesized that had alternate substituents at the 2'-position (replacement for the NH₂ group of **2**) or 4'-position (replacement for the isobutyl side chain of **2**) of the biphenyl ring system of **2** (table 3). The majority of the structural modifications were at the 4'-position and included a wide variety of alkyl, fluoroalkyl and heterocyclic ring substituents. These new analogues were tested for ET binding activity (see Murugesan *et al.* 1998, 2000, for details of this assay) and for stability in rat and human microsomal assays. Although the overall correlation was rather poor ($r^2 = 0.2$, figure 3) between rat and human results, there was a general trend seen between the values found in the two systems, i.e. compounds with poor stability in rat liver microsomes tended to have poor stability in the human liver microsomes.

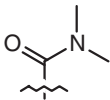
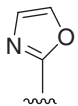
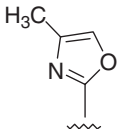
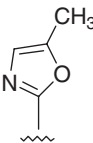
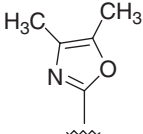
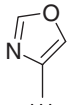
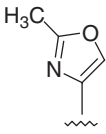
The resulting target activity versus stability results are shown in figure 4. There was a wide range of target activity and liver microsomal stability found for this set of analogues. Compound **3** had significant improvements in both ET binding activity and stability in rat liver microsomes. In addition, the stability in human liver microsomes was significantly improved when going from **2** (1.3 nmol product metabolized min⁻¹ mg⁻¹ protein) to **3** (0.2 nmol product metabolized min⁻¹ mg⁻¹ protein).

Clearance and biliary excretion of analogues

In parallel with the *in vitro* screening phase of the programme, the pharmacokinetic parameters of select analogues were examined. The results are given in figure 5. For six of the analogues tested, there was an excellent correlation found between *in vitro* microsomal stability and *in vivo* clearance. Importantly, the

Table 3. Rates of *in vitro* rat and human liver microsomal metabolism for a series of 2'- and 4'-substituted biphenylsulfonamides.

Compound	R	R'	Rate of liver microsomal metabolism (nmol min ⁻¹ mg ⁻¹ protein)	
			RLM	HLM
1	H	H	0.9	0.2
	H	NH ₂	0.1	ND
	Isopropyl	H	2.4	1.3
	Isobutyl	H	2.9	2.0
	Isobutyl	OH	1.8	0.8
	Isobutyl	CH ₂ OH	1.9	1.0
	Isobutyl	CH ₃	2.0	0.8
	Isobutyl	NHCH ₃	3.5	1.3
2	Isobutyl	NH ₂	1.5	1.3
	-NHCH(CH ₃) ₂	H	0.7	0.4
	-OCH(CH ₃) ₂	H	1.5	0.3
	-OCF ₂ H	H	1.4	0.3
	-CH(OH)CH(CH ₃) ₂	H	1.4	1.4
	-CF ₂ CH(CH ₃) ₂	H	1.7	1.1
	<i>t</i> -butyl	H	1.8	ND
		H	3.2	0.9
	5-isobutyl	H	2.4	1.3
			1.7	0.7
	<i>n</i> -butyl	H	1.4	0.7
	2,2 dimethylpropane	H	2.2	0.9
		H	1.8	0.8
	-CF ₃	H	1.3	0.3
		H	1.4	0.5

Compound	R	R'	Rate of liver microsomal metabolism (nmol min ⁻¹ mg ⁻¹ protein)	
			RLM	HLM
3		H	0.4	0.3
		H	0.5	0.2
		H	1.9	1.2
		H	0.7	1.6
		H	1.6	1.5
		H	1.6	0.1
		H	0.7	1.4
		H	0.1	0.1
4		H	0.8	0.3
		H	0.6	0.3
		CH ₂ NHCOCH ₃	0.2	0.2

ND, not determined; RLM, rat liver microsomes; HLM, human liver microsomes.

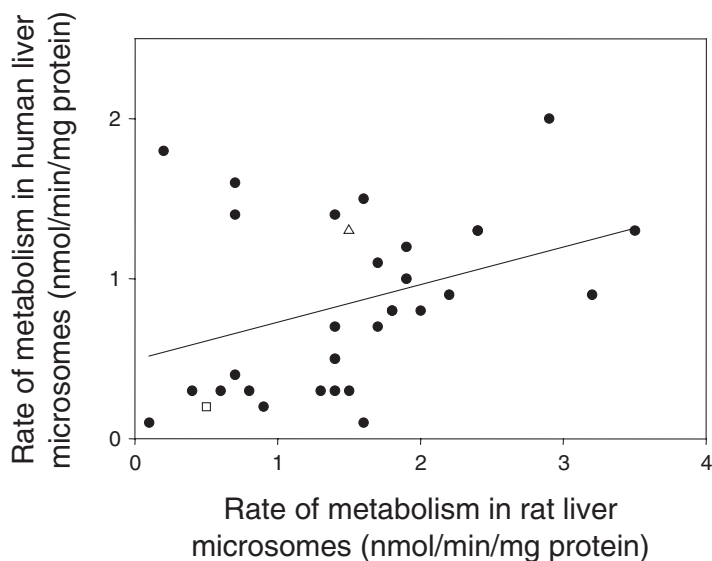


Figure 3. Plot of the rate of metabolism in rat and human liver microsomes metabolism for a series of 2'- and 4'-substituted biphenylsulfonamides. Key compounds **2** and **3** are depicted by an open triangle (Δ) or an open square ($\square$), respectively.

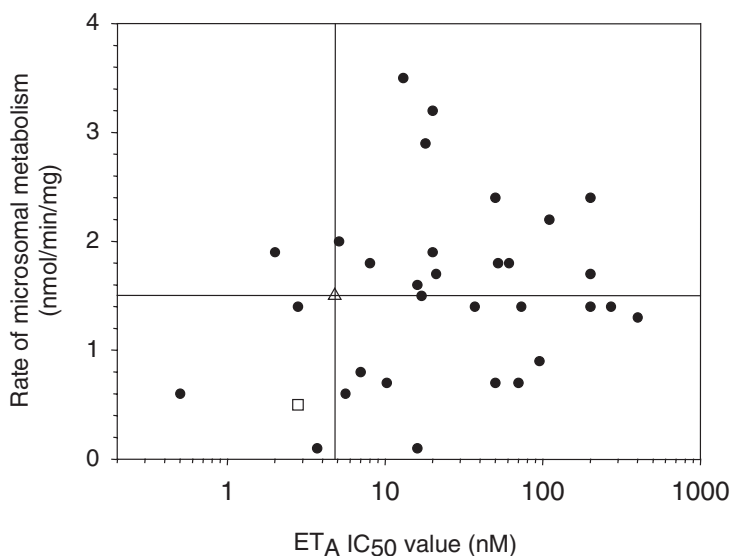


Figure 4. Plot of the ET_A IC_{50} values vs. the rat liver microsomal rate of metabolism for a series of 2'- and 4'-substituted biphenylsulfonamides. Key compounds **2** and **3** are depicted with an open triangle (Δ) or an open square ($\square$), respectively.

correlation held true for the compound identified with both improved potency and *in vitro* microsomal stability, compound **3**. Compound **3** had very low direct excretion into bile, similar to findings with **2** (table 2).

A very promising analogue subsequently synthesized was compound **4**. It displayed a much higher clearance rate than predicted from *in vitro* metabolism

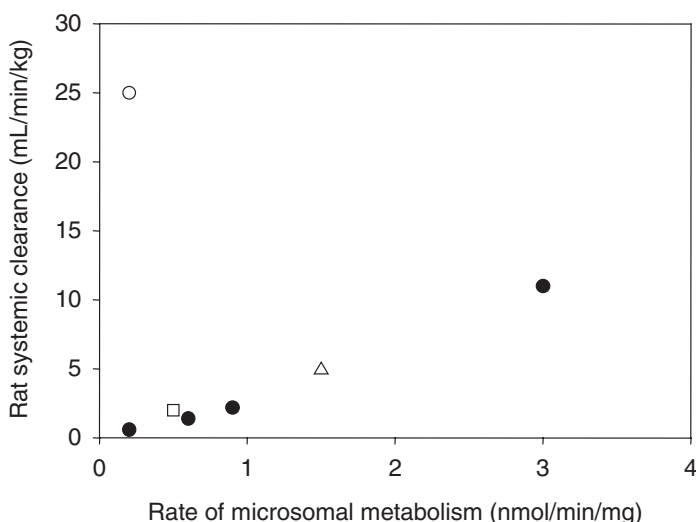


Figure 5. Plot of the rat liver microsomal rate of metabolism vs. the *in vivo* clearance for a series of analogues of **2**. Key compounds **2**, **3** and **4** are depicted with an open triangle ($\triangle$), an open square ($\square$), or an open circle ($\circ$), respectively.

Table 4. Pharmacokinetic parameters of compound **3** in rats.

Parameter	Units	Route of administration		
		i.v.		Oral
Dose	$\mu\text{mol kg}^{-1}$	10	100	10
AUC $_{\infty}$	$\mu\text{M} \times \text{h}$	72 ± 28	840 ± 110	31 ± 15
Cl	$\text{ml min}^{-1} \text{kg}^{-1}$	2.6 ± 1.0	2.0 ± 0.3	
V_{ss}	l kg^{-1}	0.08 ± 0.03	0.19 ± 0.04	
$t_{1/2}$	h	2.0 ± 0.5	2.3 ± 0.4	
MRT	h	0.51 ± 0.08	1.6 ± 0.4	
T_{max}	h			0.40 ± 0.10
C_{max}	μM			24 ± 10
Bioavailability	%			43

Rats were administered either a single i.v. or oral dose of **3** and blood samples collected for 12 h. Results are presented as mean $\pm$ SD ($n=3$ for all routes).

studies (figure 5). Compound **4** had a clearance of $25 \text{ ml min}^{-1} \text{kg}^{-1}$ and a volume of distribution of 0.4 l kg^{-1} in rats. The oral bioavailability for this compound was 8%. The compound was administered to bile duct cannulated rats and examination of the bile revealed that much of the rapid clearance of the compound was due to direct elimination of the parent compound via biliary excretion (table 2).

Pharmacokinetics of compound **3**

The pharmacokinetic parameters of **3** are listed in table 4. The compound was dosed at 10 and $100 \mu\text{mol kg}^{-1}$ and found to have similar pharmacokinetic parameters at both doses. Compound **3** is cleared less rapidly than **2** and this, along with a lower volume of distribution, yields a substantial increase in AUC (figure 6). The compound also had a good exposure after oral administration with a C_{max} of $24 \mu\text{M}$ and an oral bioavailability of 43%.

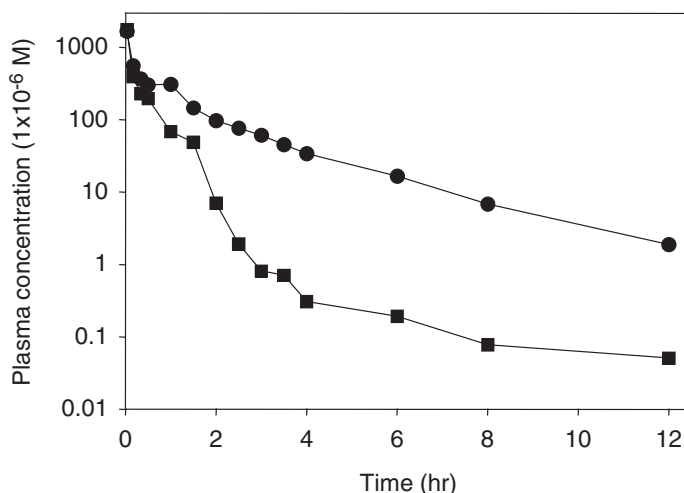


Figure 6. Plasma concentration vs. time profile for **2** (■) or **3** (●) after i.v. administration (both compounds administered at 100 μ mol/kg). Data are derived from $n = 3$ animals.

Discussion

The simultaneous optimization of the pharmacokinetic and potency properties of compounds during the discovery phase has become routine practice in modern drug discovery. The current study provides an example of a series of ET antagonists that were simultaneously optimized via structure–activity and structure–metabolism relationships for ET binding and *in vitro* microsomal metabolism.

Work with an initial, potent ET antagonist lead compound (**2**) showed that plasma levels of the compound declined rapidly after administration to rats and that the compound was rapidly metabolized in rat and human *in vitro* systems. The elimination half-life was reasonable (>10 h), but the elimination phase did not begin until the plasma concentrations of the compound had dropped over four orders of magnitude from the initial concentrations and fell below 0.2 μ M, a profile that would make maintenance of efficacious plasma concentrations difficult. In addition, the mean residence time for this compound (0.7 h) was far less than the half-life, indicating that the bulk of the elimination of the drug took place before entry into the terminal elimination phase.

The rapid decline in plasma concentrations was likely due to a number of factors, including extensive tissue distribution and metabolic clearance. To improve the plasma concentration profile, i.e. increase total plasma concentrations and decrease peak-to-trough ratios (ratio of the highest to lowest plasma concentrations over a defined dosing interval), the factors involved in the metabolic clearance were investigated further.

The metabolic clearance of the compound was shown to be predominately via oxidative metabolism. This is based on the low recovery of parent in bile and urine (table 2) over a 12-h collection along with the substantial decline in plasma concentrations over that period. Also, there were a number of oxidative metabolites present in bile, and although an absolute recovery of drug-related material could not be determined, the only clearance pathway represented in excreta was metabolic clearance via oxidative metabolism. However, the decline in plasma

concentrations seen in the study could be due to tissue uptake of parent or non-oxidative metabolite and although it is less likely, this possibility cannot be ruled out.

The oxidative metabolism of the compound *in vitro* was also quite extensive with rapid rates of metabolism found in both rat and human liver microsomes. The major metabolites were the same *in vivo* and *in vitro*, so the *in vitro* system was used to generate metabolites for full structure elucidation. The determination of the isobutyl side chain as the major site of oxidative biotransformation led to the start of structure–activity and structure–metabolism studies to try to maintain potency while minimizing metabolism and clearance.

Liver microsomal stability and ET binding were selected as the primary optimization parameters because of the known metabolism liability and because the compound was shown to be highly permeable and was predicted to have excellent *in vivo* absorption (data not shown). For these reasons, an *in vitro* rat and human liver microsomal stability screening programme was instituted along with the determination of ET binding. The screening was done at 400 μ M to facilitate rapid analysis with HPLC–UV detection. The high concentrations also allowed for metabolite peaks to be integrated and used for determination of rate, thus allowing rates to be determined with less than 20% parent depletion. Although these rates are determined at concentrations likely to be greater than the K_m for the cytochrome P450 enzymes involved in the metabolism, the good correlation of *in vitro* rates with *in vivo* clearance values (figure 5) indicates that either (1) the K_m values for these compounds are very high and this concentration is still within the linear portion of the velocity versus concentration plot or (2) that the K_m values for the set of closely related analogues are fairly consistent and the larger variations are in V_{max} values.

Human liver microsome stability was the primary parameter optimized, although the compound with the best overall characteristics had a reduced rate of rat and human liver microsomal metabolism. The poor overall correlation between the rat and human values ($r^2=0.2$, figure 3) points to the importance of determining both parameters in order to describe adequately the behaviour in the pharmacology model as well as predicting the behaviour in human. To define the correlation of *in vitro* and *in vivo* results, pharmacokinetic parameters of select analogues were determined for key compounds during the progression of the discovery programme (figure 5).

Medicinal chemistry efforts focussed on the replacement of the butyl side chain of compound **2**. The goal of this search was to find compounds that fell into the lower left quadrant of the plot shown in figure 4, i.e., compounds that had both increased potency and increased metabolic stability. A wide structural search for replacements for the butyl side chain showed that both potency and microsomal metabolism could be substantially changed. This search was mainly driven by medicinal chemistry approaches to define structure activity relationships and improve the receptor binding of the compounds. Alkyl substituted compounds were generally metabolized very rapidly, especially in comparison with the non-substituted compound **1**. This was true for compounds with or without substitution at the 2-position. In general, small heterocyclic substituted compounds had relatively low rates of microsomal metabolism and very good potency. Oxazoles looked especially promising and the 2'-substituted oxazole, compound **3**, was taken on for further study. Besides having a good balance of potency and ET binding the

compound had a more facile synthesis than some other equally promising compounds (i.e. other oxazole isomers).

Compound **3** showed improved *in vitro* properties and importantly showed significant improvement in pharmacokinetic parameters (figure 6). The compound had a decreased rate of metabolism in rat liver microsomes ($1.5\text{--}0.5\text{ nmol min}^{-1}\text{ mg}^{-1}$ protein) in comparison with **2** that translated to an approximately twofold decrease in rat clearance (4.9 to $2.0\text{ ml min}^{-1}\text{ kg}^{-1}$). The drop in metabolic rate in rat liver microsomes for **3** was also observed in human liver microsomes, but was more significant ($1.3\text{--}0.2\text{ nmol min}^{-1}\text{ mg}^{-1}$ protein) in this system. The decrease in clearance for **3** led to a substantial increase in plasma concentrations in the rat (figure 6). Also, **3** showed compelling pharmacodynamic properties in the rat. The compound gave an excellent i.v. and oral dose response in inhibition of the big-endothelin pressor response and lowered mean arterial pressure in DOCA salt hypertensive rats for 12 h following a $30\text{ }\mu\text{mol kg}^{-1}$ oral dose (Murugesan *et al.* 2000). Although **3** has improved properties, it is still not completely bioavailable and this is likely due to first-pass metabolism. Examination of the bile of rats administered this compound reveals a number of oxidative metabolites, with the major metabolites formed from biotransformation of the oxazole portion of the molecule (Chando *et al.* 1999). This demonstrates that, at least for **3**, the preferred orientation for binding to the cytochrome P450 active site presents the same part of the molecule to the haem as was the case for **2**.

Further structural work uncovered a very promising analogue, compound **4**. It showed improved *in vitro* characteristics even better than **3**, but *in vivo* tests showed there was a significant drop in pharmacokinetic performance for this compound (figure 5). Subsequent studies found that the very rapid clearance of the new analogue was not due to metabolic clearance but mediated by direct biliary clearance. Further, the rapid biliary clearance of the parent compound accounted for 62% of the total plasma clearance of the compound, which was measured as $25\text{ ml min}^{-1}\text{ kg}^{-1}$. Therefore, the compound was cleared by direct biliary excretion at a rate of $15.5\text{ ml min}^{-1}\text{ kg}^{-1}$, a rate far in excess of normal bile flow. This indicates that the excretion must be due to active transport and further exploration of this will be the subject of a separate paper. This surprising result underscores the need continuously to correlate any *in vitro* model with *in vivo* data because even within a chemotype, slight structural modifications can lead to very different *in vivo* drug disposition profiles. The finding also makes predictions of human clearance very difficult because the *in vitro* models for prediction of biliary clearance are still relatively new (Liu *et al.* 1999) and have not been widely applied. The advancement to clinical development of a compound with the properties of **4** would entail a high degree of risk because of the lack of understanding of clearance mechanisms in animals for this compound and the difficulties in making extrapolations to human for compounds cleared via biliary elimination.

The discovery of **3** was aided through the derivation of structure-metabolism relationships for the lead compound and analogues that were derived in parallel with the structure-activity relationships. The application of these techniques is most successful when the pathways of metabolic clearance are established with a lead compound. In addition, a key component in this type of study is the periodic revalidation of the relationship between the *in vitro* and *in vivo* parameters, in this case between microsomal metabolism and systemic clearance. As demonstrated with **4**, because of the multifactorial nature of systemic clearance, optimization of

an *in vitro* parameter will not always lead to optimization of *in vivo* properties, even for very closely related structural analogues.

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Stereoselective hepatic disposition of a diastereomeric pair of $\alpha_v\beta_3$ antagonists in rat

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Received 11 April 2003

1. The study investigated mechanisms underlying the stereoselective hepatic disposition observed in rats of a zwitterionic diastereomeric pair ((3*S*)-3-[(3*R* or 3*S*)-2-oxo-3-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propyl]pyrrolidin-1-yl]-3-quinolin-3-ylpropanoic acid) with different lipophilicities.

2. In a recirculating isolated rat liver system, the more hydrophilic diastereomer **II** possessed biliary clearance, CL_b , and bile-to-liver concentration ratio higher (about 10–30-fold) than the lipophilic zwitterion **I**, whereas both **I** and **II** exhibited comparably high concentration ratios between liver and perfusate. Although MK-571, a known multidrug resistance protein (MRP) inhibitor, significantly inhibited the CL_b of both compounds, it did not inhibit their canalicular transport, as evident by unchanged concentration ratios between bile and liver of either **I** or **II**.

3. Following an intravenous infusion of **I** or **II** to Sprague–Dawley rats, the biliary clearance calculated either based on plasma ($CL_{b,p}$) or liver concentration ($CL_{b,l}$), of **II** was much higher than that of **I** (about 5–50-fold). In rats lacking multidrug resistance protein 2 (Mrp2) (Eisai hyperbilirubinemic rat, EHBR), the biliary excretion rate and $CL_{b,p}$ of **II** were also higher than the corresponding values for **I**. However, both $CL_{b,p}$ or $CL_{b,l}$ of either **I** or **II** were not reduced in EHBR, as compared with control SD rats.

4. In the *in vitro* rat canalicular membrane vesicle study, **I** and **II** exhibited no differences in their inhibitory effect on the Mrp2 mediated ATP-dependent [^3H]DNP-SG initial uptake (no inhibition at 10 μM and only about 40% inhibition at 100 μM).

5. Collectively, these results suggested that (1) the difference in the hepatic disposition between the two isomers was due primarily to the difference in their transport mechanism across the canalicular membrane and (2) Mrp2 did not play a major role in the observed differences in the biliary excretion of the diastereomers **I** and **II** in rats.

Introduction

Compound **I** ((3*S*)-3-[(3*R*)-2-oxo-3-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propyl]pyrrolidin-1-yl]-3-quinolin-3-ylpropanoic acid) and its diastereomer **II** ((3*S*)-3-[(3*S*)-2-oxo-3-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propyl]pyrrolidin-1-yl]-3-quinolin-3-ylpropanoic acid) are zwitterions at physiological pH, but with marked differences in lipophilicity; $\log P$ (octanol/buffer pH 7.4) = 1.04 for **I** versus $\log P$ = –0.05 for **II**. Both **I** and **II** are potent antagonists of the $\alpha_v\beta_3$ receptor, which has been shown to play a key role in bone resorption processes (Hartman and Duggan 2000). Recently, pronounced differences in the

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absorption and disposition between the two isomers have been reported in rats (Prueksaritanont *et al.* 2002). The lipophilic diastereomer **I** was shown to be absorbed better than **II**, while **II** was shown to exhibit a higher (about fivefold) biliary clearance (CL_b) than **I**. Based on a combination of *in vitro* and *in vivo* approaches, it was concluded that **I**, but not **II**, was a good substrate for P-glycoprotein (P-gp), and that P-gp was not an important determinant for the observed differences in either the intestinal absorption or hepatic disposition of this diastereomeric pair (Prueksaritanont *et al.* 2002). Whereas the higher absorption of **I**, as compared with **II**, could be attributable primarily to its higher intrinsic permeability, the underlying mechanism responsible for the observed differences in the biliary excretion between the diastereomeric pair was not examined in Prueksaritanont *et al.* (2002).

Hepatobiliary excretion of drugs from blood to bile usually involves two key sequential events: uptake from blood to hepatocytes via the sinusoidal membrane, and secretion into bile across the canalicular membrane. Once inside the cells, compounds could also be transported back to blood via a sinusoidal efflux. To date, a wide variety of carrier proteins have been shown to be present on both the sinusoidal and canalicular membranes of hepatocytes (Kullak-Ublick *et al.* 2000, Ayrton and Morgan 2001). Of the identified canalicular transporters, MRP2/Mrp2 (human and rat isoforms, respectively), a member of the multidrug resistance protein (MRP) family, and P-gp have been shown to play an important role in the biliary excretion of several compounds (Schinkel *et al.* 1995, Meijer *et al.* 1997, Smit *et al.* 1998, König *et al.* 1999a, Ayrton and Morgan 2001). Substrates of P-gp are primarily, but not limited to, cationic drugs (Ford and Hait 1990, Schinkel *et al.* 1996), whereas those for MRP2/Mrp2 are mostly anionic drugs and drug conjugates (König *et al.* 1999a, Payen *et al.* 2000). Interestingly, whereas substrates for P-gp are usually lipophilic in nature, substrates for MRP2/Mrp2 are usually hydrophilic. Consistent with this notion, only **I**, but not **II**, was shown to be a substrate for P-gp (Prueksaritanont *et al.* 2002). Accordingly, it remains a possibility that the hydrophilic diastereomer **II** serves as a Mrp2 substrate, and that Mrp2 might play a significant role in the observed difference in the hepatic disposition between the diastereomeric pair in rats.

Thus, in view of the above considerations, the present study sets out to investigate the underlying mechanism responsible for the observed differences in the biliary excretion between the diastereomeric pair. First, the hepatic disposition of **I** and **II** was characterized using an isolated rat liver perfusion and *in vivo* rat models. A possible involvement of rat Mrp2 in the stereoselective hepatic disposition was then explored using a combination of *in situ*, *in vivo* and *in vitro* approaches. The latter study involved the isolated rat liver perfusion model in the presence of MK-571, a known inhibitor for MRP (Gekeler *et al.* 1995, Leier *et al.* 1996); *in vivo* in Eisai hyperbilirubinemic rats (EHBR), known to lack Mrp2 in the liver; and *in vitro* using rat liver canalicular membrane vesicles (CMV). The results described in this paper pointed to the difference in canalicular transport as a major contributing factor to the observed difference in the hepatic disposition between the two diastereomers, and suggested that like P-gp, Mrp2 played a minor role, and that other yet to be identified canalicular transporter(s) played an important role in the stereoselective hepatic disposition of the diastereomers **I** and **II** in rats.

Materials and methods

Materials

I and its diastereomer **II** (>98% purity), and MK-571 were synthesized at Merck Research Laboratories (West Point, PA, USA). [^3H]2,4-dinitrophenyl glutathione ([^3H]-DNP-SG) and cold DNP-SG were synthesized and purified according to the methods of Kobayashi *et al.* (1990). Radiolabelled positions on [^3H]DNP-SG were [glycine-2- ^3H] in the glutathione moiety of the molecule. The purity of [^3H]DNP-SG was >98%, as assessed by TLC (Oude *et al.* 1989). [Glycine-2- ^3H]-glutathione (44.8 Ci mmol $^{-1}$, purity >97%) and [^3H (G)]-taurocholic acid (3.47 Ci mmol $^{-1}$, purity >97%) were purchased from New England Nuclear Corp. (Boston, MA, USA). Taurocholic acid (sodium salt), 1-chloro-2,4-dinitrobenzene, glutathione *S*-transferase (from rat liver), ATP (disodium salt) and AMP (sodium salt) were from Sigma Chemical Co. (St Louis, MO, USA). Creatine phosphate and creatine phosphokinase (from rabbit muscle) were from Boehringer Mannheim GmbH (Mannheim, Germany). Solvents used for analysis were of analytical or HPLC grade (Fisher Scientific, Pittsburgh, PA, USA).

Male Sprague-Dawley (SD) rats or EHBR (200–320 g) were obtained commercially from Taconic Laboratories (Germantown, NY, USA), and Japan SLC, Inc. (Shizuoka, Japan), respectively.

Liver perfusion studies

The studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Male SD rats (250–320 g), with an indwelling cannula implanted in the portal vein and vena cava for perfusion of medium and in the common bile duct for bile sampling, were used for studies. *In situ* liver perfusion was carried out in a recirculating mode at 37°C within a thermostatically controlled cabinet. The perfusion medium was an erythrocyte- and albumin-free Krebs-bicarbonate buffer (100 ml, pH 7.4) containing **I** or **II** (10 μM), and was continuously gassed with humidified carbogen (5% CO_2 , 95% O_2). This concentration of **I** and **II** was in a comparable range with that obtained in our previous *in vivo* study (Prueksaritanont *et al.* 2002). The perfusion was pumped into the liver through the portal vein at 30 ml min $^{-1}$. A constant infusion of sodium taurocholate (1.4 $\mu\text{mol ml}^{-1}$) into the perfusion medium was used to maintain adequate concentrations of bile acid. Perfusate (0.4 ml) was sampled directly from the perfusate reservoir at 2, 5, 10, 15, 25, 30, 35, 40, 45, 50 and 60 min. Bile was collected at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 60 min in a preweighed vial containing 5 μl 1 M hydrochloric acid to minimize the hydrolysis of a glucuronide conjugate of **I** or **II** (Prueksaritanont *et al.* 2002). All samples were stored frozen until analysis by HPLC. The viability of each liver preparation was assessed by monitoring bile flow (>6 $\mu\text{l min}^{-1}$), the pH of the perfusate (7.3–7.5) and the gross appearance of the organ (uniformly pink to brown).

For inhibition studies, MK-571 (50 μM) was added to the reservoir at 20 min after the addition of **I** or **II**. At this concentration, MK-571 has been shown to exhibit a maximal and specific inhibitory effect on MRP-mediated transport, but without cell toxicity, in a cell-based assay in the presence of albumin, a condition closer to an *in vivo* situation (Gekeler *et al.* 1995). Bile, perfusate and liver samples were collected at the same time intervals as above.

In vivo studies

The studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Male SD rats or EHBR (250–320 g), with an indwelling cannula implanted in a carotid artery (for drug administration), jugular vein (for blood sampling) and the common bile duct (for bile sampling), were used. **I** or **II** was infused through the carotid artery at 10 mg min $^{-1}$ kg $^{-1}$ for 2 h following an intravenous bolus dose of 0.35 or 0.15 mg kg $^{-1}$, respectively. Blood and bile samples were obtained for every 20 min during infusion. At the end of infusion, the liver was excised, rinsed and homogenized with saline. Aliquots of plasma, bile and liver homogenate were mixed with acetonitrile (2:1 v/v) to precipitate proteins. Following centrifugation, the supernatant was analysed by the HPLC methods as described below.

In vitro transport inhibition studies

CMV were prepared as described (Takenaka *et al.* 1995, Yamazaki *et al.* 1997) according to the method of Kobayashi *et al.* (1990) and were suspended in 50 mM Tris/HCl buffer (pH 7.4) containing 250 mM sucrose. Protein concentration was determined by the method of Bradford (1976), using the Bio-Rad protein assay kit with bovine serum albumin as a standard. The CMV employed maintained characteristics (integrity) of bile canalicular membranes, based on Western blot analysis for P-glycoprotein (P-gp; Abcb1a, Abcb1b and Abcb4), using a C219 monoclonal antibody (Lin *et al.* 1999), and ATP-dependent uptake of [^3H]taurocholic acid (TCA), a prototypical marker substrate for rat bile salt export pump (sister P-gp, Abcb11, Meier and Stieger 2002).

The transport study using CMV was performed by a rapid filtration method reported by Yamazaki *et al.* (1996). Radioactivity retained on the filter (0.45 mm GVWP, Millipore Corp., Bedford, MA, USA) was determined and the uptake amount was normalized with respect to the amount of membrane

protein (pmol mg^{-1} protein). In brief, CMV ($10\mu\text{g}$ protein) was incubated with ATP (5mM) plus ATP-regenerating system (10mM creatine phosphate, $100\mu\text{g ml}^{-1}$ creatine phosphokinase) or AMP (5mM) in the medium containing [^3H]-DNP-SG ($1\mu\text{M}$). The effect of **I**, **II** and unlabeled DNP-SG on [^3H]-DNP-SG ($1\mu\text{M}$) uptake was determined at 2 min in the presence and/or absence of ATP.

Analytical procedure

An HPLC method with fluorescence detection as described (Prueksaritanont *et al.* 2002) was used for the determinations of **I** and **II** in plasma, bile and liver. In brief, perfusate, plasma, bile and liver homogenate samples, with or without appropriate dilutions, were analysed after protein precipitation with acetonitrile (1:1 v/v). The supernatant was chromatographed on a Spherisorb SCX column ($4.6 \times 250\text{ mm}$, $5\mu\text{m}$), with the mobile phase (A = acetonitrile, B = 25 mM phosphate buffer, pH 6) delivered at 1 ml min^{-1} (27% A for 5 min, then to 60% A in 1 min, and held at 60% A for 6 min). The effluent was monitored at λ_{ex} and λ_{em} of 330 and 395 nm, respectively. For all matrices examined, the intra- and interday assay precision (%CV) and accuracy was $< 15\%$ for analytes **I** and **II**.

Data analysis

Total clearance (CL) of **I** and **II** was calculated as the dose divided by the area under the perfusate concentration–time curve from time zero to infinity, AUC (liver perfusion studies), or as the dosing infusion rate divided by the average plasma concentration at steady-state (C_{pss}) (*in vivo* studies). CL_{b} was calculated as the amount excreted in bile divided by the AUC obtained during the same collection period. For *in vivo* studies, the CL_{b} defined based on plasma ($\text{CL}_{\text{b,p}}$) or liver concentration ($\text{CL}_{\text{b,l}}$) was calculated by dividing the biliary excretion rate by C_{pss} or liver concentrations at the end of infusion, respectively. In liver perfusion studies, concentrations of **I** and **II** in the perfusate, bile and liver homogenate at 60 min were used to calculate the concentration ratios in liver to perfusate and in bile to liver.

Statistical analysis was performed using ANOVA (Statview, Abacus Concepts, Berkeley, CA, USA). $p < 0.05$ was considered as statistically significant.

Results

Liver perfusion studies

In an isolated rat liver, both compounds were taken up rapidly into the liver, as evident by a sharp decline in perfusate concentrations during the first 10 min (figure 1A). At 10 min, the perfusate concentrations of **I** and **II** were only about one-tenth of their starting concentrations. Thereafter, the perfusate concentration of **I** declined slower than those of **II**, resulting in about a fivefold higher concentration than that of **II** at the end of experiment (60 min) (figure 1A). As was observed previously in this species (Prueksaritanont *et al.* 2002), the CLs of both compounds were comparable, whereas the biliary excretion of the hydrophilic zwitterion **II** was more extensive and faster than that of **I** (table 1). Over the entire study period, the values for biliary recovery, biliary excretion rate and CL_{b} of **II** were about 7–10-fold higher than the corresponding values for **I** (figure 1B, C and table 1). At the end of the study (60 min), **I** was found in the liver at higher levels (about threefold) than **II** (table 1). Consequently, bile-to-liver concentration ratios of **II** were much higher (about 30-fold) than that of **I**, whereas liver-to-perfusate concentration ratios of the two diastereomers were comparable (table 1). In the case of **II**, but not **I**, bile-to-liver and liver-to-perfusate concentration ratios were both greater than 1 (about 30 and about 2900 for **II**, and about 1 and 2300 for **I**, respectively). In addition, the total recovery (bile + liver + perfusate) of unchanged drug after 60 min perfusion for **I** was significantly lower than that for **II**, suggesting that **I** was metabolized to a greater extent than **II**. In the case of **II**, the total recovery was $> 70\%$, suggesting modest metabolism. These results were in good agreement with those obtained previously in rats (Prueksaritanont *et al.* 2002).

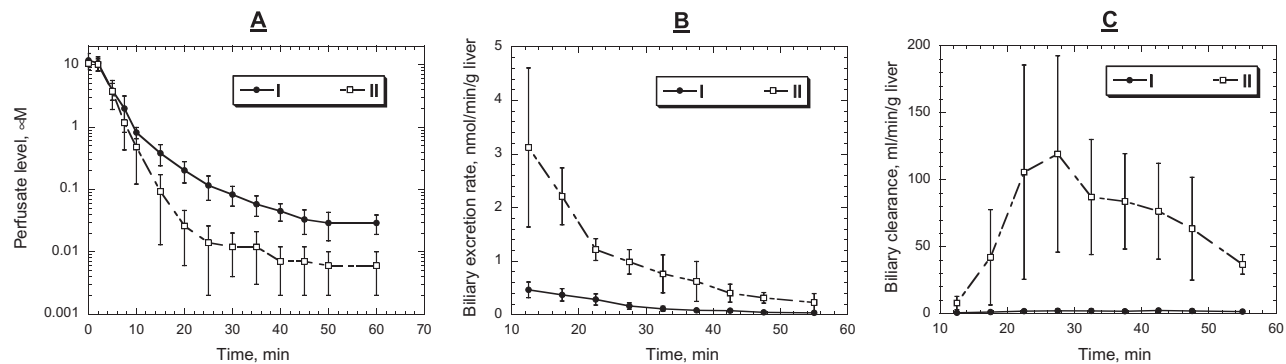


Figure 1. Perfusate concentration–time profiles (A), biliary excretion rate–time profiles (B) and biliary clearance–time profiles (C) of **I** and **II** in isolated rat liver perfusion. Results are mean $\pm$ SD, $n=4$. **I** and **II** were administered as a bolus dose of 1 μ mol (equivalent to an initial concentration of 10 μ M) at $t=0$ min.

Table 1. Comparison of parameters of **I** and **II** between control livers perfused with **I** or **II** (10 μ M) only and livers with MK-571 (50 μ M) co-infused after 20-min perfusion with **I** or **II**.

Parameter	Unit	Time ^a (min)	Control		With MK-571	
			I	II	I	II
CL	ml min ⁻¹ g ⁻¹ liver	0–60	1.9 $\pm$ 0.4	2.0 $\pm$ 0.8	1.2 $\pm$ 0.2**	1.6 $\pm$ 0.7
CL _b	ml min ⁻¹ g ⁻¹ liver	0–60	0.15 $\pm$ 0.03	1.2 $\pm$ 0.7*	0.12 $\pm$ 0.02	1.4 $\pm$ 0.7
		25–60	1.4 $\pm$ 0.5	74.9 $\pm$ 36*	0.26 $\pm$ 0.02**	12.5 $\pm$ 6.9**
Liver content	nmol g ⁻¹ liver	60	49.6 $\pm$ 6.7	15.3 $\pm$ 5.6*	42.8 $\pm$ 2.6	16.2 $\pm$ 2.4
Bile/liver concentration ratio		60	1.0 $\pm$ 0.1	28 $\pm$ 5*	0.9 $\pm$ 0.2	37 $\pm$ 8
Liver/perfusate concentration ratio		60	2288 $\pm$ 990	2948 $\pm$ 1080	184 $\pm$ 9**	405 $\pm$ 162**
Amount excreted in bile	% of dose	0–60	8 $\pm$ 2	58 $\pm$ 10*	8 $\pm$ 2	72 $\pm$ 17
Total recovery ^b	% of dose	0–60	53 $\pm$ 11	73 $\pm$ 5*	57 $\pm$ 8	90 $\pm$ 8

Data are the mean $\pm$ SD, n = 3 (with MK-571) to 4 (control).

^aTime at/during which measurement was taken or pharmacokinetic parameters were calculated.

*Statistically different (p < 0.05) from **I**.

**Statistically different (p < 0.05) from control.

^bRecovery of parent drug in perfusate + bile + liver.

MK-571 markedly increased the perfusate concentrations (up to 10-fold), and significantly decreased the biliary excretion (about 70–80%) of both **I** (figure 2A, B) and **II** (figure 2C, D). As a result, CL_b obtained after the addition of MK-571 (CL_b obtained during 25–60 min) of the two compounds was significantly decreased (>90%) by MK-571 (table 1). However, for both **I** and **II**, MK-571 did not affect the liver content or the liver-to-bile concentration ratio, whereas it significantly decreased (about 90%) the liver-to-perfusate concentration ratio (table 1). In the case of **I**, but not **II**, a significant decrease of about 40% in CL also was observed in livers perfused with MK-571.

In vivo studies

In control SD rats, plasma levels of both **I** and **II** appeared to reach the same steady-state level (about 700–800 nM) within 10 min into the infusion under the present study protocol (figure 3A, B). However, the biliary excretion rate of **II** was much higher than that of **I** over the entire study period (figure 3C, D). As was observed in the isolated rat liver perfusion system and in our earlier *in vivo* study

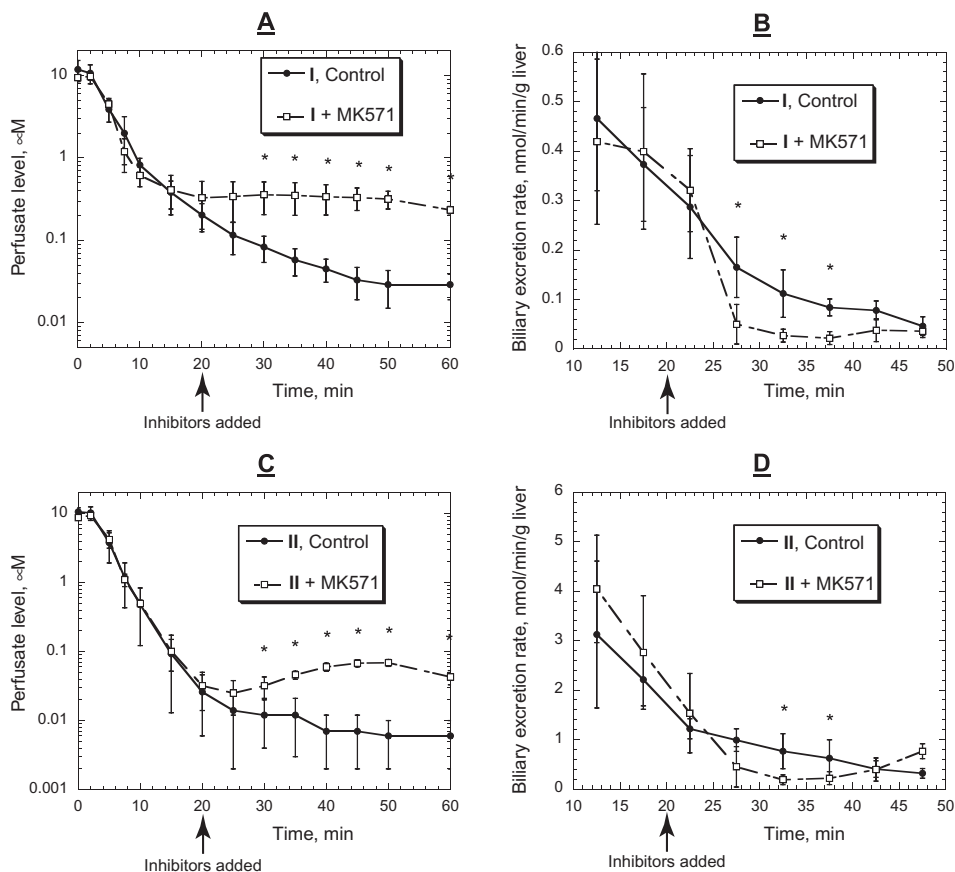


Figure 2. Perfusate concentration–time profiles of **I** (A) and **II** (C) and biliary excretion rate–time profiles of **I** (B) and **II** (D) in isolated rat liver perfusion in the absence and presence of MK571. Results are mean $\pm$ SD, $n = 3$ –4. **I** and **II** were administered as a bolus dose of 1 μ M (equivalent to an initial concentration of 10 μ M) at $t = 0$ min, and MK571 (5 μ M) was added to the perfusate at $t = 20$ min. *Statistically different from control.

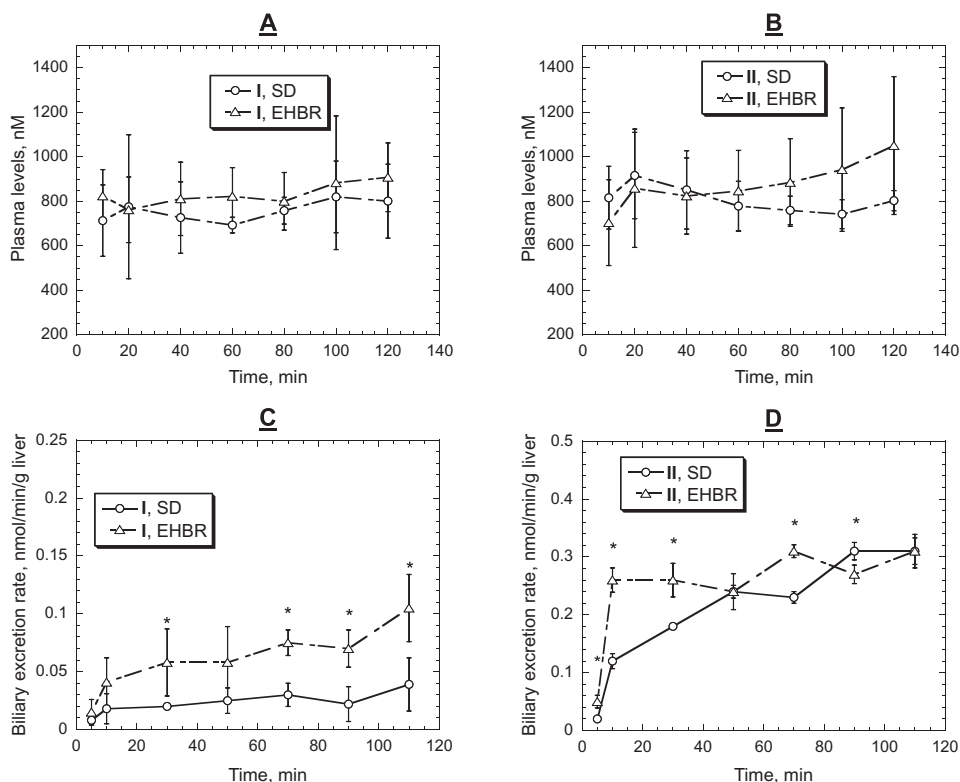


Figure 3. Plasma concentration–time profiles of **I** (A) and **II** (B) and biliary excretion rate–time profiles of **I** (C) and **II** (D) in control SD rats and EHBR. Results are means $\pm$ SD ($n=4$) obtained following an intravenous bolus dose of 0.35 or 0.15 mg kg⁻¹, respectively, followed by an intravenous infusion of **I** or **II** at 10 mg min⁻¹ kg⁻¹ for 2 h to rats. *Statistically different from control SD rats.

following an intravenous bolus injection (Prueksaritanont *et al.* 2002), CLs of the two isomers in SD rats were comparable with each other (as well as to those observed in the earlier liver perfusion and *in vivo* studies), whereas CL_b was about eightfold higher for **II** than for **I** (table 2). Additionally, the liver content obtained at the end of infusion of **I** was significantly higher (about fourfold) than that of **II**. As a consequence, CL_{b,l} of **II** was about 30-fold higher than that of **I** (table 2).

In EHBR, both compounds also reached a steady state at comparable plasma levels with those observed in control rats (figure 3A, B and table 2). As was the case in control SD rats, the biliary excretion rate and CL_b of **II** in EHBR was higher, although to a lesser degree, than the corresponding values of **I** (figure 3C, D and table 2). However, for both compounds, CL, biliary excretion rate and CL_b obtained in EHBR were not decreased as compared with those in SD rats (table 2). Instead, in the case of **I**, the biliary excretion rate and CL_b were significantly (about twofold) higher in EHBR than in control rats. Additionally, the levels of both diastereomers in liver in EHBR were significantly decreased (about 60–80%), resulting in a significant increase (about 4–15-fold) in CL_{b,l}, as compared with their respective values in control rats (table 2).

Table 2. Pharmacokinetic parameters of **I** and **II** in SD rats and EHBR received intravenous administration of **I** (0.35 mg kg^{-1}) or **II** (0.15 mg kg^{-1}) followed by constant-rate infusion of **I** or **II** ($10 \text{ mg min}^{-1} \text{ kg}^{-1}$).

Parameter	Unit	SD (control)		EHBR	
		I	II	I	II
$C_{\text{pss}}^{\text{a}}$	nM	743 ± 119	789 ± 82	830 ± 146	894 ± 226
Biliary excretion rate ^b	$\text{nmol min}^{-1} \text{ kg}^{-1}$	1.4 ± 0.5	$12.2 \pm 3.7^*$	$3.9 \pm 0.7^{**}$	15.1 ± 4.5
Liver concentration ^c	$\text{nmol g}^{-1} \text{ liver}$	33.7 ± 11	$8.5 \pm 0.5^*$	$6.1 \pm 1.6^{**}$	$3.2 \pm 0.6^{**}$
CL^{d}	$\text{ml min}^{-1} \text{ kg}^{-1}$	33.6 ± 6	30.2 ± 4	28.8 ± 3	29.2 ± 7
$\text{CL}_{\text{b},\text{p}}^{\text{e}}$	$\text{ml min}^{-1} \text{ kg}^{-1}$	1.9 ± 0.5	$15.6 \pm 5^*$	$4.8 \pm 1.3^{**}$	17.5 ± 6
$\text{CL}_{\text{b},\text{l}}^{\text{f}}$	$\text{ml min}^{-1} \text{ kg}^{-1}$	0.05 ± 0.02	$1.5 \pm 0.5^*$	$0.71 \pm 0.2^{**}$	$5.1 \pm 0.8^{**}$

Data are mean $\pm$ SD ($n = 4$).

^aAverage C_{pss} obtained during 20–120 min.

^bAverage biliary excretion rate obtained over a 2-h infusion.

^cObtained at the end of infusion (2 h).

^dCalculated by dividing infusion rate by average C_{pss} .

^eCalculated by dividing biliary excretion rate by average C_{pss} .

^fCalculated by dividing biliary excretion rate by liver concentration.

*Statistically different from **I**.

**Statistically different from SD rats.

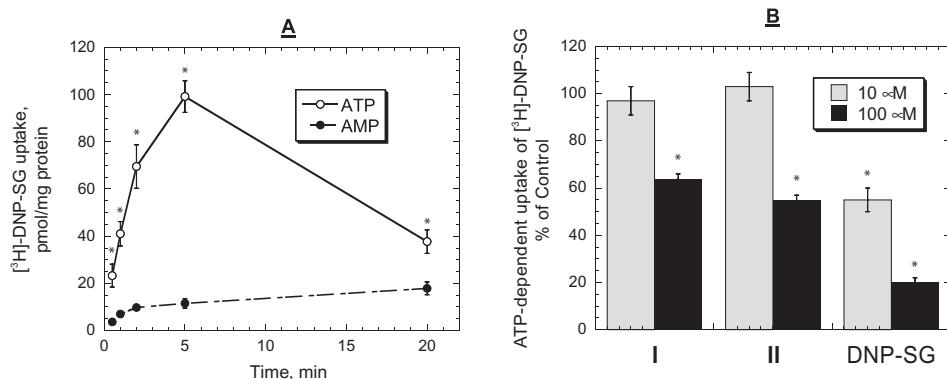


Figure 4. Uptake of $[^3\text{H}]\text{DNP-SG}$ as a function of time by CMV in the presence and/or absence of ATP (A), and effect of **I**, **II** or DNP-SG on ATP-dependent uptake of $[^3\text{H}]\text{DNP-SG}$ by CMV. CMV ($10 \mu\text{g}$ protein) was incubated with ATP (5 mM) plus the ATP-regenerating system or AMP (5 mM) in the medium containing $[^3\text{H}]\text{DNP-SG}$ ($1 \mu\text{M}$). Effect of **I**, **II** and unlabeled DNP-SG on $[^3\text{H}]\text{DNP-SG}$ ($1 \mu\text{M}$) uptake was determined at 2 min in the presence and/or absence of ATP. ATP-dependent uptake was calculated by subtracting the uptake in the presence of ATP from that in the presence of ATP. Each symbol and bar represents the mean $\pm$ SD of three independent experiments. *Statistically different from AMP control (A) or control without inhibitors (B).

In vitro transport inhibition studies

Figure 4A shows the uptake time-courses of $[^3\text{H}]\text{DNP-SG}$, a prototypical substrate for rat Mrp2 (Abcc2, Yamazaki *et al.* 1996), by CMV in the presence and absence of ATP. The uptake was stimulated significantly by the presence of ATP, demonstrating that CMV prepared has maintained functional activity of rat Mrp2.

To examine whether **I** and **II** exhibit differences in their inhibition potency against rat Mrp2-mediated transport, the effect of **I** and **II** on the initial uptake of $[^3\text{H}]\text{DNP-SG}$ was evaluated. As shown in figure 4B, both **I** and **II** exhibited no inhibition at $10 \mu\text{M}$ and about 40% inhibition at $100 \mu\text{M}$ ATP-dependent (Mrp2

mediated) [^3H]DNP-SG initial uptake. No significant inhibition was observed in ATP-independent uptake of [^3H]DNP-SG by **I** or **II** (data not shown). Unlabeled DNP-SG showed concentration-dependent self-inhibition on the ATP-dependent [^3H]DNP-SG uptake (55 and 20% at 10 and 100 μM , respectively) (figure 4B). These results demonstrated that both **I** and **II** were equally weak inhibitors of rat Mrp2.

Discussion

The present study used combination approaches to characterize first the hepatic disposition of a diastereomeric pair with markedly different lipophilicity, and subsequently to investigate the possible involvement of Mrp2 in the stereoselectivity observed in their hepatic disposition. The isolated liver perfusion was selected as one of the study models since it enables the assessment of drug uptake from the sinusoidal membrane and secretion to the canalicular membrane by hepatocytes in their normal architecture and polarity. In this system, the hydrophilic diastereomer **II** was shown to undergo biliary excretion more extensively and rapidly than **I**, similar to the *in vivo* situation (Prueksaritanont *et al.* 2002). Despite the marked difference in the lipophilicity, both compounds were shown to be comparably and avidly taken up by liver cells, resulting in comparably high (>2000) liver-to-perfusate concentration ratios at the end of 60-min perfusion. In contrast, the significantly higher concentration ratio between bile and liver of **II** (about 28) than **I** (about 1) suggested that **II** was excreted from liver into bile much more efficiently than **I**. The relatively high liver/perfusate concentration gradient for both **I** and **II** is suggestive of a possible involvement of carrier-mediated system(s) for both diastereomers at the sinusoidal membrane, whereas the much higher concentration gradient between bile and liver of **II** points to a possible involvement of a carrier-mediated transport process of **II** at the canalicular membrane. Therefore, the marked difference observed in the biliary excretion of the diastereomeric pair appeared to be due largely to differences in their transport mechanism through the canalicular, and not the sinusoidal membrane.

Results from the isolated rat liver system also suggested that Mrp2 was not a major contributing factor to the observed difference in the hepatic disposition of **I** and **II**. This suggestion was based on the finding that MK-571, a potent inhibitor of MRP, did not inhibit the concentration ratio between bile and liver for both **I** and **II**, indicating that MK-571 had a minimal inhibitory effect on the canalicular transport of the diastereomers. Considering that Mrp2 is a canalicular transporter, this finding implied minimal involvement of Mrp2 in the canalicular transport and hence biliary excretion of either **I** or **II**. Instead, it was apparent that MK-571 might have an inhibitory effect on their sinusoidal uptake into the liver, based on the significant decreases in the liver-to-perfusate concentration ratios of both isomers in the presence of MK-571 (table 1). Presumably, this effect caused decreases in the biliary excretion rate and CL_b of **I** and **II**, which was observed after the addition of MK-571. In theory, these decreases could also be explained by increased sinusoidal efflux of **I** and **II** by MK-571. To date, there has not been, however, published information about the effect of MK-571 on any known sinusoidal uptake or sinusoidal efflux transporters. It is noteworthy that the finding of significantly decreased CL , but not CL_b , both obtained over the entire study period (60 min; table 1) for the diastereomer **I** suggests that MK-571 might also

inhibit enzymes that catalyse the metabolism of **I**. This was not the case for the hydrophilic diastereomer **II**, which is less susceptible to biotransformation than **I** (Prueksaritanont *et al.* 2002).

The aforementioned proposal based on the isolated liver perfusion model that the difference in the CL_b between the two diastereomers was due primarily to the difference at the canalicular transport level was further supported by the present results obtained *in vivo* in rats. In control SD rats, the $CL_{b,l}$ for **II** was much higher than that for **I**, consistent with the concept that **II** was more efficiently exported than **I** from liver to bile across the canalicular membrane. Apparently, this efficient canalicular transport of **II** contributed to its high biliary excretion rate and CL_b observed, as compared with **I** (table 2). Also consistent with the isolated liver perfusion results, the findings that the biliary excretion rate, CL_b and $CL_{b,l}$ for both compounds in rats lacking Mrp2 (EHBR) were not significantly lower than those in the control rats effectively supported the fact that Mrp2 did not play a significant role in the biliary excretion of the two diastereomers. Interestingly, significant decreases in the liver content of both **I** and **II** were observed, resulting in a significant increase in $CL_{b,l}$ in EHBR. In the case of **II**, the decrease in the liver content was not associated with a significant increase in biliary excretion rate. This unexpected finding might be explained in part by a possible increase in the sinusoidal efflux of **II**, considering that Mrp3, a sinusoidal efflux transporter, has been shown to be induced in EHBR (Hirohashi *et al.* 1998, König *et al.* 1999b, Akita *et al.* 2001), and that plasma levels of **II** in EHBR tended to be higher, although statistically insignificant, than those in control rats (figure 3A). Presently, it is not known whether there are also decreased sinusoidal uptake transporter activity/expression in EHBR, which might explain the observed decreases in the liver content of **I** or **II**.

Consistent with the above observation that Mrp2 played a minor role in the marked difference in the hepatic disposition observed for the two diastereomers in rats, the *in vitro* canalicular membrane vesicle study showed no differences in the inhibitory effect between **I** and **II** on the Mrp2-mediated ATP-dependent [^{3} H]DNP-SG transport. Owing to the unavailability of an in-house *in vitro* rat Mrp2 model and radiolabelled **I** and **II**, an experiment to investigate directly the susceptibility of either compound as a Mrp2 substrate was not carried out in the present study. Nevertheless, the finding that both compounds exhibited comparably low inhibitory potential for DNP-SG, a known substrate for Mrp2, suggested that they might also be poor substrates for rat Mrp2.

In summary, the data from both the isolated rat liver perfusion and *in vivo* infusion in rats agreed well with each other that the hydrophilic diastereomer **II** was excreted into bile much faster and more extensively than **I**, and that the difference in the CL_b observed between the two isomers was primarily due to the difference at the level of canalicular transport. To the authors' knowledge, similar stereoselectivity in the hepatic disposition has not been reported for other diastereomers with different lipophilicity. In addition, further studies using a combination of *in vivo* and *in vitro* approaches suggested that Mrp2 did not, but rather other yet to be identified canalicular transporter(s) play an important role in the marked difference observed in the hepatic disposition for this diastereomeric pair in rats. The present and previous findings that both Mrp2 and P-gp, the important and well-characterized canalicular transporters known to date, were not involved in the hepatic excretion of **I** and **II** highlight complexities and limited

current understanding of the hepatic elimination processes for xenobiotics. These findings also exemplify the importance of compound physicochemical properties on xenobiotic transport, an addition to metabolism, in determining compound disposition.

Acknowledgements

The authors thank Mr James Perkins and Dr Robert S. Meissner for the synthesis of **I** and **II**, and Ms Yuan Meng and Mr Bennett Ma for assistance in HPLC analysis.

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Sulfation of apomorphine by human sulfotransferases: evidence of a major role for the polymorphic phenol sulfotransferase, SULT1A1

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Received 30 May 2003

1. The relative roles of various members of the human sulfotransferase (SULT) enzyme family in the metabolism of apomorphine, a dopamine receptor antagonist used in the treatment of Parkinson's disease and, more recently, erectile dysfunction, were examined. In humans, sulfation is the major route of metabolism of this drug.

2. Using recombinant SULTs expressed in *Escherichia coli*, *R*(-)-apomorphine sulfation was studied using the universal barium precipitation assay in the presence of [³⁵S] 3'-phosphoadenosine 5'-phosphosulfate and SULTs 1A1, 1A2, 1A3, 1B1, 1C2, 1E1 and 2A1. It was shown that SULTs 1A1, 1A2, 1A3 and 1E1 all sulfated apomorphine to varying extents. Low activity with SULT1B1 was only seen at the highest concentration (100 µM) and no activity with SULT1C2 or SULT2A1 was observed.

3. Kinetic analysis using purified recombinant SULTs showed that 1A1, 1A3 and 1E1 all had similar $V_{\max}/K_m$ values, although SULT1E1 had a slightly lower K_m at around 1 µM compared with approximately 4 µM for the other SULTs.

4. By correlating apomorphine sulfation (at 10 µM) in a bank of 28 liver cytosols with SULT activity towards 10 µM 4-nitrophenol (SULT1A1) and 0.2 µM 17β-oestradiol (SULT1E1), a strong correlation with SULT1A1 activity was clearly demonstrated, suggesting this enzyme was primarily responsible for hepatic apomorphine sulfation.

5. These findings were confirmed using immuno-inhibition experiments with antibodies against SULT1A and SULT1E1, which showed preferential inhibition of apomorphine sulfation in human liver cytosol by anti-SULT1A.

6. The results strongly implicate SULT1A1 as the major enzyme responsible for hepatic apomorphine metabolism. As SULT1A1 is subject to a common functional polymorphism, sulfation phenotype may be an important determinant of susceptibility to side-effects of apomorphine and/or efficacy of treatment.

Introduction

Apomorphine is a dopamine receptor agonist, active against both D1 and D2 receptors, that is used in the treatment of advanced Parkinson's disease (O'Sullivan and Lees 1999). The compound exists as two stereo-isomers, *S*(+)- and *R*(-), although the *R*(-)-apomorphine is used in the clinic. It is most successful in alleviating the end-of-dose deterioration experienced by many later stage Parkinson's patients receiving levodopa — the so-called wearing-off effect, or 'off' period (Pietz *et al.* 1998). It is generally accepted that those

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patients with unpredictable off periods receive the greatest benefit with apomorphine (O'Sullivan and Lees 1999). The drug is subject to very rapid metabolism and inactivation when taken orally, and therefore requires to be administered via parenteral routes such as intravenous, subcutaneous (e.g. APO-go[®]), sublingual or intranasal (Jankovic and Marsden 1998). Significant advances have been made in delivery systems for apomorphine, and it is now usually administered subcutaneously by continuous infusion pump (O'Sullivan and Lees 1999). More recently, apomorphine has been licensed in a number of countries including the UK (Uprima[®]) for use in treating erectile dysfunction, and is available as tablets for sublingual administration. However, apomorphine is associated with significant side-effects, the most important of these being nausea and vomiting, orthostatic hypotension and the exacerbation of peptic ulcer disease and angina (Pietz *et al.* 1998). In man, the majority (approximately 60%) of sublingual apomorphine is excreted in the form of sulfate conjugate(s) (Argiolas and Hedlund 2001), and extensive hepatic and intestinal sulfation (Gancher *et al.* 1989) contributes to the poor oral bioavailability (only 1.7%) of the drug (Gancher *et al.* 1991).

Until recently, little was known of apomorphine sulfation *in vitro*. Vietri *et al.* (2002) demonstrated that apomorphine was sulfated in human liver and duodenal cytosols and suggested, by correlation analysis, that the major phenol sulfotransferase, SULT1A1, was involved. They also demonstrated the potential for drug-drug and drug-diet interactions since the sulfation of apomorphine could be inhibited *in vitro* by mefanamic and salicylic acids and quercetin (Vietri *et al.* 2002, 2003). Sulfation is an important metabolic pathway for numerous xenobiotics and endogenous chemicals including steroid hormones, iodothyronines and catecholamines (Coughtrie 2002). The reactions are catalysed by members of the cytosolic sulfotransferase enzyme family, all of which use the universal sulfuryl donor molecule 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Humans have at least 10 functional sulfotransferase (*SULT*) genes coding for 11 different SULT enzymes with broad but often overlapping substrate specificities (Coughtrie 2002, Strott 2002). The availability of high-efficiency expression systems, principally in *E. coli*, has dramatically improved our understanding of the SULTs and the biology of sulfation, and these and other tools provide us with the ability to identify the major sulfotransferases involved in xenobiotic metabolism. It is clear that the phenol sulfotransferase SULT1A1 is an important enzyme in xenobiotic metabolism since it has broad substrate specificity (with a high affinity for many compounds) and it is expressed widely in the body, particularly in the liver. For example, it has been shown that SULT1A1 is the major enzyme involved in the metabolism of the oral anti-hyperglycemic drug troglitazone (Honma *et al.* 2002). Importantly, SULT1A1 is subject to a common functional polymorphism (Raftogianis *et al.* 1997, Coughtrie *et al.* 1999), with the variant allele (*SULT1A1**2, allele frequency 32% in Caucasians) coding for an allozyme with a single amino acid change (Arg₂₁₃His), which is unstable compared with the wild-type protein (Raftogianis 2001). Homozygotes for the *SULT1A1**2 allele typically have around 10% of the SULT1A1 activity of *SULT1A1**1 homozygotes.

The present paper reports detailed reaction phenotyping analysis on the sulfation of apomorphine in humans *in vitro*, which implicates the polymorphic SULT1A1 as the major enzyme responsible for the sulfation of this drug.

Materials and methods

Chemicals

Protein purification columns and media were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). PAP³⁵S (>98% radiochemical purity) was obtained from PerkinElmer Life Sciences (Cambridge, UK), and PAPS (>99% purity) was purchased from H Glatt, German Institute for Human Nutrition, Potsdam, Germany. Scintillation fluid (Emulsifier Safe) was from PerkinElmer Life Sciences. Apomorphine hydrochloride hemihydrate ($\geq 99\%$ purity, all *R*(-) enantiomer) was from Sigma (Gillingham, UK) and all other chemicals were from Sigma, VWR International (Poole, UK) or Roche Diagnostics (Lewes, UK).

Expression and purification of human SULTs

The cloning, expression in *E. coli* and purification of numerous human SULTs were reported (Dajani *et al.* 1998, 1999a, Taskinen *et al.* 2003). Briefly, cDNAs for SULTs 1A1, 1A2, 1A3, 1B1, 1C2, 1E1 and 2A1 were cloned into the bacterial expression vectors pET11a or pET17b as described, and *E. coli* strains BL21(DE3) or BL21(DE3) pLysS transformed with the expression constructs. Bacterial cultures (100 ml) were grown in LB broth (0.1 mg ml⁻¹ ampicillin) and induced with IPTG (1 mM final concentration) when cultures reached an optical density reading of 0.5. Cultures were grown for an additional 15 h, then centrifuged at 5000*g* and the pellet frozen at -70°C. Chloramphenicol (34 µg ml⁻¹ final concentration) was also included in the culture medium for BL21 pLysS cells. Cell-free extracts (CFX) were prepared by lysing cell pellets with 40 mM Tris-HCl (pH 8.0) and lysozyme (0.5 mg ml⁻¹ final concentration). BL21 pLysS cells do not require the addition of lysozyme. Lysed cells were then centrifuged (14 000*g* for 30 min at 4°C), and the supernatant removed and stored at -70°C. Purification was carried out by anion exchange chromatography on Q-Sepharose followed by affinity chromatography on ADP-agarose (Dajani *et al.* 1999b). Purity was assessed visually by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining, and batches showing a single polypeptide species were used.

SULT enzyme assays

Sulfation was assayed according to Foldes and Meek (1973). Reaction mixtures (final volume 160 µl) comprised 6.25 mM potassium phosphate (pH 7.4), PAPS (20 µM final concentration, containing 0.04 µCi PAP³⁵S), recombinant SULT enzyme or tissue cytosolic protein and substrate, unless otherwise indicated. Apomorphine stock solution (5 mM in deionized water) was prepared fresh each day and protected from light. Assays were performed in duplicate with a blank reaction containing deionized water in place of the substrate. Reactions were started by adding the PAPS/PAP³⁵S mixture following pre-incubation for 2 min and incubated for 10 min at 37°C. Unreacted PAPS/PAP³⁵S was precipitated with 200 µl 0.1 M barium acetate, 200 µl 0.1 M barium hydroxide and 200 µl 0.1 M zinc sulfate and centrifuged for 5 min at 14 000*g*. The supernatant (500 µl) was mixed with 3 ml scintillation fluid and radioactivity quantified by liquid scintillation spectrometry. Assays were optimized for protein and incubation time. For determination of kinetic parameters, assays were carried out in the presence of 20 µM PAPS using at least seven different concentrations of apomorphine between 0.5 and 50 µM. Data were analysed and kinetic parameters determined with the software packages Hyper.exe v.1.1 (J. S. Easterby, Department of Biochemistry, University of Liverpool, Liverpool, UK) and Prism v 3.0 (GraphPad, Inc., San Diego, CA, USA).

Tissue samples and preparation of cytosolic fractions

Human liver, kidney, jejunum and ileum were obtained from the UK Human Tissue Bank (Leicester, UK). Ethical approval for use of these tissues locally was granted by the Tayside Committee on Medical Research Ethics. All actions were performed at 4°C. Tissue (previously frozen at -70°C) was placed in ice-cold 10 mM HEPES (pH 7.4) containing 0.25 M sucrose and 3 mM 2-mercaptoethanol. The tissue was cut with scissors and homogenized (20% w/v) in a Teflon-glass homogenizer (Jencons Scientific Ltd, Leighton, UK). Homogenates were centrifuged at 10 000*g* for 15 min and the resulting supernatant subjected to an additional centrifugation step at 100 000*g* for 1 h. The resulting cytosolic fraction was aliquoted and stored at -70°C.

Antibody inhibition experiments

Preparation and characterization of sheep antisera directed against purified recombinant human SULTs 1A3 (referred to here as anti-SULT1A, since it cross-reacts strongly with SULTs 1A2 and 1A1 due to >93% amino acid sequence identity) and 1E1 (shows about 15% cross-reactivity with SULTs 1A1 and 1A3 by ELISA) have been described (Rubin *et al.* 1999, Richard *et al.* 2001, Stanley *et al.* 2001). A pool (10 µl) of six human liver cytosols was first incubated with a mixture (10 µl) of antiserum and pre-immune serum in varying proportions between 0:10 and 10:0 for 15 min at room temperature.

The SULT enzyme assay was then carried out as described above, with a reduction of 10 μ l in the volume of buffer used.

Protein assays

Protein concentrations were determined by the method described by Bradford (1976), with bovine serum albumin as standard.

Results

A panel of seven *E. coli* cell-free extracts expressing individual recombinant human SULTs was first screened for the ability to sulfate apomorphine at concentrations of 1, 10 and 100 μ M. The results of this initial screening showed that SULTs 1A1, 1A2, 1A3 and 1E1 all sulfated apomorphine to varying extents (table 1), with SULTs 1A1, 1A3 and 1E1 all capable of sulfation at 1 μ M substrate. Low activity with SULT1B1 was only seen at the highest concentration (100 μ M) and there was no activity observed with either SULT1C2 or SULT2A1. Using this information, a comparative kinetic analysis of apomorphine sulfation by purified recombinant human SULTs 1A1, 1A3 and 1E1 was carried out. It was believed that SULT1A2 was not expressed to any significant extent in any tissue due to aberrant splicing of the mRNA (Dooley *et al.* 2000), so we did not proceed further with this isoform. Figure 1 shows the results of this kinetic analysis and indicates that all three isoforms have similar specificity constants ($V_{\max}/K_m$) for apomorphine. SULT1E1 had the lowest K_m (suggestive of highest affinity), although the rate was much lower than for either SULT1A1 or SULT1A3.

Thus, these experiments did not clearly indicate which SULT isoform might be responsible for the sulfation of apomorphine in human tissues, so the sulfation of the drug was then analysed in cytosolic fractions prepared from human liver, kidney and intestine (jejunum, ileum). The samples were first assayed with 10 μ M apomorphine at a variety of protein concentrations to assess the activity and optimize the protein concentrations per assay (data not shown). In addition, to confirm suitability and viability of these tissues, they were assayed for SULT activity using widely accepted isoform-specific probe substrates: 4-nitrophenol (10 μ M) for SULT1A1, dopamine (10 μ M) for SULT1A3 and 17 β -oestradiol (0.2 μ M) for SULT1E1. Figure 2 shows the results of these assays, which confirm that the tissues were viable and also that SULT1A3 is not expressed in the adult

Table 1. Sulfation of apomorphine by recombinant human SULTs expressed in *E. coli*.

SULT isoform	Enzyme activity (nmol min ⁻¹ mg ⁻¹)		
	1 μ M	10 μ M	100 μ M
SULT1A1	0.39	2.65	2.63
SULT1A2	— [†]	1.24	5.06
SULT1A3	0.68	10.6	17.0
SULT1B1	—	—	0.21
SULT1C2	—	—	—
SULT1E1	0.90	9.76	6.43
SULT2A1	—	—	—

Sulfation of apomorphine at concentrations 1, 10 and 100 μ M was measured with 20 μ M PAP³⁵S using cell-free extracts prepared from *E. coli* strains expressing the various recombinant human SULTs. Data are from assays performed in duplicate, which did not differ by more than 20%.

[†]Activity below the limit of detection of the assay (about 20 pmol product formed in these assays).

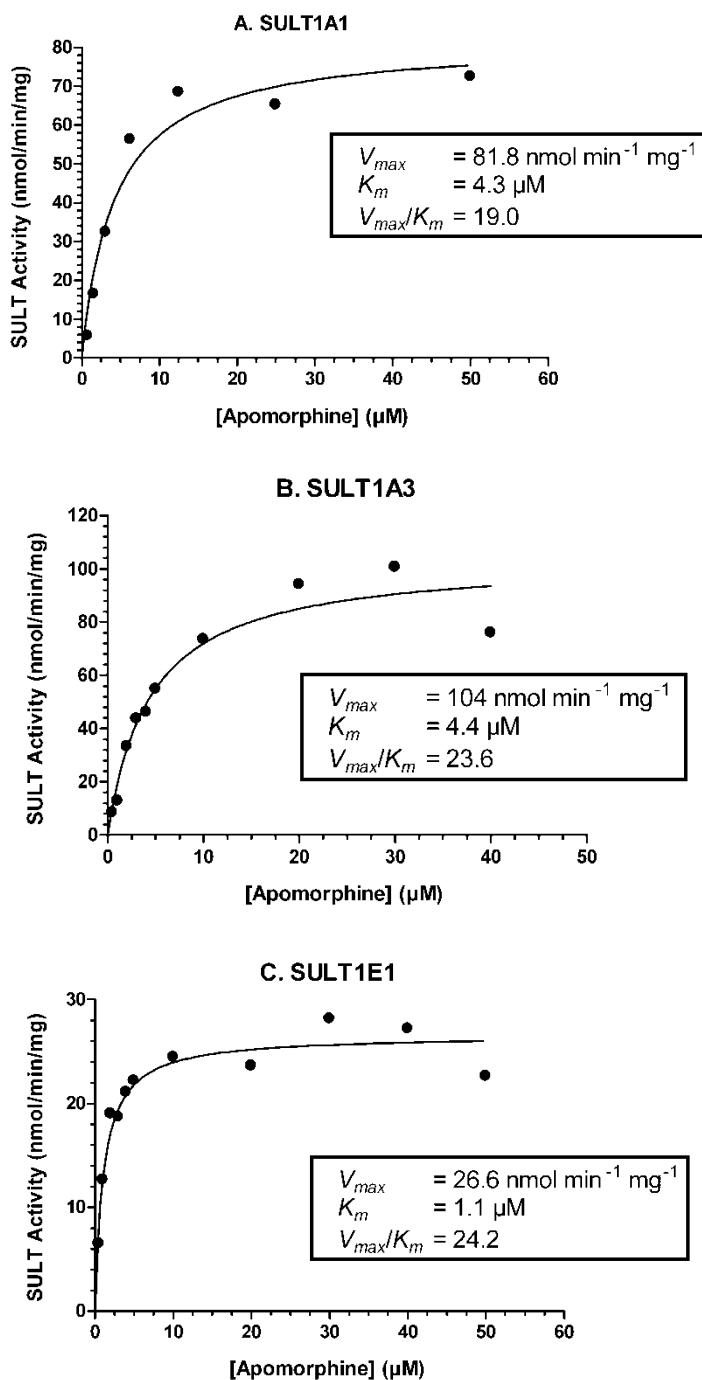


Figure 1. Kinetics of apomorphine sulfation by purified recombinant human SULTs. Sulfotransferase enzyme activity was measured in the presence of various concentrations of apomorphine and 20 μM PAPS with SULT1A1 (A), SULT1A3 (B) and SULT1E1 (C). Data points are averages of duplicate assays.

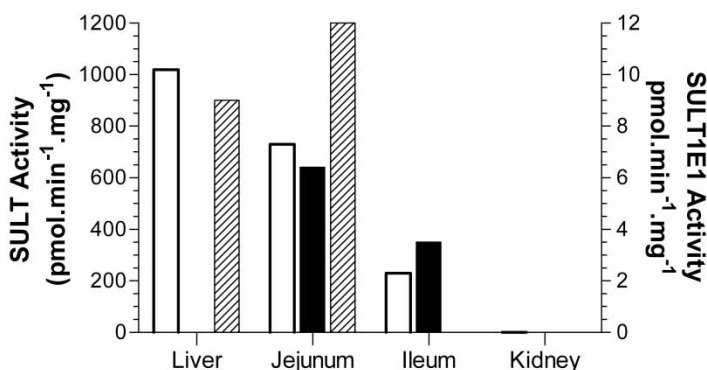


Figure 2. Sulfotransferase activities in human tissue cytosols. SULT enzyme activity was measured in cytosols prepared from liver, jejunum ileum and kidney with substrates representative of SULT1A1 (4-nitrophenol, open bars), SULT1A3 (dopamine, black bars) or SULT1E1 (17β-oestradiol, hatched bars). Data points are averages of duplicate assays.

Table 2. Sulfation of apomorphine by human tissue cytosols.

	$V_{\max}$ (znmol min ⁻¹ mg ⁻¹)	K_m (μM)	$V_{\max}/K_m$
SULT1A1 [§]	81.8	4.3	19.0
SULT1A3	104	4.4	23.6
SULT1E1	26.6	1.1	24.2
Human Liver ($n=4$, mean $\pm$ SEM)	0.57 ± 0.07	1.6 ± 0.72	0.36
Human jejunum ($n=2$)	0.79	1.4	0.56
Human ileum ($n=2$)	0.35	0.96	0.36
Human kidney ($n=4$)	N.D. [†]	N.D.	N.D.

Data are from assays performed in duplicate, which did not differ by more than 20%.

[§]Data for purified recombinant human SULTs, taken from figure 1, are shown for comparison.

[†]N.D., no apomorphine sulfotransferase activity detectable.

human liver (Rubin *et al.* 1996), and that whole kidney cytosol does not have a significant sulfation capacity (Heroux *et al.* 1989). Subsequently, kinetic analysis was performed for apomorphine sulfation in these tissue cytosol samples (table 2). Apomorphine was efficiently sulfated by cytosols from liver, jejunum and ileum, but no activity was detected in human kidney cytosol. The higher activity in jejunum was consistent with Vietri *et al.* (2002), who reported that sulfation of apomorphine in duodenum was higher than in liver. Enzyme activity (i.e. $V_{\max}$) was highest in the jejunum, and the K_m values in tissue cytosols were comparable with those observed with the purified recombinant enzymes.

As SULT1A3 is not expressed in liver, it remained possible that apomorphine might be sulfated by either SULT1E1 or SULT1A1 (or both) in this tissue. To resolve this, sulfation of apomorphine (10 μM) was assessed in 28 human liver cytosols and compared the activities with the sulfation of 10 μM 4-nitrophenol (for SULT1A1) and 0.2 μM 17β-oestradiol (for SULT1E1). As shown in figure 3, a strong correlation was observed between sulfation of apomorphine and 4-nitrophenol (Spearman $r=0.825$, $p<0.0001$), but not between apomorphine and 17β-oestradiol (Spearman $r=0.153$, $p=0.43$), suggesting that it is the SULT1A1 isoform that is primarily responsible for the sulfation of apomorphine in human liver.

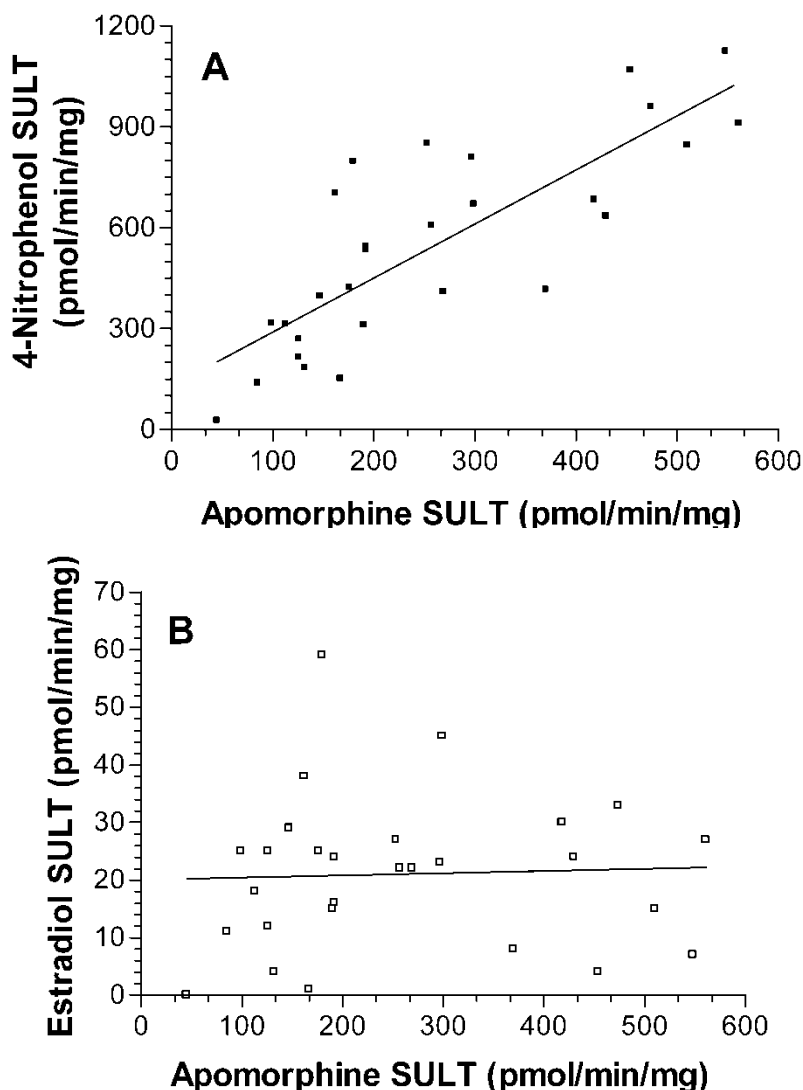


Figure 3. Correlation analysis of apomorphine sulfation in human liver cytosol. Cytosols from 28 human liver samples were assayed for sulfotransferase activity with 10 μ M apomorphine as substrate and with 10 μ M 4-nitrophenol (A, for SULT1A1) and 0.2 μ M 17 β -oestradiol (B, for SULT1E1). Data points are averages of duplicate assays. Correlation analysis was carried out using Prism 3.0.

Finally, these results were confirmed by performing immuno-inhibition experiments with sheep antiserum reactive against human SULT1A family members (Richard *et al.* 2001) and human SULT1E1 (Rubin *et al.* 1999). In these experiments, pooled human liver cytosol (from six livers) was incubated with increasing amounts of antiserum followed by assay for sulfation of 4-nitrophenol (SULT1A1), 17 β -oestradiol (SULT1E1) and apomorphine (figure 4). These data show that sulfation of apomorphine in human liver cytosol is completely inhibited by very low amounts of anti-SULT1A, as is the sulfation of 4-nitrophenol. At higher concentrations of anti-SULT1E1,

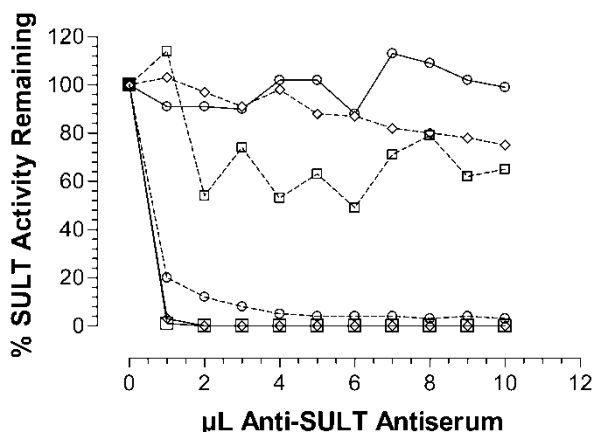


Figure 4. Immuno-inhibition analysis of apomorphine sulfation in human liver cytosol. Human liver cytosol (a pool of six different samples) was incubated with increasing amounts of anti-SULT1A (solid lines) or anti-SULT1E1 (dashed lines) antiserum (balanced with pre-immune serum) and then assayed for sulfation of 10 μ M apomorphine ($\square$), 10 μ M 4-nitrophenol ($\diamond$) or 0.2 μ M 17 β -oestradiol ($\circ$). Data points are averages of assays performed in duplicate.

sulfation of apomorphine was only inhibited by approximately 30%, and there was also some inhibition of 4-nitrophenol sulfation by this antibody. Thus, these experiments confirm that SULT1A1 is likely to be the major SULT isoform responsible for the sulfation of apomorphine in human liver.

Discussion

Sulfation is the major pathway of metabolism for apomorphine in humans (Argiolas and Hedlund 2001). The present paper has shown detailed reaction phenotyping analysis of the sulfation of apomorphine by recombinant human SULTs and in human tissue cytosols, and it was concluded that the major isoform responsible for this metabolism, in the liver at least, is SULT1A1.

The initial screening of apomorphine sulfation by recombinant human SULTs indicated that several isoforms could metabolize the drug with reasonable efficiency and high affinity (the K_m values were all below 5 μ M). It was therefore not possible to identify conclusively from these experiments the SULT isoform most likely to be responsible for apomorphine sulfation. Using a combination of correlation analysis with SULT1A1 and SULT1E1 activity in human liver cytosols and immuno-inhibition using antibodies recognizing SULT1A and SULT1E1, it was determined that SULT1A1 plays a major role in apomorphine sulfation, at least *in vitro*. The jejunum and ileum also had a high capacity to sulfate apomorphine, suggesting that in these gastrointestinal tract tissues at least, the catecholamine sulfotransferase SULT1A3 might also contribute significantly to apomorphine metabolism. This enzyme is not expressed in adult human liver, and its major site of expression is in the gastrointestinal tract (Rubin *et al.* 1996, Richard *et al.* 2001). This is unlikely to be particularly significant for apomorphine metabolism in the clinic, however, since the drug is now exclusively administered via parenteral routes such as sublingual (e.g. Uprima[®]) or subcutaneous (e.g. APO-go[®]).

The observation that SULT1A1 is probably the major enzyme involved in apomorphine metabolism in humans has potentially important implications, since SULT1A1 expression is polymorphic in the population (Raftogianis *et al.* 1997). Available evidence implicates a common non-synonymous coding region single nucleotide polymorphism (cSNP) that results in a SULT allozyme (SULT1A1*2) differing from the wild-type (SULT1A1*1) by a single amino acid substitution, Arg₂₁₃His (Raftogianis *et al.* 1997, 1999, Coughtrie *et al.* 1999). Individuals homozygous for *SULT1A1**2 have reduced SULT1A1 expression (by up to 90%) (Raftogianis *et al.* 1997), and this is believed to be a result of instability and the reduced biological half-life of the SULT1A1*2 allozyme (Raftogianis 2001). It has also been repeatedly demonstrated that SULT1A1 enzyme protein expression varies widely in the population, as measured in various tissues (Jones *et al.* 1993, Stanley *et al.* 2001). It is therefore possible that individuals who are *SULT1A1**2 homozygotes have a reduced capacity to sulfate apomorphine, and consequently may be at increased risk of side-effects associated with the use of this drug. Equally, such individuals may actually respond better to apomorphine treatment, since the drug may be more metabolically stable than in 'rapid' sulfators. It is suggested that these hypotheses should be tested in an appropriate clinical trial.

In conclusion, these studies have demonstrated the value of using a combination of approaches to identify individual SULT isoforms involved in metabolism of drugs and have confirmed that the widely expressed, but polymorphic, phenol sulfotransferase SULT1A1 is an important drug-metabolizing enzyme in humans.

Acknowledgements

The authors are grateful to Drs Alan Hood and Rana Dajani for cloning of SULT1A2 and SULT1B1 cDNAs, and to Dr Richard Roberts for helpful discussions. Work was funded by the Human Drug Conjugation Consortium (AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, F. Hoffman-La Roche, Lilly, Novartis, Pfizer, Wyeth-Ayerst).

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Bioavailability and half-life of decabromodiphenyl ether (BDE-209) in rat

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Received 3 April 2003

1. The absorption of the flame retardant decabromodiphenyl ether (BDE-209) has been shown by its detection in human plasma, but reported experimental data on its absorption in rat are inconsistent. The bioavailability and half-life were therefore determined in rat, and qualitative analyses by GC/MS of metabolites in plasma were performed. The relative amount of phenolic metabolites was determined in a rat plasma sample obtained after administration of radiolabelled BDE-209.

2. The bioavailability of parent BDE-209 was calculated to be about 26% in rat. The concentrations of phenolic radioactivity in plasma 3 and 7 days after dosing were four times higher than those of the neutral compounds, i.e. parent compound, indicating that total absorption was higher than 26%.

3. Thirteen phenolic metabolites were determined in the plasma and the major phenolic metabolites were characterized as a hydroxy-octaBDE, a hydroxy-nonaBDE and a hydroxy-methoxy-hexaBDE (guaiacol-type). The exposure to the phenolic metabolites seemed higher than the parent compound, BDE-209.

4. The initial elimination phase in plasma $t_{1/2\alpha}$ for BDE-209 was 2 h, implying a rapid distribution of BDE-209 to well-perfused tissues. The distribution volume at steady state was 1.4 l kg^{-1} , implying a low tendency for distribution to adipose tissue. The terminal $t_{1/2}$ for BDE-209 in the intravenously dosed rat was calculated as 2.5 days (58 h).

Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in textiles, rubbers, high-impact polystyrenes, polyamides and polyolefins, the latter often used in electronic equipment (WHO 1994, Alaei *et al.* 2003). Their extensive use, lipophilicity and persistency have led to their bioaccumulation and retention in both wildlife and humans (de Boer *et al.* 2000, de Wit 2002, Sjödin *et al.* 2003). Technical decaBDE, mainly consisting of one PBDE congener, decabromodiphenyl ether (BDE-209), is the most commonly used PBDE with a world-wide production of 54 000 tons in 1999 (BSEF 2000). Despite the extensive use of BDE-209, it is still rarely analysed, mainly due to analytical difficulties. However, when analysed, the levels have hitherto been low in both wildlife and humans (de Boer *et al.* 2000, de Wit 2002, Sjödin *et al.* 2003) except for BDE-209 in guillemot egg (Sellström *et al.* 2001). In contrast the levels of the lower brominated PBDEs, e.g. tetraBDE, pentaBDE and heptaBDE, are higher (de Boer *et al.* 2000, de Wit 2002, Sjödin *et al.* 2003). This may be caused by a high UV degradation rate of BDE-209

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(Watanabe and Tatsukawa 1987, Sellström *et al.* 1998, Eriksson *et al.* 2001), high adsorption to particles and/or low bioavailability (WHO 1994). However, BDE-209 concentrations are comparatively high in occupationally exposed individuals (Sjödin *et al.* 2003). After 4 weeks vacation from work, the plasma levels of BDE-209 had decreased substantially compared with those of 2,2',4,4',-tetraBDE (BDE-47) and (BDE-183). This suggests that BDE-209 is more rapidly eliminated from the body 2,2',3,4,4',5',6-heptaBDE than the other PBDE congeners. Calculations of the half-life for BDE-209 and BDE-183 in these latter workers showed a 10-fold difference with values of 7 days for BDE-209 and 86 days for BDE-183 (Hagmar *et al.* 2000); but recently a more thorough study has indicated half-lives of 14 days for BDE-209 (Jakobsson and Thuresson, personal communication).

BDE-209, measured as total radioactivity, is eliminated relatively rapidly in rats, i.e. approximately 74% of an intravenously (i.v.) administered ^{14}C -labelled BDE-209 was recovered in faeces within 72 h (El Dareer *et al.* 1987). However, BDE-209 with a molecular weight of 959 Da was reported to be poorly absorbed from the gut (Norris *et al.* 1975, El Dareer *et al.* 1987). BDE-209 related radioactivity is recovered almost quantitatively in faeces within 3 days after an oral dose (El Dareer *et al.* 1987, Mörck *et al.* 2003). Comparison of average tissue concentrations after i.v. versus oral dosing to rats in a toxicity study suggested that only $0.33 \pm 0.19\%$ of the dose was absorbed (NTP 1986). However, more recently it has been reported that a low dose of radiolabelled BDE-209 given to bile duct-cannulated rats was absorbed to at least 10%, judged by the excretion in bile (Mörck *et al.* 2003). In the same study, it was reported that 75% of an oral dose to rats was excreted in faeces within 24 h and mainly as phenolic, aqueous and lipid-bound metabolites (53% of the dose), suggesting that BDE-209 is absorbed and rapidly metabolized (Mörck *et al.* 2003).

The inconsistent data on absorption of BDE-209 (NTP 1986, Mörck *et al.* 2003), and the indications of rapid metabolism (Mörck *et al.* 2003), encouraged the present study. The main goal was to calculate the bioavailability and plasma half-life of BDE-209 in the most commonly used laboratory animal, the rat.

Material and methods

Chemicals

Unlabelled decabromodiphenyl ether (BDE-209) was purchased from Fluka Chemie (Buchs, Switzerland) and recrystallized in toluene to a purity of > 98% (the impurities were identified as lower brominated BDEs). Decabromo- ^{14}C -diphenyl ether (specific activity of 15 Ci mol^{-1} , purity > 98%) was synthesized as described (Mörck *et al.* 2003). The internal standard (IS) for quantification of BDE-209 was 2,2',3,4,4',5'-hexabromodiphenyl ether (BDE-138), synthesized as described by Örn *et al.* (1996). Diazomethane was synthesized according to the method described by Fieser and Fieser (1967).

Solvents

n-Hexane and dichloromethane (distol grade) were purchased from Fisher Scientific (Loughborough, UK), methyl-*tert*-butyl ether (MTBE) (HPLC grade) was purchased from Rathburn Chemicals (Walkenburn, UK) and 2-propanol (2-PrOH) of proanalysis (*p.a.*) grade from Prolabo (Cedex, France). Dimethylamide (DMA) was purchased from Sigma Aldrich Chemie (GmbH, Schnelldorf, Germany). Potassium hydroxide (KOH) *p.a.* grade was obtained from Eka Nobel (Bohus, Sweden), and potassium chloride (KCl), silica gel 60 (0.063–0.200 mm), sulphuric acid (H_2SO_4) and hydrochloric acid (HCl), all of *p.a.* grade, from Merck (Darmstadt, Germany).

Dose formulation

Unlabelled BDE-209 was dissolved in DMA to a concentration of 4.5 mg BDE-209 ml⁻¹ DMA and mixed with polyethylene glycol (PEG 400). Water was added drop-wise to the DMA/PEG solution with vigorous stirring. The proportion of DMA/PEG400/water was 4/4/1 (v/v/v) and the final concentration of BDE-209 was 2 µmol ml⁻¹.

Instruments

Gas chromatography/mass spectrometry (GC/MS) analyses were performed with either a Finnigan TSQ 700 or a Finnigan SSQ 710 instruments (Thermo Finnigan, Bremen, Germany). Both MS instruments were connected to a Varian 3400 gas chromatograph equipped with a DB-5HT capillary column (15 m, 0.25-mm i.d., 0.1 µm film thickness; J&W Scientific, Folsom, CA, USA). Helium was used as carrier gas (pressure 5 psi). The injections were performed on-column using a septum-equipped programmable injector fitted with a high-performance insert. The injector temperature was programmed as 60°C, rate 150°C min⁻¹ up to 300°C and kept there for 10 min. The transfer line temperature was 290°C. The oven temperature was programmed as: 80°C (1 min), 15°C min⁻¹ to 300°C (10 min). The instrument was operated in electron capture negative ionization (ECNI) mode with a primary electron energy of 70 eV using methane (≥ 99.995% purity, containing < 5 ppm O₂; AGA, Stockholm, Sweden) as the electron thermalization buffer gas. The ion source temperature was 200°C and the pressure was 5.5 torr. For full-scan experiments, m/z 30–1000 was scanned and for selective ion monitoring (SIM), the bromine isotope ions at m/z 79 and 81 were selected.

Radioactivity measurements were performed on a Wallac 1409 scintillation counter using emulsifier scintillator 299 (Packard Instruments, Meriden, CT, USA) and Opti Scint Hisafe 2 (Wallac Oy, Turku, Finland) as the scintillation cocktail.

Animals: pharmacokinetic study

Thirty-six male Sprague–Dawley rats (200–220 g each, B&K, Sollentuna, Sweden) were kept in cages (three rats per cage) under a 12/12 h light and dark cycle with access to food (R70 Lactamin, from Lab For, Sigtuna, Sweden) and water *ad libitum*. One group (A) of rats (18) was dosed orally by gavage and the second group (B) (18) was dosed i.v. via the tail vein. The dose of BDE-209 was 2 µmol kg⁻¹ (dose volume 0.2 ml/rat) for both groups. The blood sampling schedule was the same for orally and i.v. dosed rats. Three rats were used at each sampling occasion; group 1 was sampled 1 and 24 h after dosing; group 2 after 3 and 48 h; group 3 after 6 and 72 h; group 4 after 96 h; group 5 after 120 h; and group 6 after 144 h. The first sample for rats sampled twice was 1 ml blood taken orbitally and the remaining blood samples (5 ml) were taken by cardiac puncture. The blood was collected into tubes and the samples immediately centrifuged to obtain plasma, which was stored at –18°C.

Plasma samples from a metabolism study

Plasma samples from a previous study on the metabolism of radiolabelled BDE-209 (Mörck *et al.* 2003) were used for qualitative analysis. In this study, eight male Sprague–Dawley rats (200–210 g; Harlan Sprague–Dawley, WI, USA) were dosed orally by gavage with ¹⁴C-labelled BDE-209 (dispersed in a suspending vehicle, consisting of Lutrol F127/soya phospholipone/water (3 µmol kg⁻¹, 15 Ci mol⁻¹ [555 GBq mol⁻¹], dose volume 1 ml kg⁻¹). The rats were kept in metabolism cages (one rat per cage) for 3 or 7 days at a 12/12 h light and dark cycle, with food and water *ad libitum*. Four rats were killed after 3 days and the remaining four rats after 7 days. Blood was collected by cardiac puncture (approximately 5 ml) and immediately centrifuged to obtain plasma and stored at –18°C.

Sample extraction, clean-up and quantification

The extraction and clean-up steps used were described by Hovander *et al.* (2000). Briefly, plasma was transferred to screw-capped test tubes and diluted with KCl (1%, w/v) to give a total weight of 5 g and isopropanol and HCl (6 M, 1 ml) was added. For samples included in the pharmacokinetic evaluation, the internal standard, BDE-138 (1.5, 3.0 or 7.5 pmol), was also added. The diluted plasma was extracted three times with hexane/MTBE. The extract was washed with KCl (1%, w/v). The extract was concentrated to dryness, lipid weight determined gravimetrically, and the extract redissolved in hexane (4 ml). Neutral and phenolic compounds were separated by partitioning with KOH in ethanol (Hovander *et al.* 2000).

For quantification of BDE-209, the fraction containing unchanged compound and neutral metabolites was purified on a silica: H₂SO₄ (1 g, 2/1, w/w) column, eluted with hexane/dichloromethane (10 ml) and dichloromethane (5 ml) (Sjödén *et al.* 1999). After adjustment of the volume to a suitable concentration for GC/MS analysis, unchanged BDE-209 in the samples was quantified by GC/MS (SIM), using a six-point calibration curve. ICIS2 software (Thermo Finnigan, Bremen, Germany) was used for collecting and processing the GC/MS data. For qualitative analysis of metabolites in the phenolic fraction from the pharmacokinetic study, all samples 1–144 h after the oral administration were pooled and samples from the i.v. treatment were pooled similarly. The pooled fractions containing

phenolic metabolites were concentrated and treated with diazomethane. The samples were purified on a silica gel column (Hovander *et al.* 2000) and the methylated metabolites eluted with dichloromethane. The metabolites were analysed by GC/MS in ECNI mode full-scan m/z 30–1000.

The radioactivity content in the plasma samples from the metabolism study (Mörck *et al.* 2003) was determined in plasma before and after separation into phenolic and neutral fractions, respectively. Phenolic compounds were derivatized by addition of an excess of acetic acid anhydride and triethylamine, incubated at room temperature for 1 h, and the acetyl derivatives were extracted with *n*-hexane (Rydberg *et al.* 1996). The parent compound and neutral metabolites were analysed by GC/MS in ECNI mode full-scan m/z 30–1000. Acetylated phenolic metabolites were monitored by scanning the ions m/z 79 and 81.

Pharmacokinetic calculations

The following definitions are used: bioavailability = fraction of administered parent compound reaching systemic circulation; absorption = fraction of dose being absorbed from the gut; exposure = concentration in plasma. The pharmacokinetic calculations were based on the mean plasma concentration–time curves using WinNonlin Pro 3.1 (Pharsight Co., Mountain View, CA, USA). Non-compartmental and compartmental analyses were performed on oral and i.v. data, respectively. The maximum plasma concentration ($C_{\max}$) as well as the time point for $C_{\max}$ ($t_{\max}$) after oral administration was determined. The terminal half-life ($t_{1/2\alpha}$) was calculated from the terminal slope of the logarithm of the total concentration–time curve by linear regression. The total area under the plasma concentration–time curve (AUC) was calculated by the use of the linear trapezoidal rule up to the last datum point plus the residual area up to infinity calculated by integration. The percentage of the total AUC that was extrapolated (AUC_{extr}) was also determined. Total plasma clearance (CL) was calculated by $CL = \text{dose} / AUC_{\text{i.v.}}$ and the apparent volume of distribution at steady state by $V_{\text{ss}} = (\text{dose} * AUMC_{\text{i.v.}}) / (AUC_{\text{i.v.}})^2$. The $AUMC_{\text{i.v.}}$ is the area under the first moment curve calculated up to the last datum point by the trapezoidal rule and then to infinity by means of integration. The systemic exposure after oral administration (F) was calculated by dividing the AUC_{oral} with the $AUC_{\text{i.v.}}$. Using weighted ($1/y^2$) least-squares non-linear regression, a tri-exponential function was fitted to the plasma–concentration time data after the i.v. administration $C = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where α , β and γ are the corresponding slopes A , B and C the intercepts for the respective phases.

Results

In figure 1, the mean plasma concentration–time curves after both oral and i.v. administration of $2 \mu\text{mol kg}^{-1}$ ($0.4 \mu\text{mol/rat}$) BDE-209 are shown together with the individual plasma concentrations. The disposition of BDE-209 after the i.v. dosing was best described by a three-compartment model with $t_{1/2,\alpha}$, $t_{1/2,\beta}$ and $t_{1/2,\gamma}$

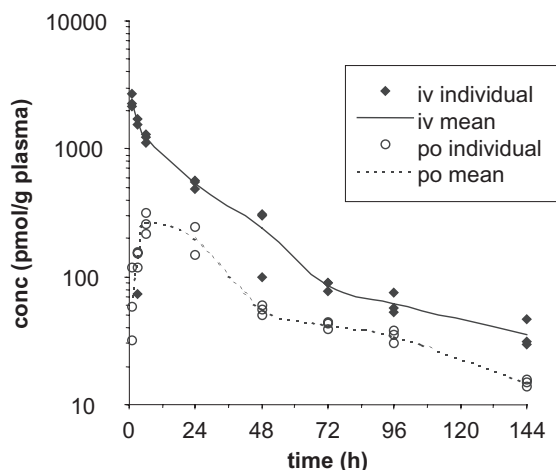


Figure 1. Mean plasma ($n=3$) concentration–time curves and individual data points of BDE-209 after i.v. (◆) and oral (○) administration of $2 \mu\text{mol/kg}$ ($0.4 \mu\text{mol/rat}$). The dotted line represents the mean after oral administration, the solid line after i.v. administration.

Table 1. Pharmacokinetic data characterizing the disposition of BDE-209 following intravenous and oral bolus doses of 0.4 μmol ($2 \mu\text{mol kg}^{-1}$) using a three-compartment model.

	Intravenous	Oral
C_{max} (nmol ml^{-1})	3.5	0.26
t_{max} (h)	—	6
$t_{1/2,\gamma}$ (h)	58	51
$t_{1/2,\beta}$ (h)	12	6.9
$t_{1/2,\alpha}$ (h)	1.6	—
AUC ($\text{nmol ml}^{-1} \cdot \text{h}$)	47	12
F (%)	—	26
CL ($\text{ml min}^{-1} \text{ kg}^{-1}$) (250 g)	0.60	—
V_{ss} (l kg^{-1}) (250 g)	1.4	—

values of 1.6, 12 and 58 h, respectively (table 1). The β -phase represents 56% of the total AUC, whereas the γ -phase accounts for 34% and the α -phase for only 10% of the total AUC. The CL was calculated as $0.6 \text{ ml min}^{-1} \text{ kg}^{-1}$ and V_{ss} as 1.4 l kg^{-1} . The oral bioavailability (F) was calculated to be 26%.

The mean C_{max} after oral dosing was determined as 264 pmol ml^{-1} in the 6-h sample after dosing and the terminal half-life $t_{1/2\gamma}$ was 51 h. The average concentration in the 72-h plasma sample, obtained after oral administration of BDE-209, were 42 pmol g^{-1} fresh weight (f.w.) (range $39\text{--}45 \text{ pmol g}^{-1}$ f.w.). The average lipid content in the plasma samples from orally and i.v. dosed rats was 0.5%. The mean plasma concentration on lipid weight (l.w.) basis was thus 8.4 nmol g^{-1} l.w. in the 72-h plasma sample.

Metabolite analysis

GC/MS analysis (pooled samples) of the neutral compounds in plasma from orally and i.v. dosed rats showed that the major compound was unmodified BDE-209. In all samples, traces ($< 0.5\%$ peak area cf. BDE-209) of three nonaBDEs (metabolites 1–3, M1–3) were also found.

The pooled phenolic fractions were analysed after derivatization with diazo-methane both by scanning the bromine isotopes (m/z 79 and 81) and by full-scan MS (m/z 30–1000). In the SIM analyses (m/z 79 and 81), 13 peaks containing brominated compounds were detected in both the orally and i.v. dosed rats. The relative amount of metabolites was similar for both administration routes. By full-scan MS of the pooled samples, only three phenolic metabolites were present in sufficiently high concentration to allow interpretation of the mass spectra.

In electron-capture negative ionization, halogenated diphenyl ethers are cleaved on either side of the ether bridge resulting in two observable fragments, each containing the ether oxygen, and no molecular ion can be determined (Campbell *et al.* 1985, Haglund *et al.* 2000). The metabolites detected followed this pattern and the observed fragments are listed in table 2. Two phenolic metabolites were characterized as methyl derivatives of mono-hydroxylated octa- and nonaBDE (M4, M5) (figure 2). The mass spectra had one ion corresponding to a fragment containing the ether oxygen, four bromine atoms and one methoxy group (addition of 30 mass units) and the second ion corresponded to a fragment containing the ether oxygen plus four or five bromine atoms, respectively (M4 and M5; table 2). The third phenolic metabolite (M6) had one fragment containing the ether oxygen, two bromine atoms and an additional substituent corresponding to

Table 2. Major fragments of the methylated phenolic metabolites in pooled plasma samples observed by GC/MS (ECNI mode) from the orally and intravenously dosed rats. Tentative metabolite structures are shown in figure 2.

Metabolites in the phenolic fraction		
Metabolite	Fragment 1 (m/z)	Fragment 2 (m/z)
M4	434.8	404.8
M5	434.8	482.6
M6	306.9	404.8

58 mass units and the second fragment containing the ether oxygen and four bromine atoms on the phenyl ring (M6, table 2). This fragmentation is similar to that observed in a previous study of decaBDE metabolism where the use of acetylation as derivatization method in addition to methylation suggested the metabolite structure to be a methyl derivative of a guaiacol-type structure (the structure is shown in figure 2) (Mörck *et al.* 2003).

Distribution of radioactivity

The plasma sample taken in the metabolism study, at 3 days (about 72 h) after an oral dose of ^{14}C -labelled BDE-209 had a total concentration of 50 pmol $^{14}\text{C g}^{-1}$ f.w. After 7 days (168 h), the concentration was 20 pmol $^{14}\text{C g}^{-1}$ f.w. The average lipid content in the plasma was 0.2% resulting in concentrations of radioactivity on a lipid weight basis to be 22 and 8.8 nmol $^{14}\text{C g}^{-1}$ l.w. at 3 and 7 days, respectively. Separation into phenolic and neutral compounds revealed that 21% of the total radioactivity on day 3 and 19% day 7 represented neutral compounds. According to GC/MS (ECNI), the neutral fraction primarily contained unchanged BDE-209 and traces (<0.5% of the BDE-209 peak area) of three nonaBDEs (M1–3). The phenolic fraction could not be purified enough to allow interpretation of the mass spectra.

Discussion

In the present study, the pharmacokinetic properties of BDE-209 in the rat were evaluated using unlabelled BDE-209 and the metabolites in plasma were qualitatively analysed as pooled samples. As a supplementary study, qualitative analysis of two plasma samples from a study on the excretion and metabolism of ^{14}C -labelled BDE-209 in the rat were performed. The dose and the vehicle were similar but not identical, and the animal experiments performed at different laboratories, and could thus give rise to differences in the results.

The pooled phenolic metabolites in the samples were analysed by GC/MS. Two of the observed metabolites were tentatively identified to be hydroxy-nonaBDE and hydroxy-octaBDE (M4 and M5, figure 2). The mass spectrum of the third metabolite (M6, figure 2) was similar to that previously observed for BDE-209 metabolites and was interpreted to correspond to a guaiacol-structure (hydroxy- and methoxy-group on adjacent carbons) and six bromine atoms. Reductive debromination, resulting in the loss of bromine atoms, may be the first step in the metabolic pathway (M1–3). Such reaction occurs also for hexabromobenzene (Koss *et al.* 1982). Subsequent oxidation would result in the phenolic metabolites M4 and M5. The guaiacol metabolites were most probably

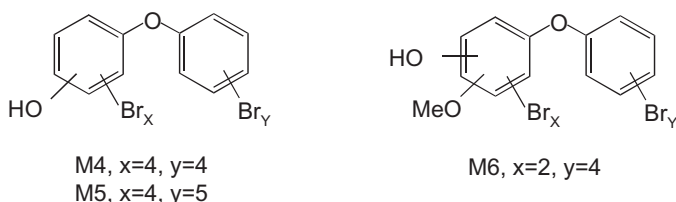


Figure 2. BDE-209 metabolites in plasma tentatively identified by GC/MS (ECNI).

formed via an arene oxide and dihydrodiol and further re-aromatized, or via two subsequent oxidations and finally methylated. The metabolic pathway for BDE-209 is discussed by Mörck *et al.* (2003).

The plasma sample from the metabolism study provided information on total radioactivity and relative amount of phenolic and neutral compounds in the plasma samples. At both 3 and 7 days after the oral dose, the levels of phenolic metabolites (based on radioactivity) were four times higher than that of the neutral, i.e. parent BDE-209. A possible explanation for the high concentration of phenolic metabolites relative to BDE-209 could be reversible binding to the thyroxine hormone transporting protein transthyretin. Hydroxylated metabolites of PCB, with the hydroxy-group in the *para*-position and chlorine atoms on the adjacent carbons, have affinity for transthyretin that results in the retention of the metabolites in blood (Lans *et al.* 1993, Bergman *et al.* 1994, Brouwer *et al.* 1998). Lower brominated diphenyl ethers have been shown to produce metabolites *in vitro* that also have affinity for transthyretin (Meerts *et al.* 2000). Based on the structural requirements described for binding to transthyretin (Lans *et al.* 1993), it is plausible that at least hydroxy-nonaBDE and hydroxy-octaBDE also have affinity for this protein.

Absorption of BDE-209 after oral administration has previously been reported to be low ($< 0.5\%$ of the dose) (Norris *et al.* 1975, NTP 1986, El Dareer *et al.* 1987). The present result demonstrated that about 26% of BDE-209 was bioavailable. The presence of phenolic metabolites compared with neutral compounds indicate that the absorption of BDE-209 is higher than the 26% calculated for the parent BDE-209, suggesting that BDE-209 undergoes first-pass metabolism before reaching the circulation. A higher absorption is also suggested by the results of an earlier metabolism study in rats (Mörck *et al.* 2003) where 65% of the dose was recovered as metabolites in faeces within 3 days. Possible explanations for the discrepancy between the bioavailability of BDE-209 (26%) and the amount of metabolites (65%) is probably an extensive first-pass effect in the gastrointestinal tract and/or the liver but could also in part be due to metabolism by the microflora.

The V_{SS} of 1.4 l kg^{-1} was unexpectedly low for a lipophilic compound such as BDE-209. However, it corresponds well with the low distribution of radioactivity to adipose tissue observed after administration of ^{14}C -BDE-209 to rats ($3 \mu\text{mol kg}^{-1}$, 15 Ci mol^{-1}) (Mörck *et al.* 2003). Instead, the highest radioactive concentrations were found in well-perfused organs such as the liver. The most common site for lipophilic compounds is otherwise the lipid-rich tissues, e.g. adipose tissue (Klasson Wehler *et al.* 1993, 1996, Örn and Klasson Wehler 1998, Hakk *et al.* 2002). BDE-209 showed surprisingly low distribution to adipose tissue with a liver-to-adipose tissue ratio of 5 at day 3 (Mörck *et al.* 2003) compared with 0.02–0.05 for PCBs at steady state (Öberg *et al.* 2002) and tetrabrominated

diphenyl ether, BDE-47 with a liver-to-adipose ratio of <0.01 (Örn and Klasson Wehler 1998).

The excretion of BDE-209 has been reported to be rapid with 90% of the total radioactivity given orally excreted in faeces within 3 days and the excreted radioactivity consisted of 65% of dose as metabolites (Mörck *et al.* 2003). The initial α -phase with a $t_{1/2\alpha}$ of about 2 h showed a rapid distribution of BDE-209 to well-perfused tissues such as the liver (Mörck *et al.* 2003). In the present study, the terminal $t_{1/2\gamma}$ was approximately 2.5 days (58 h) and although this is short relative to other halogenated aromatic compounds such as penta-octaBDEs and PCBs (Tanabe *et al.* 1981), it should be remembered that the plasma levels of the phenolic metabolites are four times higher than the parent BDE-209 level at days 3 and 7. Thus, the exposure to phenolic metabolites seems to be higher than to the parent compound, BDE-209.

Halogenated aromatic pollutants such as PCBs and PBDEs are generally readily absorbed via the gastrointestinal tract and the route of excretion is mainly in the faeces (Klasson Wehler *et al.* 1993, 1996, Hakk *et al.* 2002). These compounds are rapidly distributed to lipid-rich tissues and they are often metabolized very slowly and thereby retained in the body for a long time. The physical properties of BDE-209, $\log P > 7$, a molecular weight of 959 Da and full bromination would be expected to result in bioaccumulation once it was absorbed. However, the current study provides evidence that BDE-209 is well absorbed after oral administration and is rapidly eliminated primarily by metabolism. The terminal half-life of BDE-209 in the rat is approximately 2.5 days (58 h) and it is not retained in adipose tissue. Metabolism converts the BDE-209 to a large number of phenolic metabolites that were observed in plasma, including a guaiacol-type metabolite. The dominating metabolites in plasma were hydroxy-nonaBDE and hydroxy-octaBDE. The retention of the phenolic metabolites may be of significance as there may be a risk for interaction with the thyroxine hormone transporting protein transthyretine, similar to that reported for PCB metabolites (Brouwer *et al.* 1998) and for *in vitro* generated metabolites of lower brominated diphenyl ethers (Meerts *et al.* 2000). This however requires further studies.

Acknowledgement

The skilful technical assistance for GC/MS analysis by Ioannnis Athansiadis and linguistic improvement of the manuscript by Associate Professor Hugh Salter (AstraZeneca R&D, Södertälje, Sweden) are gratefully acknowledged. The project was supported financially by the Cancer and Allergy Foundation, Stockholm, Sweden.

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Pharmacokinetics, metabolism and excretion of an inhibitor of inducible nitric oxide synthase, L-NIL-TA, in dog

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Received 13 March 2003

1. The pharmacokinetics, metabolism and excretion of L-NIL-TA, an inducible nitric oxide synthase inhibitor, were investigated in dog.

2. The dose of [^{14}C]L-NIL-TA was rapidly absorbed and distributed after oral and intravenous administration (5 mg kg^{-1}), with C_{max} of radioactivity of $6.45\text{--}7.07\text{ }\mu\text{g equivalents g}^{-1}$ occurring at $0.33\text{--}0.39\text{-h}$ after dosing. After oral and intravenous administration, radioactivity levels in plasma then declined with a half-life of 63.1 and 80.6-h , respectively.

3. Seven days after oral and intravenous administrations, 46.4 and 51.5% of the radioactive dose were recovered in urine, 4.59 and 2.75% were recovered in faeces, and 22.4 and 22.4% were recovered in expired air, respectively. The large percentages of radioactive dose recovered in urine and expired air indicate that [^{14}C]L-NIL-TA was well absorbed in dogs and the radioactive dose was cleared mainly through renal elimination. The mean total recovery of radioactivity over 7 days was approximately 80% .

4. Biotransformation of L-NIL-TA occurred primarily by hydrolysis of the 5-aminotetrazole group to form the active drug L-N6-(1-iminoethyl)lysine (NIL or M3), which was further oxidized to the 2-keto acid (M5), the 2-hydroxyl acid (M1), an unidentified metabolite (M2) and carbon dioxide. The major excreted products in urine were M1 and M2, representing 22.2 and 21.2% of the dose, respectively.

Introduction

L-N6-(1-iminoethyl)lysine-5-tetrazole-amide (L-NIL-TA) is a selective inhibitor of inducible nitric oxide synthase (iNOS) and under development for the treatment of osteoarthritis (OA) (Hallinan *et al.* 2002). It has been proposed that nitric oxide (NO) both activates cartilage degradative enzymes and inhibits chondrocyte proteoglycan synthesis (Hickery *et al.* 1994, Taskiran *et al.* 1994, Järvinen *et al.* 1995). NO is produced in large amounts by chondrocytes upon pro-inflammatory cytokine stimulation (Charles *et al.* 1993, Järvinen *et al.* 1995). In contrast to normal cartilage, osteoarthritic cartilage spontaneously produces NO, likely reflecting the *in situ* stimulation of chondrocytes by cytokines (Amin *et al.* 1995, Pelletier *et al.* 1996). Furthermore, high levels of nitrite/nitrate have been found in the synovial fluid and serum of arthritis patients (Farrell *et al.* 1992), and both the mRNA and protein for inducible NO synthase, an enzyme responsible for NO production, have also been detected in synovial tissue from OA patients (Sakurai

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et al. 1995, McInnes *et al.* 1996). It has been hypothesized that NO contributes to the development of arthritic lesions (Stefanovic-Racic *et al.* 1993, Evans *et al.* 1995, Cannon *et al.* 1996). This hypothesis is based on data showing that in addition to inhibiting the synthesis of cartilage matrix macromolecules (Taskiran *et al.* 1994, Järvinen *et al.* 1995), NO also enhances matrix metalloprotease (MMP) activity (Murrell *et al.* 1995). Moreover, NO was shown to reduce the synthesis of the IL-1 receptor antagonist (IL-1Ra) in chondrocytes (Pelletier *et al.* 1996), a process possibly responsible for the enhanced IL-1[β] effect on these cells. In experimental models of inflammatory arthritis, treatment with compounds that inhibit iNOS activity, either non-selectively or in a few cases selectively, has been reported to reduce the severity of arthritis (McCartney-Francis *et al.* 1993, Stefanovic-Racic *et al.* 1993, 1994, 1995, Connor *et al.* 1995, Evans *et al.* 1995, Hobbs *et al.* 1999).

L-NIL-TA is designed to be cleaved in blood to the active drug, L-N6-(1-iminoethyl) lysine (L-NIL), which inhibits inducible nitric oxide synthase (Moore *et al.* 1994). The effectiveness of a selective inhibitor of iNOS, L-NIL, has been confirmed in attenuating the progression of experimental OA. The results suggested that L-NIL may act by reducing the activity of metalloproteases in cartilage and the production of IL-1[β] by synovium, both of which are known to play a major role in the pathophysiology of OA structural changes (Pelletier *et al.* 1998, Salerno *et al.* 2002). Furthermore, in an experimental dog model of OA, the selective inhibition of iNOS by L-NIL and the subsequent decreased production of NO results in a marked decrease in production of major catabolic factors such as MMP, IL-1[β] and peroxynitrite, as well as a reduction in the inducible form of cyclooxygenase (COX-2) expression (Pelletier *et al.* 1999a, b).

The present study investigated the pharmacokinetics, metabolism and excretion of L-NIL-TA in dogs following a single oral or intravenously (i.v.) administration. Routes of excretion were determined and metabolites isolated from urine and their structures were identified.

Materials and methods

Chemicals

L-NIL-TA and radiolabelled [^{14}C]L-NIL-TA (figure 1) were synthesized by Pfizer, Inc. (Skokie, IL, USA). The specific activity of [^{14}C]L-NIL-TA in dosing solution was $4.8\ \mu\text{Ci mg}^{-1}$ and the radiochemical purity was approximately 98.1%, as determined by HPLC with a in-line radioactivity detector. L-N6-(1-iminoethyl)lysine (M3), 2-hydroxy-6-[(1-iminoethyl)amino]hexanoic acid (M1) and 2-keto-6-[(1-iminoethyl)amino]hexanoic acid (M5) were synthesized at Pfizer, and the structures were confirmed by MS and NMR (figure 1). All other chemicals and reagents were analytical grade and were commercially available.

Animal studies

For oral administration, six male and six female pure-bred beagle dogs (HSD/Ridgman Farms, Inc., Mt. Horeb, WI, USA), approximately 8 months old and weighing 8–10 kg, were administered [^{14}C]L-NIL-TA in Mega-pure water at a dose of $5\ \text{mg kg}^{-1}$ via a gavage tube. The dogs were acclimated for approximately 2 weeks before use. Three male and three female dogs were housed in individual stainless steel cages for blood collection. The remaining six dogs were housed in individual metabolism cages designed for the separation and collection of expired air, urine and faeces. Animals were provided certified canine diet #5007 (PMI Feeds, Inc., St Louis, MO, USA) and water *ad libitum*. For i.v. administration, six female pure-bred beagle dogs, approximately 10 months old and weighing 8–9 kg, were administered [^{14}C]L-NIL-TA i.v. in saline (0.9% sodium chloride) at $5\ \text{mg kg}^{-1}$. Three female dogs were housed in individual stainless steel cages for blood collection. Three female dogs were housed in metabolism cages designed for the collection of expired air, urine and faeces.

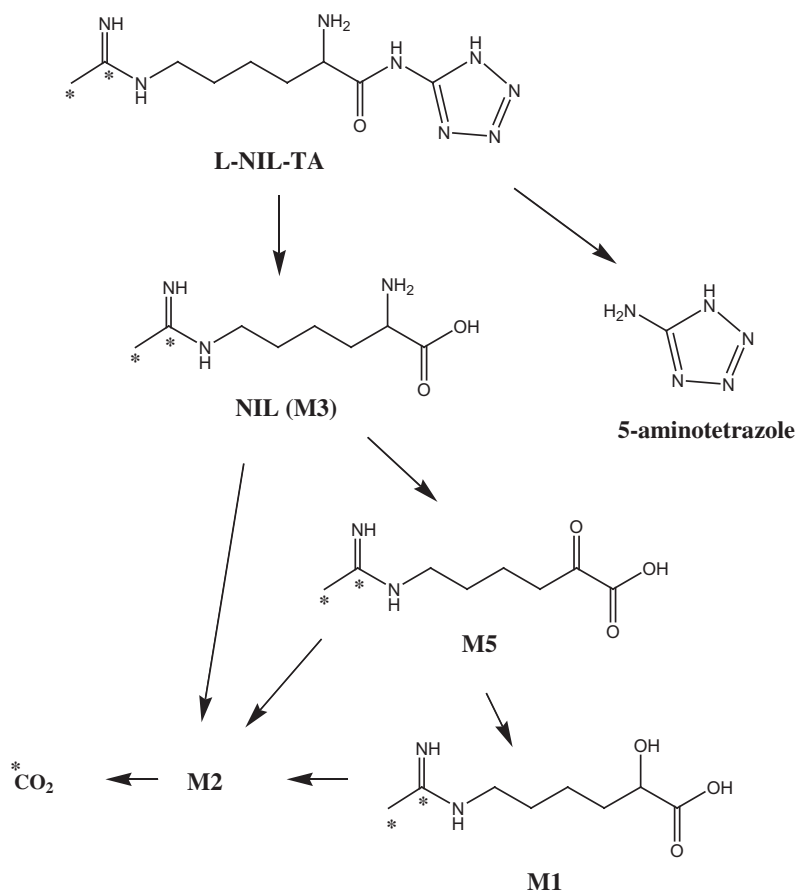


Figure 1. Proposed biotransformation pathways of L-NIL-TA in dogs (* denotes the Positions labeled with ^{14}C).

Blood samples (approximately 2 ml) were collected from the jugular vein before dosing and at 0.33, 0.67, 1, 1.5, 2, 3, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168-h post-dose and transferred to heparinized tubes. The blood was placed on wet ice or in Kryoracks immediately following collection. Blood samples were centrifuged at approximately 1300 g at 4°C for 10 min. All urine and faeces were collected from the dogs at -18 to 0-h predose and at 24-h intervals for 7 consecutive days after dose administration. Urine was collected into containers surrounded by dry ice. Faeces were frozen on dry ice immediately at the end of each collection interval. The weight of the urine and faecal samples was recorded and stored at approximately -20°C until analysed for total radioactivity and metabolic profile. After the last excreta collection, the cages were washed with a 1% trisodium phosphate solution and wiped with gauze pads. The gauze (cage wipe) and cage wash were saved for radioanalysis. Expired volatile compounds and carbon dioxide trapping solutions were collected predose and at 24-h intervals through 168-h post-dose. Expired air was passed through an activated charcoal trap to recover volatile organic compounds and then through a carbon dioxide trapping solution (1:1 2-ethoxyethanol:ethanolamine) to recover radiolabelled carbon dioxide. Carbon dioxide-trapping solutions were transferred to plastic containers at the time of collection and the weight of each sample was recorded.

Radioactivity analyses

Plasma and red blood cells (RBC). Duplicate aliquots (approximately 0.1 g) of plasma were placed in 20-ml glass scintillation vials along with liquid scintillation cocktail (AquaassureTM, PerkinElmer Life Sciences, Boston, MA, USA) and radioactivity was determined by a Model 1900 TF liquid scintillation counter (LSC) (PerkinElmer Life Sciences). RBC samples were homogenized by stirring several times with a spatula. Duplicate aliquots (approximately 0.1 g) were weighed in a CombustaconeTM with pads (Packard Instrument Co., Downers Grove, IL, USA), combusted, and analysed by LSC.

Urine. The total weight of the urine samples was recorded. Duplicate aliquots (approximately 0.25 g) of urine were placed in liquid scintillation vials along with liquid scintillation cocktail and radioactivity was determined by LSC.

Faeces. Faecal samples were thawed at ambient temperature and weighed. Approximately two-to-three times the sample weight of water was added. The faecal samples were homogenized in a StomacherTM bag using a StomacherTM Laboratory Blender 400 (A. J. Seward, London, UK). Duplicate aliquots (approximately 0.25 g) of each faecal homogenate were placed in a CombustoconeTM with pads and allowed to dry overnight. The samples were combusted using a Packard Sample Oxidizer (PerkinElmer Life Sciences). The combustion products were trapped in 7 ml CarbosorbTM and mixed with 9 ml PermafluorTM (PerkinElmer Life Sciences) for determination of radioactivity by LSC.

Cage wash. The total weight was recorded. The samples were homogenized by shaking lightly. Duplicate aliquots (approximately 0.5 g) were weighed into scintillation vials and analysed by LSC.

Cage wipe. Sufficient water was added to cover the sample and the weight was recorded. The samples were allowed to extract overnight. The samples were shaken gently and duplicate aliquots (approximately 0.5 g) were weighed into scintillation vials and analysed by LSC.

Expired air. The total weight of the trapping solution was recorded. The samples were homogenized by shaking and duplicate aliquots (approximately 0.5 g) were weighed into vials. Methanol (approximately 5 ml) was added, the aliquot shaken, then PermafluorTM (approximately 10 ml) was added and the samples analysed by LSC.

Volatiles. The two charcoal tubes were split into four to six samples, the charcoal combusted and analysed by LSC.

Quantitative metabolite profiling

Plasma. The dog plasma samples (approximately 1 ml) were extracted with acetonitrile at a ratio of 3:1. The samples were acidified with 2 N HCl (80 μ l), mixed by vortexing for 2 min and then centrifuged at 2000g for 10 min. Supernatants were removed to a separate tube and acetonitrile:water (50:50, v/v) was added to the pellet at a ratio of 1:1. The pellet and acetonitrile:water were vortexed and centrifuged as indicated above. Supernatants from the two extractions were combined. The recovery of radioactivity following the extraction procedure was $99.5\% \pm 5.6\%$ ($n = 12$). The supernatants were dried under a gentle stream of nitrogen at room temperature and the residues were reconstituted in mobile phase and injected directly onto HPLC system to determine the metabolic profile.

Urine. The urine samples from each dog (5% of the 0–168-h samples) were pooled. An aliquot (10 ml) of each pooled urine sample was concentrated under nitrogen to 1.5 ml. Samples were vortexed, sonicated for 10 min and then centrifuged at 1000g for 15 min. The supernatant was removed and 100 μ l of the urine samples were injected onto the HPLC system to determine the metabolic profile. The percentage of the dose excreted as each metabolite in urine was calculated as the per cent of dose recovered in the urine sample $\times$ the fraction of total HPLC radioactivity of each metabolite peak.

HPLC

HPLC analysis of dog plasma and urine samples was performed using a 1050 series autosampler and pump (Agilent Technologies, Palo Alto, CA, USA) equipped with a YMC Basic HPLC column (4.6 $\times$ 250 mm, 5 μ m, YMC, Inc., Wilmington, NC, USA). An isocratic system was run with 2% acetonitrile and 98% buffer consisting of 50 mM sodium phosphate and 50 mM pentanesulfonic acid sodium salt (pH 3) over 35 min at a flow rate of 1 ml min⁻¹. The HPLC eluent was mixed with Flo-Scint TM III at a ratio of 1:3 (v/v) and the C-14 radioactivity was detected with a Flo-One\Beta A-500 radioactive detector (all PerkinElmer Life Sciences). The radioactive peaks were collected at 3–5, 5–6, 12–14 and 16–18 min for dog urine using a fraction collector. The fractions were evaporated to dryness under a stream of nitrogen and the residues were extracted with 0.5 ml acetonitrile. After centrifugation, the supernatants were transferred and evaporated to dryness in preparation for LC-MS analysis.

LC-MS/MS

LC-MS/MS analyses were performed on a system consisting of an Agilent 1100 autosampler and HPLC pump coupled to a Micromass Quattro II triple quadrupole mass spectrometer (Altrincham, UK). The separation was performed on a Zorbax SB-Phenyl HPLC column (4.6 $\times$ 250 mm, 4 μ m, Agilent Technologies, Chadds Ford, PA, USA) that was eluted isocratically with 100% mobile phase A

(0.4% trifluoroacetic acid in water) for 8 min at a flow rate of 1.0 ml min^{-1} , then programmed with a linear gradient system to 20% mobile phase A and 80% of mobile phase B (0.4% trifluoroacetic acid in methanol) over 7 min and held for another 10 min. Approximately 20% of the flow was diverted to the mass spectrometer using a 1:4 T-splitter. The remainder of the flow was mixed with Flo-Scint TM III at a ratio of 0.8:3 (v/v) and detected with a Flo-One\Beta A-500 radioactive detector. The mass spectral analysis was performed in positive-ion electrospray ionization mode with the capillary and the cone voltages at 3.6 and 27 V, respectively. Collision-induced dissociation (CID) studies were performed using a collision energy of 21 eV and at a collision cell pressure of 2×10^{-3} mBar argon.

Results

Plasma and RBC concentrations of total radioactivity

Concentrations of radioactivity in plasma and RBC versus time after oral and i.v. administrations of [^{14}C]L-NIL-TA to dogs are shown in figures 2 and 3. Mean pharmacokinetic parameters are listed in table 1. All concentrations are expressed as μg equivalents of [^{14}C]L-NIL-TA g^{-1} . [^{14}C]L-NIL-TA was rapidly absorbed after administration, with a mean maximum plasma concentration of radioactivity of $7.07 \mu\text{g}$ equivalents g^{-1} occurring at 0.39-h after oral dosing and $6.45 \mu\text{g}$ equivalents g^{-1} occurring at 0.33-h after i.v. dosing. Radioactivity levels in plasma then declined with a half-life of 63.1 and 80.8-h after oral and i.v. administrations, respectively. The AUC_{0-168} values for total radioactivity after oral and i.v. dose were 276 and $220 \mu\text{g}$ equivalents h g^{-1} , respectively. Mean concentrations of radioactivity in RBC reached a maximum at 24-h after dose administration.

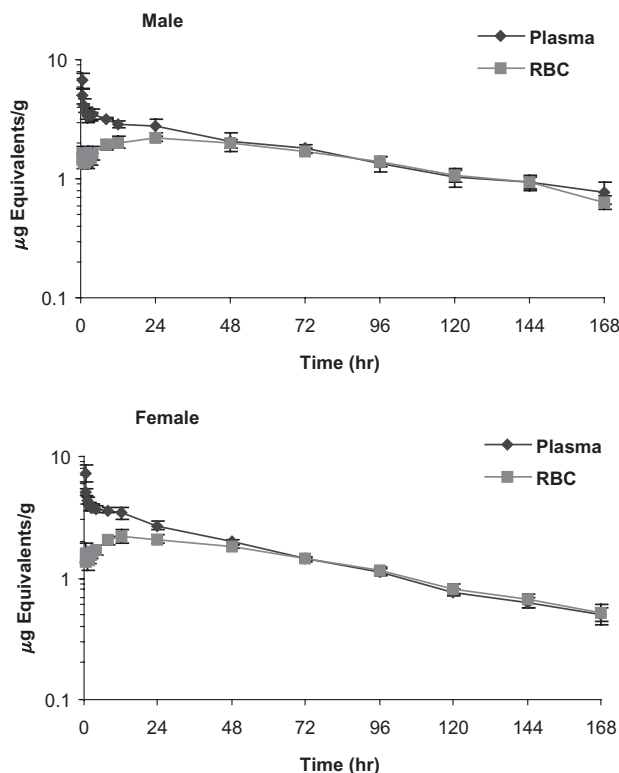


Figure 2. Plasma and RBC concentrations of total radioactivity after a single oral dose of 5 mg/kg [^{14}C]L-NIL-TA to dogs. Each point represents the mean $\pm$ SEM of three animals.

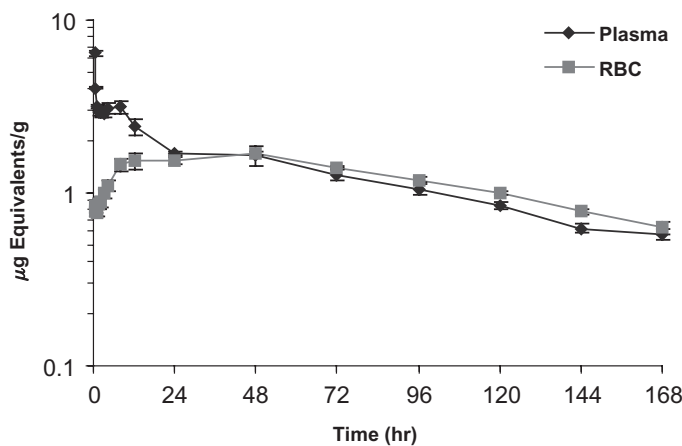


Figure 3. Plasma and RBC concentrations of total radioactivity after a single i.v. dose of 5 mg/kg [^{14}C]L-NIL-TA to dogs. Each point represents the mean $\pm$ SEM of three animals.

Table 1. Pharmacokinetics of radioactivity in plasma for dogs after a single oral and i.v. doses of 5 mg kg $^{-1}$ [^{14}C]L-NIL-TA.

Dose	Gender	$C_{\max}$ ($\mu\text{g eq g}^{-1}$)	$T_{\max}$ (h)	AUC_{0-168} ($\mu\text{g eq}\cdot\text{h g}^{-1}$)	$t_{1/2}$ (h)
Oral	Plasma				
	Male	6.70 ± 0.98	0.33 ± 0.00	288 ± 31	72.5 ± 9.3
	Female	7.44 ± 1.18	0.44 ± 0.11	263 ± 9.0	53.6 ± 4.4
	male + female	7.07 ± 0.70	0.39 ± 0.06	276 ± 15	63.1 ± 6.2
	RBC				
	Male	2.34 ± 0.07	20.0 ± 4.0	250 ± 15	86.6 ± 17.21
IV	Female	2.29 ± 0.23	14.7 ± 4.8	220 ± 9.0	73.0 ± 20.7
	male + female	2.31 ± 0.26	17.3 ± 7.4	235 ± 25	80.3 ± 30.2
	Plasma				
	Female	6.45 ± 0.22	0.33 ± 0.0	220 ± 0.20	80.6 ± 7.71
	RBC				
	Female	1.73 ± 0.07	36 ± 12	205 ± 3.7	84.9 ± 11.1

Data are the mean $\pm$ SEM of three males and females.
RBC, red blood cells.

Radioactivity concentrations in RBC were generally lower than corresponding concentrations in plasma at all time points up to and including the maximum mean concentrations seen at 24-h after dosing. However, RBC concentrations were nearly equal to plasma between 48 and 168-h post-dose. All pharmacokinetic parameters were calculated based on the mean data, which derived from values determined in individual animals.

Radioactivity in urine, faeces and expired air

The excretion data after oral and i.v. administration of [^{14}C]L-NIL-TA to dogs are summarized in tables 2 and 3. There was little difference for excretion data between oral and i.v. administration, which is indicative of extensive absorption of the oral dose. Within 7 days after administration of a 5 mg kg $^{-1}$ dose of [^{14}C]L-NIL-TA, 46.4% (oral) and 51.5% (i.v.) of the dose were recovered in urine, 22.4% was recovered in expired air for both oral and i.v., and 4.59% (oral) and 2.75% (i.v.) were recovered in faeces. A small amount of the dose (less than 1%)

Table 2. Excretion of radioactive dose in urine, faeces, cage wash, case wipe, expired air trap and volatiles after a single oral dose of 5 mg kg⁻¹ [¹⁴C]L-NIL-TA.

Collection interval (h)	Per cent of radioactive dose excreted in:			
	Urine	Faeces	Expired air trap	Volatiles
0-24	11.6 ± 1.26	0.75 ± 0.17	3.99 ± 0.38	0.04 ± 0.01
24-48	10.0 ± 1.27	0.65 ± 0.08	4.84 ± 0.51	0.08 ± 0.01
48-72	7.55 ± 0.70	0.65 ± 0.11	4.41 ± 0.57	0.13 ± 0.01
72-96	6.43 ± 0.49	0.81 ± 0.15	3.57 ± 0.42	0.16 ± 0.01
96-120	4.50 ± 0.30	0.66 ± 0.15	2.30 ± 0.27	0.15 ± 0.01
120-144	3.75 ± 0.32	0.65 ± 0.15	1.98 ± 0.29	0.14 ± 0.01
144-168	2.46 ± 0.14	0.41 ± 0.09	1.34 ± 0.19	0.12 ± 0.02
0-168	46.4 ± 2.63	4.59 ± 0.77	22.4 ± 2.32	0.82 ± 0.06
Cage wash			1.88 ± 0.15	
Cage wipe			0.99 ± 0.31	
Total			77.1 ± 2.44	

Data are the mean ± SEM of three males and females.

Table 3. Excretion of radioactive dose in urine, faeces, cage wash, case wipe, expired air trap and volatiles after a single i.v. dose of 5 mg kg⁻¹ [¹⁴C]L-NIL-TA.

Collection interval (h)	Per cent of radioactive dose excreted in:			
	Urine	Feces	Expired air trap	Volatiles
0-24	12.7 ± 2.06	0.28 ± 0.02	3.38 ± 0.09	0.02 ± 0.00
24-48	9.85 ± 0.69	0.30 ± 0.07	5.02 ± 0.31	0.07 ± 0.01
48-72	8.34 ± 0.82	0.68 ± 0.12	4.43 ± 0.41	0.10 ± 0.03
72-96	6.95 ± 0.52	0.42 ± 0.08	3.38 ± 0.21	0.09 ± 0.01
96-120	5.36 ± 0.21	0.35 ± 0.05	2.37 ± 0.38	0.13 ± 0.02
120-144	4.71 ± 0.37	0.39 ± 0.07	2.33 ± 0.49	0.14 ± 0.01
144-168	3.58 ± 0.08	0.33 ± 0.09	1.51 ± 0.34	0.09 ± 0.02
0-168	51.5 ± 4.49	2.75 ± 0.32	22.4 ± 0.90	0.57 ± 0.05
Cage wash			1.22 ± 0.17	
Cage wipe			1.25 ± 0.78	
Total			79.7 ± 4.49	

Data are the mean ± SEM of three males and females.

was excreted as volatile organics. The large percentage of the dose recovered in expired air indicated that the carbon-14 radiolabel of [¹⁴C]L-NIL-TA was located in a metabolically labile functional group within the molecule, ultimately resulting in expiration of ¹⁴CO₂. Recovery of approximately 70% of the dose in urine and expired air indicated that [¹⁴C]L-NIL-TA was well absorbed after oral administration to dogs. The comparable percentages of dose radioactivity recovered in urine and faeces of male and female dogs after oral administration indicated that there was no gender effect on the renal and hepatobiliary elimination of [¹⁴C]L-NIL-TA in dogs. However, the somewhat larger percentage of radioactivity recovered in expired air collected from female dogs (26.9%) compared with males (18.0%) over 7 days might indicate a small gender-rated difference in the metabolism of this compound. The mean total recoveries of radioactivity in urine, faeces, cage wash, cage wipe, expired air and volatiles over 7 days were 77.1 and 79.1% after oral and i.v. administration, respectively. Renal elimination of absorbed [¹⁴C]L-NIL-TA radioactivity was slow in dogs, with approximately 12% of the dose radioactivity excreted in the first 24-h after dosing. This 24-h

urinary recovery corresponds to only about one-quarter of the total 7-day recovery of radioactivity in the urine. By 72-h post-dose, approximately 30% of the dose radioactivity had been excreted in urine, corresponding to 60% of total 7-day urinary recovery of radioactivity. Similarly, about 60% of the total dose radioactivity recovered in expired air had been eliminated by 72-h after dosing.

Metabolites of L-NIL-TA present in plasma and urine

Representative HPLC profiles in plasma and urine after oral administration of [^{14}C]L-NIL-TA are shown in figure 4. Most of the radioactivity in plasma was associated with the active drug M3 (L-NIL). However, minor amounts of M2 were also observed in the dog plasma samples obtained at 8 and 24-h post-dose. There was little difference in the plasma profiles between 8 and 24-h post-dose. The major metabolites in the dog urine were M1 and M2, accounting for 21.8 and 21.3% of the dose in urine, respectively. Minor amounts of L-NIL-TA (<1%), M3 (<1%) and M5 (<2%) were also observed in the urine. There was no significant difference of the urine profiles from 0–24, 24–28 and pooled 0–168-h post-dose.

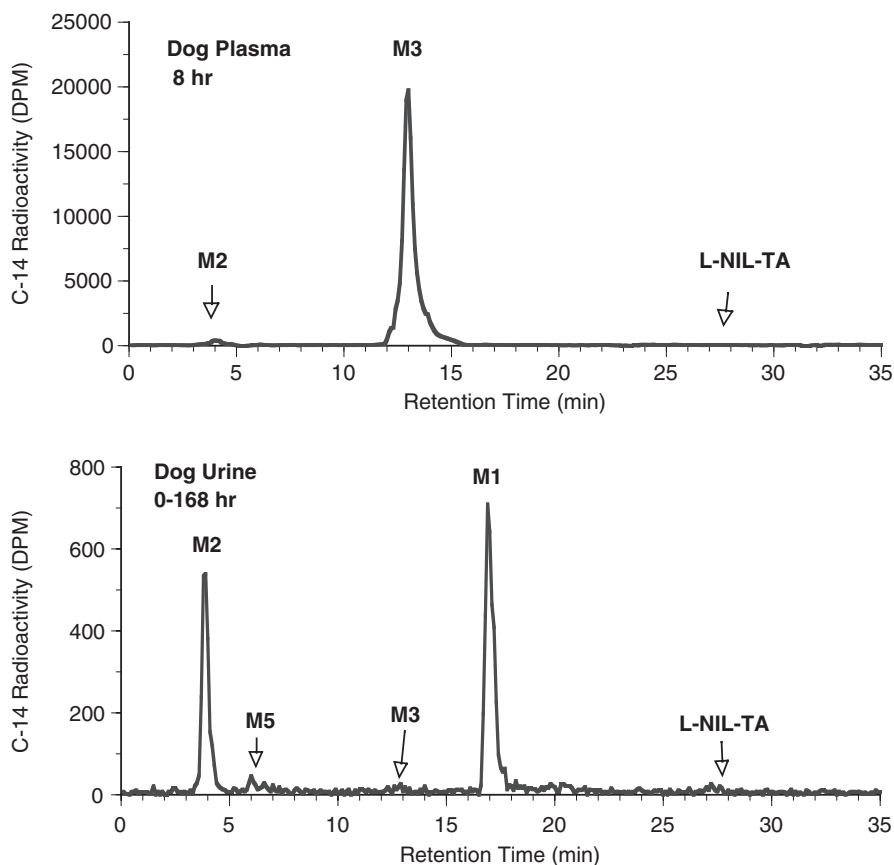


Figure 4. Representative radiochromatograms of dog urine samples from 0–168 hours after a single dose of 5 mg/kg [^{14}C]L-NIL-TA.

Structure elucidation of metabolites

The chemical structures of the metabolites were elucidated by electrospray LC-MS/MS using a combination of full and product-ion scanning techniques (Paulson *et al.* 2000a, b, Zhang *et al.* 2000). The chemical structures of the metabolites were proposed based on MS data and further confirmed by comparison of their spectra and HPLC retention times with those of synthetic standards when possible.

M1. The peak at 17 min (M1) in the dog urine HPLC radiochromatogram showed a protonated molecular ion $[M + H]^+$ at m/z 189, 1 Da (dalton) higher than L-N6-(1-iminoethyl)lysine. The CID spectrum of the m/z 189 yielded a series of product ions at m/z 143, 85, 67, 59 and 57. The sequential losses of 46 (HCO_2H), 58 (CH_3CNHNH_2) and 18 (H_2O) Da from the m/z 189 generated the product ions at m/z 143, 85 and 67, respectively. The product ion at m/z 143 lost 4 ($CH_3CNHNCH_2CH_2$) and 86 Da ($(CH_2)_4CHOH$) to yield the product ions at m/z 59 and 57, respectively (figure 5). Based on the mass spectral interpretation, M1 was proposed as 2-hydroxy-6-[(1-iminoethyl)amino]hexanoic acid and was synthesized. The CID spectrum and the HPLC retention time of M1 from dog urine were the same as those of the synthetic standard. Therefore, M1 was confirmed as 2-hydroxy-6-[(1-iminoethyl)amino]hexanoic acid.

M2. M2 was very polar and co-eluted with the void volume in the two HPLC systems used. Extensive efforts were made to retain M2 on the HPLC column in order to purify it, but without success. The mass spectrum of M2 was not determined owing to matrix ion suppression and interference of endogenous peaks.

M3. The minor peak eluting at 12.5 min (M3) had the same HPLC retention time as that of the active drug, L-N6-(1-iminoethyl)lysine. The protonated molecular ion $[M + H]^+$ of M3 at m/z 188 in its mass spectrum was consistent with that of L-N6-(1-iminoethyl)lysine. The CID spectrum of the m/z 188 generated two major product ions at m/z 143 and 84, which were identical to those in the CID spectrum of the L-N6-(1-iminoethyl)lysine standard. The product ions at 143 and 84 were generated by the losses of 45 (CO_2H) and 104 ($HCO_2H + CH_3CNHNH_2$) Da from $[M + H]^+$, respectively. Therefore, M3 was confirmed as the active drug, L-N6-(1-iminoethyl)lysine.

M5. The mass spectrum of the peak at the retention time of 5.9 min (M5) revealed an $[M + H]^+$ ion at m/z 187, 1 Da less than that of L-N6-(1-iminoethyl)lysine. The CID spectrum of the m/z 187 showed product ions at m/z 100, 84 and 67, which were formed by the losses of 87 ($CO_2H + CH_3CNH$), 103 ($CO_2H + CH_3CNH NH_2$) and 120 ($CO_2H + CH_3CNH NH_2 + H_2O$) Da from the m/z 187, respectively (figure 6). Based on the mass spectral analysis, M5 was proposed as a 2-keto analogue of M3 and was synthesized. The CID spectrum and HPLC retention time of M5 from dog urine were the same as those obtained with the synthetic standard. Therefore, M5 was confirmed as 2-keto-6-[(1-iminoethyl)amino]hexanoic acid.

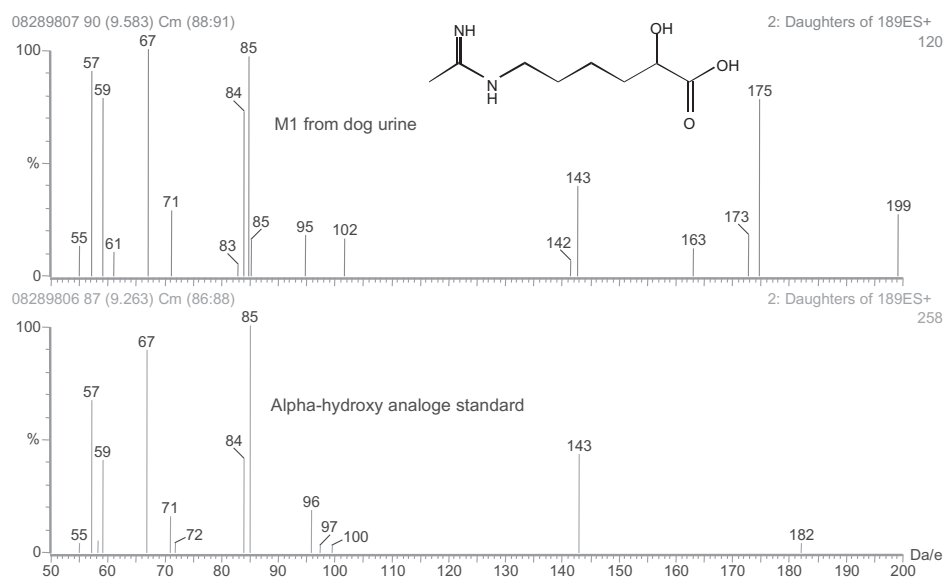


Figure 5. CID MS spectra of M1 isolated from dog urine (top) and the synthetic standard (bottom).

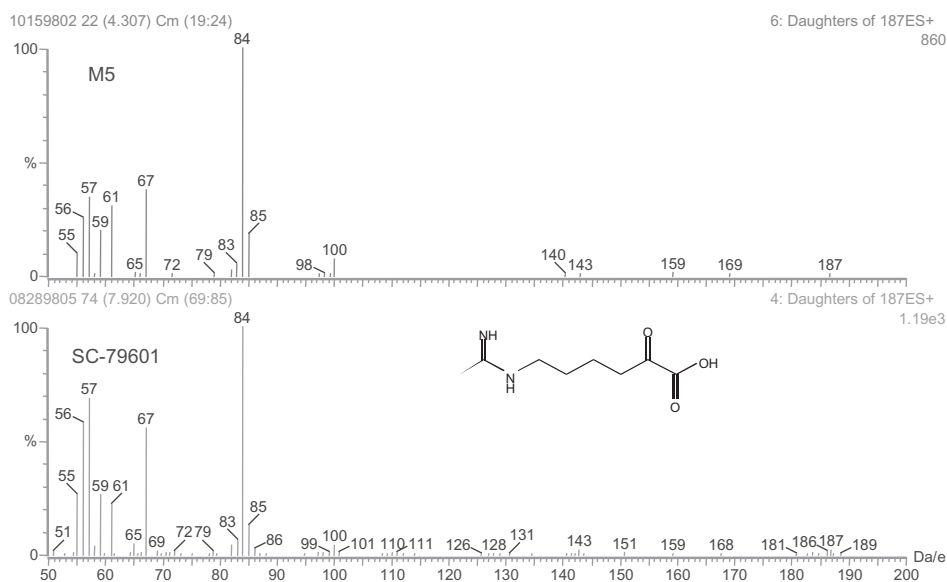


Figure 6. CID MS spectra of M5 isolated from dog urine (top) and the synthetic standard (bottom).

Discussion

After oral and i.v. administration of a 5 mg kg^{-1} dose of $[^{14}\text{C}]$ L-NIL-TA to dogs, the total recovery of the radioactive dose was approximately 80%. The majority of the radioactivity was recovered in urine (46.4% for oral, 51.5% for i.v.) and expired air (22.4% for both oral and i.v.). The large percentage of dose radioactivity recovered in expired air indicates that the carbon-14 radiolabel of $[^{14}\text{C}]$ L-NIL-TA was located in a metabolically labile functional group within the

molecule, ultimately resulting in expiration of a portion of the radiolabel as $^{14}\text{CO}_2$. Recovery of approximately 70% of the dose radioactivity in urine and expired air indicates that [^{14}C]L-NIL-TA was well absorbed after oral administration to dogs. There was no gender difference in the pharmacokinetics of total radioactivity in dogs after oral administration. Both male and female dog extensively metabolized L-NIL-TA after oral administration, with less than 1% of the radioactive dose excreted as the unchanged drug. Three metabolites were identified in dogs along with carbon dioxide. The proposed biotransformation pathways of L-NIL-TA in dogs are shown in figure 1.

L-NIL-TA is rapidly hydrolysed by esterases in dog blood by loss of 5-aminotetrazole to form the active drug M3. M3 is then oxidized at the 2-amino group to form the 2-keto metabolite M5. The reduction of M5 at the 2-keto moiety leads to the formation of the 2-hydroxy metabolite M1. *In vitro* studies were conducted to identify possible enzymes involved in the formation of these metabolites and showed that L-amino acid oxidase can catalyse the formation of M5 from M3, and that lactate dehydrogenase is involved in the reduction of M5 to M1 (W. Moore, unpublished data). M2 is a very polar metabolite that has not presently been identified. It is most likely a breakdown metabolite containing the iminoethyl group based on the positions of radioactive labelling. It appears to be further oxidized to form carbon dioxide and is then expired in air. One hypothesis for the pathway is that this occurs via an oxidative N-dealkylation process, which would result in formation of actamidine, then acetamide and then acetate, the later leading to formation of carbon dioxide. However, it was found that the HPLC retention time of M2 was different from those of actamidine and acetamid, but similar to that of acetic acid. Therefore, it is possible that M2 is an acetic acid or acetate derivative. Based on the above hypothesis, M2 might be formed from M1, M3 or M5. M1 and M2 are the major metabolites in the dog urine, accounting for 21.8 and 21.3% of the dose radioactivity in urine, respectively.

In conclusion, L-NIL-TA is eliminated primarily by a single metabolic pathway, i.e. hydrolysis by blood esterases to the active drug followed by the further oxidative deamination to a 2-hydroxy metabolite. The major route of elimination of L-NIL-TA is metabolism followed by excretion of the metabolites in the urine and expired air.

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Altered expression of sulfotransferases, glucuronosyltransferases and mrp transporters in FVB/mrp1^{-/-} mice

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Received 2 May 2003

1. Genetically altered mice increasingly are being used in toxicology and pharmaceutical development. As such, knowledge of the compensatory activity of enzymes is critical when interpreting the results of studies using these animals.

2. The present study examined alterations in hepatic phase I and II enzyme activity, and alterations in phase III (transporter) RNA expression, between FVB mice and mice lacking the multidrug resistance-associated protein 1 (mrp1) gene (FVB/mrp1^{-/-} mice). It was hypothesized that other transporters and phase I and II enzymes would be increased in the FVB/mrp1^{-/-} mice, presumably as a compensatory mechanism.

3. No differences were found in hepatic cytochrome P450 activity between FVB and FVB/mrp1^{-/-} mice, nor were there differences in the amount of total hepatic glutathione or in glutathione *S*-transferase enzyme activity.

4. However, sulfotransferase activity towards 2-naphthol was significantly increased by 2.6-fold in the FVB/mrp1^{-/-} mice, whereas glucuronosyltransferase activity towards both 4-nitrophenol and testosterone was significantly reduced 1.5-fold. In addition, mrp2 RNA expression was significantly increased by 3.4-fold and mrp5 expression was significantly increased by 1.6-fold in the FVB/mrp1^{-/-} mice.

5. Mice lacking mrp1 have significantly increased hepatic transcription of at least two other ATP-binding cassette transporters, as well as increased 2-naphthol sulfotransferase activity, presumably to compensate for the lack of mrp1.

Introduction

The members of the multidrug resistance-associated protein (MRP) subfamily are 150–210 kDa membrane-bound glycoproteins that transport glutathione, glucuronide and sulfate conjugates (for a review, see Kruh *et al.* 2001). Multidrug resistance-associated protein 1 (MRP1, also termed ABCC1) is one member of this family. It can extrude endogenous ligands, such as 17 β -oestradiol glucuronide and leukotriene C₄ (Loe *et al.* 1996), chemotherapeutic agents typically in conjunction with glutathione, such as the anthracyclines, epipodophyllotoxins and the vinca alkaloids (Loe *et al.* 1998), and heavy metals including arsenic and antimony (Chen *et al.* 1997).

The tissue expression pattern of these transporters varies from being rather ubiquitously expressed, like MRP1 (Peng *et al.* 1999) and MRP5 (Belinsky *et al.* 1998), to a more restricted pattern, such as MRP2, which is present in the liver, kidney, ileum and duodenum (Paulusma *et al.* 1997) and MRP3, which is predominantly present in the colon, pancreas, liver and kidney (Belinsky *et al.*

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1998). In addition, their subcellular localization in polarized epithelial cells varies, with MRP1 (Flens *et al.* 1996), MRP3 (Konig *et al.* 1999b) and MRP5 (Wijnholds *et al.* 2000) being localized on the basolateral side of cells, while MRP2 (Paulusma *et al.* 1997) and another member of the ATP-binding cassette family termed P-glycoprotein (P-gp) are localized on the apical side (Thiebaut *et al.* 1987). Several of these transporters appear to be coordinately regulated, increasing their expression when the expression of another member is lowered. For example, rats lacking mrp2 protein (Suzuki and Sugiyama 1998) have increased levels of mrp3 (Hirohashi *et al.* 1998) and mrp1 (Pei *et al.* 2002).

Mice lacking the gene encoding for mrp1 have been produced (Lorico *et al.* 1997, Wijnholds *et al.* 1997), in which these animals display a decreased response to leukotriene-inducing inflammatory stimuli. Mrp1 also plays a role in protection of organs from drug damage, as etoposide treatment of homozygous knockout mice causes damage to the mucosal layer of the tongue and cheek and to the basal plasma membrane of Sertoli cells (Wijnholds *et al.* 1998). We recently performed a study exposing FVB control mice and mrp1 knockout mice (termed FVB/mrp1^{-/-}) to methoxychlor and found that mrp1 protected the testicular tubules from methoxychlor toxicity (Tribull *et al.* 2003). In the course of that study, it was noticed that cytochrome P450 hydroxylation activity in the testes tended to be reduced in the FVB/mrp1^{-/-} compared with the FVB mice, presumably in an attempt to protect the developing spermatozoa. It is therefore possible that the activities and expression of other metabolizing enzymes and transporters were also altered in these mice.

Previous studies have demonstrated that phase I and II enzymes and transporters can be differentially expressed in knockout mice in an apparent compensatory manner. For example, mice lacking one or more *mdr1* genes have significantly increased expression of hepatic cyp3A proteins (Schuetz *et al.* 2000). Interestingly, these experiment performed on *mdr1a*(^{-/-}) mice housed in either the USA or Amsterdam showed differences in cytochrome P450 expression. Mice housed in Amsterdam that lacked the *mdr1a* or both *mdr1a/b* genes had increases in cyp1a and cyp2b levels (Schuetz *et al.* 2000). FXR null mice have significantly reduced levels of the sister of P-glycoprotein/bile salt export pump (SPGP/BSEP) and increased levels of cyp3a11 and cyp2b10, as well as mrp4, presumably as protective measures against hepatic bile acid toxicity (Schuetz *et al.* 2001). As genetically altered mice are being increasingly used in toxicology and pharmaceutical development, knowledge of altered activity is critical to interpreting the results of studies with these animals. The current study describes alterations in hepatic phase II enzymes and in the mrp subfamily of transporters between FVB mice and mice lacking the *mrp1* gene.

Materials and methods

FVB and FVB/mrp1^{-/-} mice

Four FVB (wild type) and four FVB/mrp1^{-/-} knockout mice (Lorico *et al.* 1997, Wijnholds *et al.* 1997) were obtained at 5 weeks of age from Taconic Farms (Germantown, NY, USA) and housed in cages kept at 25°C at 50% humidity, fed Teklad rodent chow (Sinclair, Florence, SC, USA) and water *ad libitum*. All care and treatment of the animals was conducted in accordance with AALAC guidelines and approval from the institutional animal care and use committee. The animals were used for a previous study in which they were fed 100 µl honey at 8 weeks of age for 39 days. No significant differences were observed with respect to the animals' body weight, organ weights or in serum chemistry parameters. The mice were then euthanized and their livers removed and sectioned. Half

of the sections were snap-frozen in liquid nitrogen and stored at -80°C for cytosol and microsome preparation, while the other sections were placed in TRI-Reagent (Sigma Chemical Co., St Louis, MO, USA) and stored at -80°C for total RNA preparation.

Cytosol and microsome preparation

Individual livers were thawed and homogenized in ice-cold buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, containing $2\text{ }\mu\text{g ml}^{-1}$ each aprotinin, leupeptin and pepstatin) with 15 strokes of a Dounce homogenizer. Microsomes were prepared by differential centrifugation, centrifuging the homogenate at 10000g for 10 min, transferring the supernatant and centrifuging it at 100000g for 60 min. The cytosol was stored at -80°C , and the pellet was resuspended in homogenization buffer containing 10% glycerol and stored at -80°C . Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as the standard.

Liver testosterone hydroxylation activity

Testosterone hydroxylation assays were performed by incubating 200 μg microsomal protein in 0.1 M phosphate buffer (pH 7.4) along with 40 nmol [^{14}C] testosterone (53.6 mCi mmol $^{-1}$, NEN, Boston, MA, USA) (Baldwin and LeBlanc 1992). Testosterone was regio- and stereospecifically hydroxylated by specific cytochrome P450s (Waxman *et al.* 1983), which enables the measurement of the activities of a number of different cytochrome P450s simultaneously, based on their migration by thin-layer chromatography. Reactions were initiated by the addition of 1 mM NADPH and incubated for 10 min at 37°C in a shaking water bath. The enzymatic activity was terminated by the addition of 2 ml ethyl acetate. Samples were vortexed and centrifuged to separate the upper solvent layer from the aqueous layer. The ethyl acetate layer was transferred to a new tube, the aqueous layer was extracted again and the extracts combined. The extracts were evaporated under a stream of nitrogen, dissolved in 35 μl ethyl acetate, spotted onto TLC plates and resolved as previously described. The TLC plates were exposed to autoradiography film, and individual metabolites were cut from the plates and then quantitated by liquid scintillation counting. Specific activity for each metabolite was calculated from the scintillation counting as pmol hydroxytestosterone metabolite produced $\text{min}^{-1}\text{mg}^{-1}$ microsomal protein. Statistical differences were determined by Student's *t*-test.

Conjugative enzyme activities

Glutathione *S*-transferase (gst) activity was measured by incubating cytosol in buffer (0.1 M sodium phosphate, pH 6.5, 1 mM glutathione and 2.5 mM 1-chloro-2,4-dinitrobenzene (CDNB)). CDNB is a substrate for all the cytosolic forms of GSTs. Conjugate formation was measured spectrophotometrically at 345 nm and is expressed as $\mu\text{mol min}^{-1}\text{mg}^{-1}$ protein (Habig *et al.* 1974). Total glutathione (both GSH and GSSG) was determined by incubating liver cytosol with 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.75 U glutathione reductase in buffer (143 mM sodium phosphate, 6.33 mM EDTA, pH 7.5), and measuring the rate of 5-thio-2-nitrobenzoic acid formation spectrophotometrically at 412 nm (Anderson 1985). Sulfotransferase activity was determined by the methylene blue extraction method using liver cytosolic protein, 0.2 mM 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Sigma) as a substrate, and using 0.25 mM 2-naphthol or 0.05 mM dehydroepiandrosterone (DHEA) as a sulfuryl acceptor in buffer (0.5 M sodium acetate, pH 5.5, 7.5 mM 2-mercaptoethanol). The methylene pairs with the formed sulfate, which was analysed spectrophotometrically at 651 nm and expressed in $\text{nmol mg}^{-1}\text{ protein min}^{-1}$ (Sheng *et al.* 2001). Sulfotransferase assays were linear for 30 min using up to 150 μg protein. Glucuronyltransferase activity towards testosterone was assessed using 100 μg activated microsomal protein (0.01% Triton X) with 1.8 mM [^{14}C]UDP-glucuronic acid (0.5 $\mu\text{Ci }\mu\text{mol}^{-1}$) (Perkin-Elmer Life Sciences, Boston, MA, USA) and 0.1 mM testosterone (Sigma) in buffer (50 mM Tris, pH 7.5, 10 mM MgCl_2 , 100 $\mu\text{g ml}^{-1}$ phosphatidylcholine, 8.5 mM saccharolactone). The glucuronide conjugates were extracted with acidified ethyl acetate, and the amount of conjugate formed was determined by scintillation counting. The activity was expressed in $\text{pmol min}^{-1}\text{mg}^{-1}\text{ protein}$ (Coffman *et al.* 1999). Activity remained linear for up to 2 h with 100 μg protein. Ugt activity towards *p*-nitrophenol was assessed using 2 mM UDP-glucuronic acid (Sigma), 100 μg microsomal protein and 1 mM *p*-nitrophenol in buffer (50 mM Tris, pH 7.8, 10 mM MgCl_2 , 1 mM saccharolactone). Activity was assessed spectrophotometrically at 405 nm (Peters *et al.* 1984). *p*-Nitrophenol activity remained linear for up to 60 min with 100 μg protein. All samples ($n=4$) were assayed in triplicate in two separate experiments, which were averaged together. Significant differences were determined by Student's *t*-test.

Real-time polymerase chain reaction (PCR) for transporter expression

Total liver RNA (2 μg) was treated with DNase I and then reverse-transcribed using MMLV-RT, RNasin (Applied Biosystems, Foster City, CA, USA) and 50 ng random hexamers. Transporter cDNAs were amplified by real-time PCR (Cepheid, Sunnyvale, CA, USA) using dNTPs, 0.25X Sybr green (Sigma), Taq polymerase (Qiagen, Valencia, CA, USA), along with the following primers: gapdh

forward, 5'-CATGACCACAGAGTCCATGC-3', gapdh reverse 5'-GTCATACCAGGAAAT-GAGC-3', mdr1a forward 5'-GAGAAAGCTGTCAAGGAAGC-3', mdr1a reverse 5'-GAGCTAT-CACAATGGTGGTCC-3', mrp2 forward 5'-CTGCCTCTTCAGAATCTTAG-3', mrp2 reverse 5'-CCATGATGGTGTGCAGCCTG-3', MRP3 forward 5'-GCTGGCAAGTCTTCCATGAC-3', mrp3 reverse 5'-CGTGTGCAGGTGGGACAGC-3', mrp5 forward 5'-GGAATGTATTGCC-CAGCTACC-3', and mrp5 reverse 5'-GCAGCAAACATGGCATAGAATC-3'. All PCR products had a denaturing step of 95°C for 15 s and an extension of 72°C for 30 s for 40 cycles. The annealing temperature for gapdh was 56°C, the annealing temperature for mdr1a was 56°C, mrp2 had an annealing temperature of 53°C, mrp3 had an annealing temperature of 58°C, and mrp5 had an annealing temperature of 62°C. The cycle thresholds were converted into starting number of molecules per 100 ng RNA using known concentrations of the specific gene product, which was then normalized to the number of gapdh molecules. The cDNA products used as the standards were prepared by RT-PCR and sequenced by the University of Maine's DNA sequencing facility (Orono, ME, USA) to confirm their identity, based upon sequence homology to murine gapdh (Gen Bank Accession #M32599), murine P-glycoprotein (#M33581), murine mrp2 (#AF282772), murine mrp3 (#XM_126648), and murine mrp5 (#XM_147225).

Results

Phase I enzyme activity

This study examined alterations in hepatic phase I and II enzyme activity, and alterations in phase III (transporter) RNA expression, between FVB mice and mice lacking the mrp1 gene (FVB/mrp1^{-/-} mice). To determine hepatic cytochrome P450 activity, testosterone was used as the substrate. Testosterone is regio- and stereospecifically hydroxylated by specific cytochrome P450s (Waxman *et al.* 1983). For example, the 15β/7α-, 6β- and 2α-hydroxytestosterone products correspond primarily to mouse cyp2a4/2a1, cyp3a11 and rat cyp2c11. In our study, there were no differences in hepatic enzyme activity between FVB and FVB/mrp1^{-/-} mice (table 1). This was not entirely unexpected as MRP1 is thought to be a predominantly conjugate transporter, or a protein that transports unconjugated organic anions in conjunction with a glutathione moiety (Loe *et al.* 1998).

Phase II enzyme activity

Therefore, it was next determined whether the activity of conjugative enzymes, including glutathione *S*-transferases, sulfotransferases and glucuronosyltransferases, as well as total glutathione levels, differed between the two groups of

Table 1. Cytochrome P450-mediated testosterone hydroxylation in liver microsomes of FVB and FVB/mrp1^{-/-} mice.

Hydroxytestosterone product	Specific activity (pmol min ⁻¹ mg ⁻¹ protein)	
	FVB mice	FVB/mrp1 ^{-/-} mice
15α-Hydroxytestosterone	5.31 ± 1.27	4.54 ± 0.55
16α-Hydroxytestosterone	12.48 ± 5.09	10.89 ± 2.64
7α-Hydroxytestosterone	12.00 ± 5.93	13.06 ± 2.02
6α-Hydroxytestosterone	9.25 ± 2.59	7.04 ± 1.35
6β-Hydroxytestosterone	57.26 ± 15.91	59.84 ± 8.22
16β-Hydroxytestosterone	11.49 ± 7.85	13.79 ± 2.25
2α-Hydroxytestosterone	9.29 ± 3.64	8.97 ± 1.17

Data are ± standard deviation.
Sample size was four animals for each group. No significant differences between the groups were found using the Student's *t*-test.

Table 2. Amount of total glutathione and glutathione *S*-transferase activity in liver cytosol of FVB and FVB/mrp1^{-/-} mice.

Enzyme assay	Specific activity (gst) or concentration (GSH)	
	FVB mice	FVB/mrp1 ^{-/-} mice
Glutathione <i>S</i> -transferase	0.34 ± 0.11	0.29 ± 0.03
Glutathione	52.4 ± 13.0	45.2 ± 8.5

Specific activity for glutathione *S*-transferase is $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein $\pm$ standard deviation. Glutathione is expressed in μM glutathione mg^{-1} protein $\pm$ standard deviation.

Sample size was four animals for each group and all samples were performed in triplicate. No significant differences between the groups were found using the Student's *t*-test.

mice. We had hypothesized that these would be increased in the FVB/mrp1^{-/-} mice, presumably as a compensatory mechanism. There was no difference in either total glutathione levels or gst activity as determined using 1-chloro-2,4-dinitrobenzene as a substrate (table 2). However, hepatic sulfotransferase activity towards 2-naphthol was significantly increased by 2.6-fold in the FVB/mrp1^{-/-} mice (figure 1A), whereas DHEA sulfotransferase activity was not different between the two groups (figure 1B). Interestingly, hepatic glucuronosyltransferase activity using either testosterone (figure 2A) or 4-nitrophenol (figure 2B) was significantly reduced by 1.5-fold in the FVB/mrp1^{-/-} mice. This was rather unexpected as it had been predicted that the levels of all conjugative enzymes would either not change or increase in the FVB/mrp1^{-/-} mice to compensate for the lack of mrp1 expression.

Phase III transporter expression

Finally, differences were investigated in transporter RNA expression in FVB versus FVB/mrp1^{-/-} mice using real-time PCR. It was hypothesized that the lack of mrp1 protein would increase the expression of other transporters that could be used in a similar manner for elimination. Although both mrp1 and mdr1a are both ATP-binding cassette (ABC) transport proteins, mdr1a normally transports parent compounds, whereas members of the MRP subfamily typically transport conjugates. Therefore, it was not surprising that there were no changes in hepatic mdr1a expression between FVB and FVB/mrp1^{-/-} mice (figure 3). However, mrp2 RNA expression was significantly increased by 3.4-fold (figure 3), and mrp5 expression was significantly increased by 1.6-fold in the FVB/mrp1^{-/-} mice (figure 3). Mrp3 RNA expression did not differ between the two groups (figure 3). Thus, mice lacking the mrp1 transporter significantly increased hepatic transcription of at least two other ATP-binding cassette transporters, localized on both apical and basolateral sides of the membrane, presumably to assume the function of mrp1.

Discussion

This study indicates that other transporters, including mrp2 and mrp5, as well as phase II metabolizing enzymes, are altered in mice lacking mrp1, presumably as a compensatory mechanism. The activities of hepatic phase I enzymes, as exemplified by the cytochrome P450 superfamily, remained unchanged when compared between the FVB and FVB/mrp1^{-/-} mice. In earlier studies with

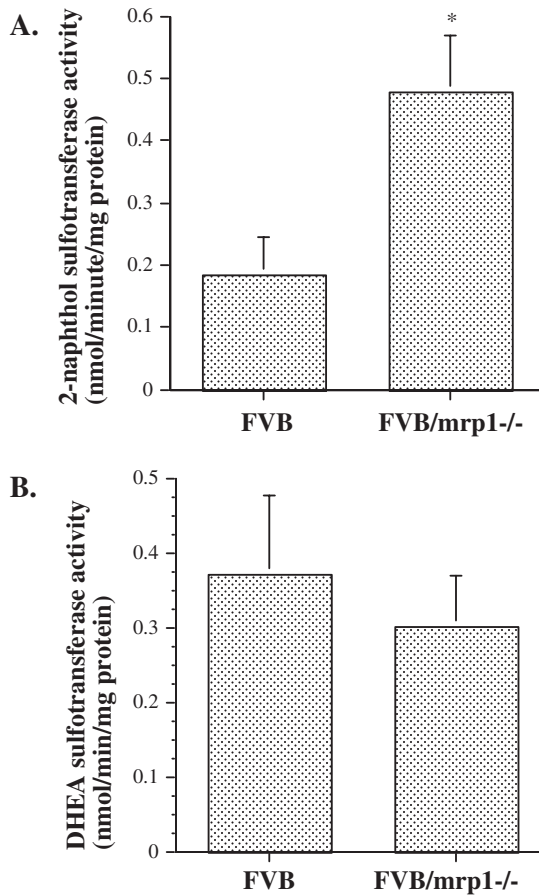


Figure 1. Hepatic aryl sulfotransferase (A) and hydroxysteroid sulfotransferase (B) activity in FVB and FVB/mrp1-/- mice. Liver cytosolic protein was incubated with 2-naphthol to determine aryl sulfotransferase activity (A) or with dehydroepiandrosterone (DHEA) to assess hydroxysteroid sulfotransferase activity (B). Activity is expressed as nmol product formed $\text{min}^{-1} \text{mg}^{-1} \pm$ standard deviation and is the average of two separate experiments, each containing four samples assayed in triplicate. Statistical differences (*) were determined by Student's *t*-test ($p < 0.05$).

these mice, the present authors had noticed significant reductions in testicular P450 activity mediated by three different cytochrome P450s, but the reductions were small, typically only a 1.4-fold reduction in activity (data not shown). Although other studies demonstrate a correlation between cytochrome P450 and P-glycoprotein expression (Schuetz *et al.* 2000), there is little information to suggest a link between MRP-type transporters and cytochrome P450s. Neither hepatic total glutathione nor glutathione *S*-transferase activity differed between FVB and FVB/mrp1-/- mice, as reported (Lorico *et al.* 1997).

However, the results of the present study did demonstrate alterations in several phase II enzyme activities. Sulfotransferase activity towards 2-naphthol was increased 2.6-fold in the FVB/mrp1-/- mice. Glucocorticoid drugs, such as dexamethasone, can significantly increase mRNA levels of the sulfotransferase in primary rat hepatocytes that is associated with 2-naphthol activity (Runge-Morris 1998). The regulation of this particular sulfotransferase was thought to be by the

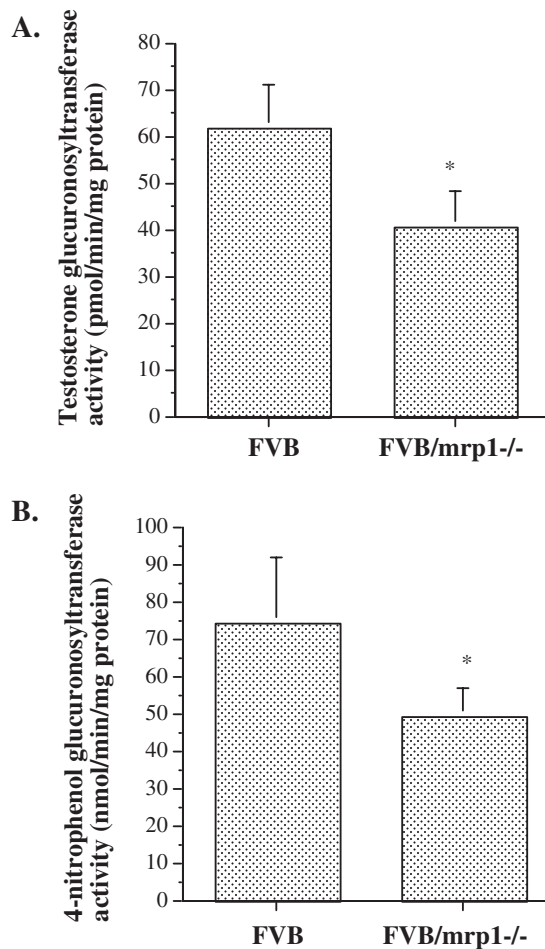


Figure 2. Hepatic steroid glucuronosyltransferase activity (A) and aryl glucuronosyltransferase activity (B) in FVB and FVB/mrp1^{-/-} mice. Liver microsomal protein (100 μ g) was incubated with testosterone (A) or with 2-nitrophenol (B) to determine glucuronosyl transferase activity. Activity is expressed as pmol product formed $\text{min}^{-1}\text{mg}^{-1} \pm$ standard deviation and is the average of two separate experiments, each containing four samples assayed in triplicate. Statistical differences (*) were determined by Student's *t*-test ($p < 0.05$).

glucocorticoid receptor, since the increase in transcript levels could be reduced by co-incubation with RU-486. In contrast, sulfotransferase activity towards DHEA did not change in the FVB/mrp1^{-/-} mice. In primary rat hepatocytes, DHEA activity was also increased by dexamethasone but could not be blocked by co-incubation with RU-486 (Runge-Morris 1998). Indeed, other studies have shown that DHEA sulfotransferase activity appears to be regulated by the pregnane X receptor (PXR) (Sonoda *et al.* 2002). Phenobarbital, a classical inducer of the constitutive androstane receptor (CAR), represses mRNA and protein levels in rat liver SULT1 and SULT 2 isoforms (Runge-Morris *et al.* 1998). Perhaps glucocorticoid receptor activity is altered in the FVB/mrp1^{-/-} mice, accounting for the induction in 2-naphthol sulfotransferase activity.

Unlike the sulfotransferases, both testosterone and 4-nitrophenol glucuronosyltransferase activities were significantly reduced in the FVB/mrp1^{-/-} mice.

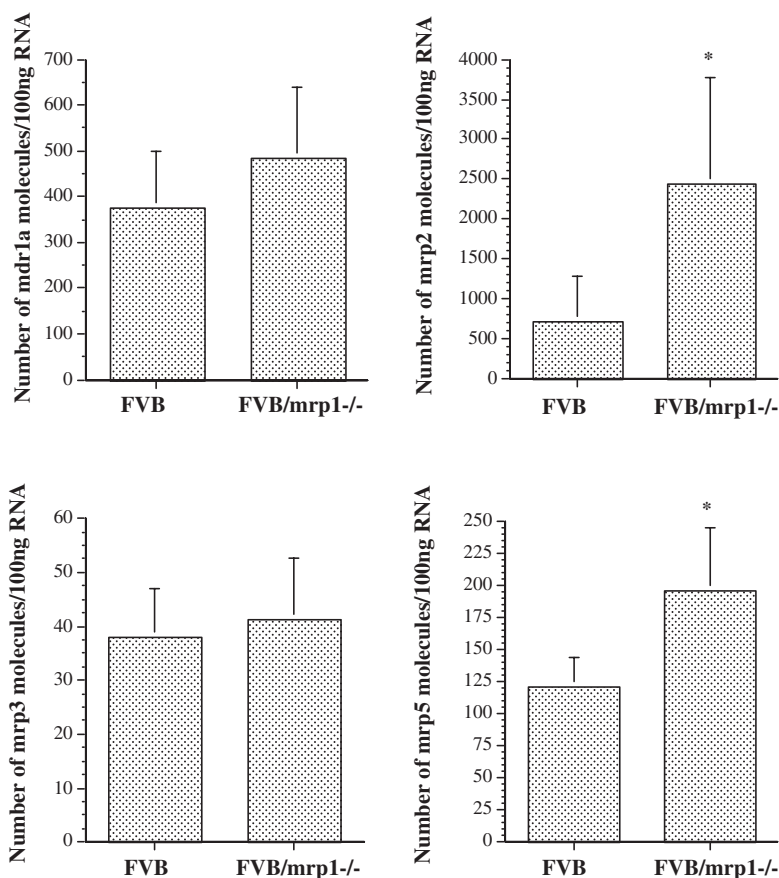


Figure 3. Hepatic mdr1a, mrp2, mrp3 and mrp5 RNA expression in FVB and FVB/mrp1^{-/-} mice. RNA expression of mdr1a, mrp2, mrp3 and mrp5 was examined by real-time polymerase chain reaction using gene-specific primers and four different cDNA samples, each run in duplicate. The cycle thresholds were converted to the number of molecules per 100 ng RNA using known transporter concentrations, which were then normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are the number of molecules per 100 ng RNA $\pm$ standard deviation and the average of two separate experiments. Statistical differences (*) were determined by Student's *t*-test ($p < 0.05$).

A variety of different endogenous compounds such as bile acids, oestradiol and other hormones, and nucleotides such as UTP and AZT, can down-regulate UGT activity (Grancharov *et al.* 2001). Dexamethasone treatment reduced testosterone glucuronosyltransferase activity and mRNA levels in rats and primary rat hepatocytes (Strasser *et al.* 1998), while 4-nitrophenol UGT activity was unchanged. Like the sulfotransferases, altered glucocorticoid receptor activity may be reducing UGT activity in the FVB/mrp1^{-/-} mice. With most of the other nuclear receptors, including PXR (Xie *et al.* 2003), CAR (Huang *et al.* 2003) and the arylhydrocarbon receptor (AhR) (Bock *et al.* 1998), one sees an induction, rather than a repression, of UGT activity. However, the Nrf2 transcription factor knockout mice also have lowered glucuronosyltransferases activity compared with their wild-type counterparts (Enomoto *et al.* 2001). Whether Nrf2 activity or glucocorticoid receptor activity is altered in the FVB/mrp1^{-/-} mice remains to be determined.

The transporters significantly upregulated in the knockout mice were mrp2 and mrp5. It was not surprising that MRP2 expression would be upregulated as this gene, along with MRP3, is the most closely related homologue to MRP1 based upon phylogenetic analysis of these transporters in humans, followed by MRP6, MRP4 and MRP5 (Borst *et al.* 1999). In addition, rats lacking mrp2 protein have increased levels of mrp1 (Pei *et al.* 2002). However, these rats also have increased expression of mrp3 (Hirohashi *et al.* 1998), whereas no significant induction in its expression was seen. We attempted to examine mrp4 expression, but could not detect its presence in the livers of the mice (data not shown). Unlike the other members of the MRP/ABCC subfamily, MRP4 is not highly expressed in human liver (Lee *et al.* 1998). Mrp5 expression was also induced in the knockout animals, which we believe is the first demonstration of this transporter being upregulated in a physiological setting. Both MRP1 (Konig *et al.* 1999a) and MRP5 (Wijnholds *et al.* 2000) prefer to transport glutathione conjugates. As their substrate specificity is similar, it is reasonable to speculate that perhaps mrp5 can compensate for the lack of mrp1's basolateral transporter function.

It was thought that perhaps the induction of hepatic sulfotransferase activity and the reduction of hepatic glucuronosyl transferase activity in the FVB/mrp1^{-/-} mice might also be due to substrate preference of the upregulated ABC transporters. MRP1 has the highest preference for glutathione conjugates, but can also transport glucuronide conjugates, such as 17 β -oestradiol glucuronide with micromolar affinity (Konig *et al.* 1999a). MRP2 has a very similar substrate specificity profile as MRP1, but transports glucuronide conjugates with slightly higher affinity (Jedlitschky *et al.* 1997). In contrast, MRP3 does not have very high affinity for either glucuronide or glutathione conjugates, although it does transport sulfate conjugates with micromolar affinity (Hirohashi *et al.* 2000). Although the substrate specificity of MRP5 is less well understood, it is also capable of transporting glutathione conjugates as well as organic anions coupled with a phosphate group (Wijnholds *et al.* 2000). Since both MRP2 and MRP5 have similar substrate specificities as MRP1, they probably were upregulated to compensate for the lack of MRP1.

Thus, the results of this study demonstrate that mice lacking mrp1 have significantly increased hepatic transcription of mrp2 and mrp5, as well as increased aryl sulfotransferases activity, presumably to compensate for the lack of mrp1.

Acknowledgements

The authors thank T. Tribull for help with the animal care and handling, and J. Warren for assistance with the hydroxylation assays. Support for the study was provided by the National Institute of Environmental Health Sciences (Grant R15 ES10110-02), the National Center for Research Resources (5G12 RR08124) and the National Science Foundation (EEC-9550502).

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Hepatic metabolism of diallyl disulphide in rat and man

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Received 12 August 2003

1. The metabolism of diallyl disulphide was investigated *in vitro* with rat and human liver cell subfractions and *ex vivo* with an isolated perfused rat liver.

2. Diallyl disulphide was oxidized to diallylthiosulphinat by rat liver microsomes with an apparent $K_m = 0.86 \pm 0.1$ mM and an apparent $V_{max} = 0.47 \pm 0.12$ nmol min⁻¹ mg⁻¹ protein (mean $\pm$ SE). Both cytochrome P450 (CYP) and flavin-containing monooxygenases were involved, with CYP2B1/2 and CYP2E1 being the most active CYP enzymes.

3. In rat and man, microsomal oxidation of allylmethyl sulphide to allylmethyl sulfoxide and allylmethyl sulphone also occurred, although at a low rate. Diallyl disulphide was also metabolized to allylglutathione sulphide and allylmercaptan. In addition, diallylthiosulphinat reacted non-enzymatically with glutathione to form allylglutathione sulphide.

4. When an isolated rat liver was perfused with diallyl disulphide, the metabolites allylmercaptan, allylmethyl sulphide, allylmethyl sulfoxide, allylmethyl sulphone and allylglutathione sulphide were detected primarily within the liver tissue, with only small amounts of metabolites found in the bile and perfusion medium. The pharmacokinetic parameters for diallyl disulphide were $t_{1/2} = 6.09$ min; $AUC_{0-\infty} = 4.77$ min mmol l⁻¹; clearance = 34.22 ml min⁻¹.

5. A scheme for the metabolism of diallyl disulphide in rat and man is proposed.

Introduction

Garlic has been used for a long time in traditional medicine, being recommended for a broad range of disorders such as headache, fever, inflammation, insomnia and cough (Rivlin 2001). More recently, investigations have been undertaken to provide a scientific basis for its medicinal use. Several studies have shown that organosulphur compounds contained in garlic were linked with its biological activities, including prevention of cardiovascular diseases and cancer, and its antibacterial, antifungal, antithrombotic, hypotensive and hypolipidaemic properties (Reuter 1995, Amagase *et al.* 2001, Milner 2001). These compounds act through mechanisms such as modulation of drug-metabolizing enzymes, inhibition of cell proliferation, inhibition of genotoxicity, immune system enhancement (reviewed by Le Bon and Siess 2000) and antioxidant activities (Phelps and Harris 1993, Prasad *et al.* 1996). Information on the bioavailability and metabolism of the organosulphur compounds from garlic is essential to explain its health benefits. Most of the garlic

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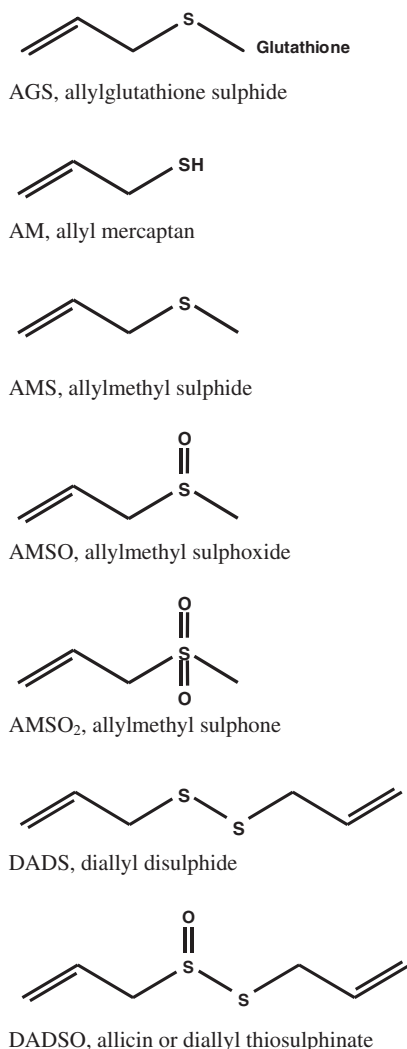


Figure 1. Molecular structures of the sulphur compounds investigated.

organosulphur compounds are derived from alliin (*S*-allyl-cysteine-sulphoxide), which is converted to diallyl thiosulphinate (DADSO) or allicin (figure 1) by the enzyme alliinase when a garlic clove is crushed or cut. DADSO is an unstable compound that breaks down into numerous organosulphur products such as ajoene and various sulphides (Block 1992). Among these sulphides, diallyl disulphide (DADS) (figure 1) is one of the major volatile degradative garlic compounds that accounts for 40–60% of garlic oil. However, metabolic studies of DADS are scarce, although a few have been reported. Studies in mice using radioactive DADS indicated that the uptake of radioactivity peaked in the liver 90 min after intraperitoneal (i.p.) injection and rapidly disappeared (Pushpendran *et al.* 1980). With similar kinetics, it was recently reported that after administration of DADS to rats, the metabolites allylmercaptan (AM), allylmethyl sulphide (AMS), allylmethyl sulfoxide (AMSO) and allylmethyl sulphone (AMSO₂) (figure 1) were detected

in tissues such as stomach, plasma, liver and urine (Germain *et al.* 2002). The pharmacokinetic parameters were determined for all these compounds except for DADS, which disappeared too rapidly in the body. In this latter *in vivo* experiment, the observed metabolites were in good agreement with previous observations in rat hepatocytes, in which DADS was reported to be converted to AM and AMS (Sheen *et al.* 1999). In a study using perfused rat liver, DADS was metabolized to DADSO and AM (Egen-Schwind *et al.* 1992). In an other system using human microsomes, DADS was oxidized efficiently to DADSO (Teyssier *et al.* 1999), which could react rapidly and non-enzymatically with glutathione (Miron *et al.* 2000). In man, AM and DADS have been detected in breath after garlic consumption (Laakso *et al.* 1989, Minami *et al.* 1989, Cai *et al.* 1995, Taucher *et al.* 1996). AMSO and AMSO₂ have been detected in an *in vivo* rat study. With regard to these results, the hepatic metabolism seems to be predominant for the sulphur compounds DADS and DADSO (Egen-Schwing *et al.* 1992, Teyssier *et al.* 1999). AM and AMS are synthesized in the liver. They could be also transformed by the liver. For AMSO and AMSO₂, there is less evidence that the liver plays a role in their metabolism. They were detected in different tissues during an *in vivo* study (Germain *et al.* 2002). However, during the perfusion of a liver with DADS these compounds were not detected (Egen-Schwing *et al.* 1992). Then the role of the liver in the metabolism of AMSO, AMSO₂ AM and AMS is not clearly established.

The present aim is to determine DADS metabolic pathways in the liver by three approaches: (1) an *in vitro* study of phase I and II enzymes from rat liver tissues; (2) an *in vitro* study of phase II human liver enzymes (Teyssier *et al.* 1999); and (3) an isolated rat liver perfused with DADS to determine the pharmacokinetic parameters of DADS, and to identify the metabolites formed in the liver. Based on these results, a possible metabolic pathway in the liver is proposed in rat and man.

Materials and methods

Materials

DADS (purity 80%), AMS and AM were obtained from Aldrich Chemical Co. (Strasbourg, France). DADS was further purified by distillation to 92.2% before use and the impurities were identified by HPLC as diallyl sulphide and diallyl trisulphide. Pure DADSO was provided by Professor Auger (Institut de Recherche sur la Biologie de l'Insecte, Université F. Rabelais, Tours, France). AMSO and AMSO₂ were synthesized by oxidation of AMS with sodium metaperiodate in a methanolic solution thawed on ice overnight (Furniss *et al.* 1989, Germain *et al.* 2002). The reaction gave a mixture of AMSO (85%) and AMSO₂ (15%) as estimated by GC-MS. [glucuronyl-¹⁴C]-UDPGA (272.5 mCi mmol⁻¹ specific activity) and [glycine-2-³H]-GSH (44.8 Ci mmol⁻¹ specific activity) were purchased from NEN (Boston, MA, USA). [³H-CH₃]-S-adenosyl-methionine (85 Ci mmol⁻¹ specific activity) was purchased from Isotopchim (Ganagobie-Peyruis, France). All other chemicals and reagents were of the highest commercial quality available.

Animals and treatments

Weaning male SPF Wistar rats (4 weeks old) were purchased from Janvier (Le genest Saint Isle, France). They were maintained for 4 weeks in a controlled animal area (22°C with a 12-h light/dark period) and fed with a purified diet during the last week (Ref. 20118S from Harlan, Gannat, France). For cytochrome P450 monooxygenase (CYP) *in vivo* induction, inducers were administered to some rats at the end of this period as described (Teyssier and Siess 2000).

Human liver samples

Human liver samples were provided by Professor Favre (Département de Chirurgie Digestive Thoracique et Cancérologique, General Hospital, Dijon, France) according to a protocol approved by the local ethics committee. Samples were provided from women and men with a mean (± SE) age

of 58.5 ± 7.4 years, operated because of hepatic carcinoma or metastases. They did not follow a chemotherapy or radiotherapy. The samples were frozen in liquid nitrogen and stored at -80°C until further use for subcellular fraction preparation.

Subcellular fraction preparation

The animals (average body weight 201 g) were sacrificed by cervical dislocation, followed by exsanguination 24 h after the last treatment (16 h of fasting). Human and rat hepatic subcellular fractions were prepared as described (Haber *et al.* 1994). Microsomal and cytosolic protein contents were quantified by the method of Bradford (1976) using bovine serum albumin fraction V as standard. The method was adapted for automatic measurements performed with a Cobas Fara II analyser (Roche Instruments, Basel, Switzerland). CYP was assayed according to Omura and Sato (1964).

Phase I experiments

For microsomal monooxygenases reactions, liver microsomes corresponding to 300 pmol CYP were incubated with 1.5 mM substrate, 1 mM NADPH, 50 mM Tris-HCl, pH 7.0, in a 500 μl final volume. The reaction rate was linear over 60 min with respect to protein concentration and incubation time. After 30 min (60 min for AMS) at 37°C , the reaction was stopped by the addition of acetonitrile (or dichloromethane for AMS). After protein precipitation, the mixture was centrifuged and the supernatant analysed by HPLC (see below for HPLC analysis). The AMS incubation medium was analysed by GC-MS after dichloromethane extraction.

Heat inactivation of microsomes (Kitchell *et al.* 1978) was performed by pre-incubation in Tris-HCl buffer for 10 min at 37°C in the absence of NADPH, whereas control microsomes (non-inactivated) were pre-incubated in the presence of NADPH. DADS and NADPH were then added to both samples before incubation for 30 min. A positive control of the complete FMO thermal inactivation was done by measuring methimazole oxidase activity. The ethoxycoumarin deethylase activity was used to check the absence of thermal effect on CYP (Edwards *et al.* 1984). These two activities were measured as described by Teyssier and Siess (2000).

Phase II experiments

For determination of cytosolic glutathione *S*-transferase (GST) activity, the incubation mixture contained 0.75 mg cytosolic protein, 1.5 mM substrate (DADS, DADSO or AM), and 5 mM [^3H]-GSH or cold GSH in 50 mM Tris, pH 7.0, in a 500 μl final volume. After 30-min incubation at 37°C , the reaction was stopped by the addition of 1 M HCl and the supernatant obtained after centrifugation was analysed by HPLC.

For determination of microsomal UDP-glucuronyl transferase activities, the incubation mixtures with the substrates DADS, DADSO or AM were conducted as described (Teyssier and Siess 2000). The products of the reaction were analysed by HPLC.

For cytosolic methyl transferase reactions, the incubation mixture consisted of 50 mM Tris-HCl, pH 7.4, 1.2 mM MgCl_2 , 200–1000 μM *S*-adenosyl-methionine and 0.05 μCi [^3H - CH_3]-*S*-adenosyl-methionine, 1 mg ml^{-1} cytosolic or microsomal protein, 1 mM dithiothreitol and 1.5 mM substrate (DADS, DADSO or AM) in a final volume of 500 μl . The incubation was carried out at 37°C for 30 min to 2 h and was stopped by addition of acetonitrile. After elimination of precipitated proteins by centrifugation, the supernatant was analysed by HPLC. The same assays were also conducted in the absence of MgCl_2 or dithiothreitol. The positive control used phenylthiol and luteolin as substrates. The reaction was validated by HPLC detection of the methylated compounds.

Determination of kinetic constants

The kinetic constants of phase I or II enzymes were determined with DADS at concentrations of 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 8 mM under the conditions specified above. Apparent K_m and $V_{\max}$ were estimated by fitting the Michaelis–Menten equation using a non-linear regression program (SAS software, Cary, NC, USA).

Chemical inhibition study

The substrate concentration was identical in separate inhibition experiments and as described above (1.5 mM). The concentrations used for each inhibitor are indicated with their specificities. They were chosen as low as possible to get an inhibition in the range of the specificity of the inhibitor. The inhibitors used were α -naphthoflavone (CYP1A1/2; 0–20 μM ; Teyssier and Siess 2000), chlorzoxazone (CYP2E1; 0–2.0 mM; Takana 2001), diethyldithiocarbamate (CYP2E1; 0–100 μM ; Shu and Hollenberg 1997), nifedipine (CYP3A1/2; 0–500 μM ; Bourrié *et al.* 1996), orphenadrine (CYP2B1/2; 0–500 μM ; Teyssier and Siess 2000), quinidine (CYP2D1; 0–20 μM ; Teyssier and Siess 2000), tolbutamide (CYP2C6; 0–500 μM ; Gardner *et al.* 1997), tranlycypromine (CYP2B1/2 and CYP2E1; 0–500 μM ; Belanger and Atitse-Gbeassor 1982) and troleandomycin (CYP3A1/2; 0–500 μM ; Teyssier and Siess 2000). Inhibitors were added to the incubation mixtures before initiation of the reaction. With the

mechanism-based inhibitors 1-aminobenzotriazole and troleandomycin, samples were pre-incubated for 10 min at 37°C before the addition of DADS. All inhibitors were dissolved in an ethanolic solution and controls received the equivalent concentration of pure ethanol.

Liver perfusion

Liver was isolated from rats of around 300 g body weight as reported (Teyssier and Siess 2000). Surgery was performed to collect the bile (with a PE10 cannula) during liver perfusion with a Krebs-Henseleit bicarbonate buffer (pH 7.4). After a single-pass equilibration period, the experiment was performed with recirculation of a new solution of perfusate (volume of 150 ml) containing 1 mM DADS. Perfusate samples (0.5 ml) were collected at 0, 5, 10, 15, 20, 30, 40, 50, 60, 75 and 90 min following initiation of DADS perfusion, and subsequently analysed by HPLC by direct injection as described below. The viability of the liver was assessed by its visual appearance, by checking the perfusate flow and by measuring lactate dehydrogenase activity in the perfusate. At the end of the experiment, the liver, bile and perfusate were stored at -80°C until further analyses. They were then thawed on ice at 4°C for 1 h, and the liver was homogenized in twofold volumes of water. A total of 100 µl HPLC standard *p*-cymene (200 ng ml⁻¹) was added to each sample. Proteins and cell debris were eliminated by addition of trichloroacetic acid followed by centrifugation. The collected supernatant was extracted three times with distilled dichloromethane. The aqueous phase obtained was analysed by HPLC whereas the organic layer, enriched with a second standard (nonane at 200 ng ml⁻¹), was analysed by GC-MS.

The DADS pharmacokinetic parameters were calculated with Kinetica[®] software (InnaPhase, Champs sur Marne, France) using a non-compartmental method. The elimination rate constant (k_{el}) was determined by linear regression of the log perfusate concentrations in the elimination phase β . The area under the concentration versus time curve (AUC) was calculated by the trapezoidal rule and extrapolated from the last point to infinity with k_{el} . The half-life ($t_{1/2}$) was determined from the phase β in the log-linear regression plot. Clearance was deduced from the dose/AUC ratio and the distribution volume from the clearance/ k_{el} ratio.

HPLC analysis

HPLC analysis was carried out using a Waters (Saint Quentin-en-Yveline, France) system equipped with a pump (model 600), an autosampler (model 717), a photodiode array UV detector (model 996) or an UV detector (model 2487) associated with a Serie A-100 Flo-One detector from Radiomatic and an Uptisphere 5 µm ODB column (4.6 × 150 mm; GL Sciences, Inc., Tokyo, Japan) thermostated at 25°C. The flow rate was 0.7 ml min⁻¹. Data were processed by Waters Millennium software. Analysis of the metabolites after microsomal oxidation of DADS and assays of methylation of AM were performed in an isocratic phase that consisted of 70:30 (v/v, acetonitrile/water). Allylglutathione sulphide (AGS, figure 1) was analysed using an isocratic phase consisting in 30:70 (v/v, acetonitrile/1% acetic acid in water). The spectra were obtained with a photodiode array UV detector from 190 to 300 nm, or with a Flo-One detector associated with an UV detector (at 254 nm) for radiolabelled compounds. DADSO and AM were quantified at 254 nm using linear concentration standard curves for each metabolite. For DADSO, the HPLC detection was linear from 12 ± 0.465 to 1.5 ± 0.014 nmole, the latter being the limit of detection. For AM, the detection was linear from 7.0 ± 1.46 to 0.5 ± 0.14 nmole (detection limit). The quantification of AGS was carried out with the standard curve of chemically synthesized AGS from radiolabelled GSH. The detection was linear from 10 ± 0.25 to 0.1 ± 0.05 nmole (detection limit).

Mass spectrometry analysis

Mass spectrometry analysis of AGS was carried out in the positive-ion mode on an Esquire LC mass spectrometer equipped with an electrospray interface (Bruker, Strasbourg, France). To detect AGS, the phase II incubation medium, after protein precipitation, was directly analysed by direct infusion at 200 µl h⁻¹. Analyses were performed at a capillary voltage of 4 kV, and helium (constant velocity fixed at 35 cm s⁻¹) was used at an operating pressure of 30 psi. In-source collisions were used to induce some fragmentation of selected precursor ions.

GC-MS analyses of AM, AMS, AMSO and AMSO₂ were carried out using an Agilent 6973 quadrupole mass spectrograph equipped with an Agilent 6890 gas chromatograph. Analyses were performed in a positive-ion mode with an electronic impact of 70 eV. Helium was employed as carrier gas. A DB-1701 (J&W Scientific, Folsom, CA, USA) capillary GC column (30 m × 0.32 mm, 1 µm film thickness) was used. The samples were injected onto the column at 35°C. After 2 min, the oven temperature was raised to 180°C at 5°C min⁻¹, and then at 10°C min⁻¹ to 220°C. The samples were detected in scan (from 29 to 220 *m/z*) and in selected ion monitoring (SIM) modes. Detection of AM, AMS, AMSO, AMSO₂ and DADS was performed with the ions at *m/z* 74, 88, 104, 120 and 146, respectively. Purity was continuously checked by the control of the ratio between this identifying ion and another one specific for each compound. As already shown, the concentration of each compound in

the biological samples was calculated by the ratio of the area of the organosulphur compound and the product of the *p*-cymene (internal standard for extraction) and the nonane area (internal standard for final volume) (Germain *et al.* 2002).

Results

In vitro oxidation of DADS and its metabolites in rat

To gain an insight into DADS biotransformation, the reaction products arising from phase I and II systems were investigated separately. In the presence of NADPH, liver microsomes catalysed DADSO formation from DADS, with kinetics fitting the Michaelis–Menten equation. The apparent K_m was 0.86 ± 0.1 mM and the apparent V_{max} was 0.47 ± 0.12 nmol min⁻¹ mg⁻¹ protein (means $\pm$ SE of four samples). To determine which monooxygenase is responsible of the DADSO formation, the effects of FMO inactivation by heat or CYP inhibition were examined using 1-aminobenzotriazole, an ubiquitous CYP suicide inhibitor. Heat inactivation led to a 48% inhibition of activity (figure 2) and aminobenzotriazole produced up to 65% inhibition suggesting the contribution of both types of monooxygenases in DADS oxidation to DADSO.

To identify precisely which CYP enzymes were involved in this reaction, the effects of specific chemical inhibitors (figure 3) and inducers (figure 4) were evaluated. Extensive inhibition was observed with orphenadrine (specific inhibitor of CYP2B1/2), chlorzoxazone and diethyldithiocarbamate (inhibitors of CYP2E1) and tranlylcypromine (inhibitor of both CYP2B1/2 and CYP2E1). The rate of DADSO production by microsomes from animals treated with inducing agents was compared with that observed with microsomes isolated from untreated animals. DADSO formation was increased significantly ($p < 0.05$) in microsomes prepared from livers of rats pretreated with phenobarbital (CYP2B inducer and lightly CYP2C7, CYP3A1/2), 3.37-fold greater. The other treatments inducing CYP1A (methylcholanthrene), CYP2E1 (acetone), CYP3A (dexamethasone) or CYP4A

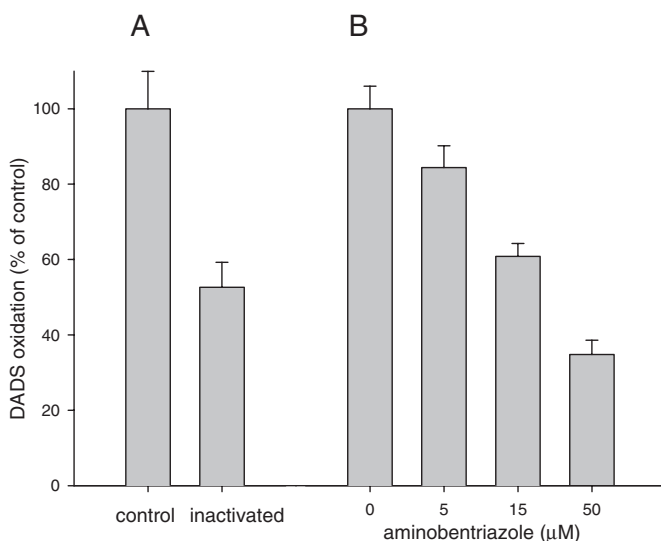


Figure 2. Effects of inactivation of FMOs or CYPs on DADS oxidase activity. (A) Thermal inactivation of FMOs; (B) chemical inhibition. Results are the percentage of control (without inactivation) expressed as means $\pm$ SE ($n = 4$).

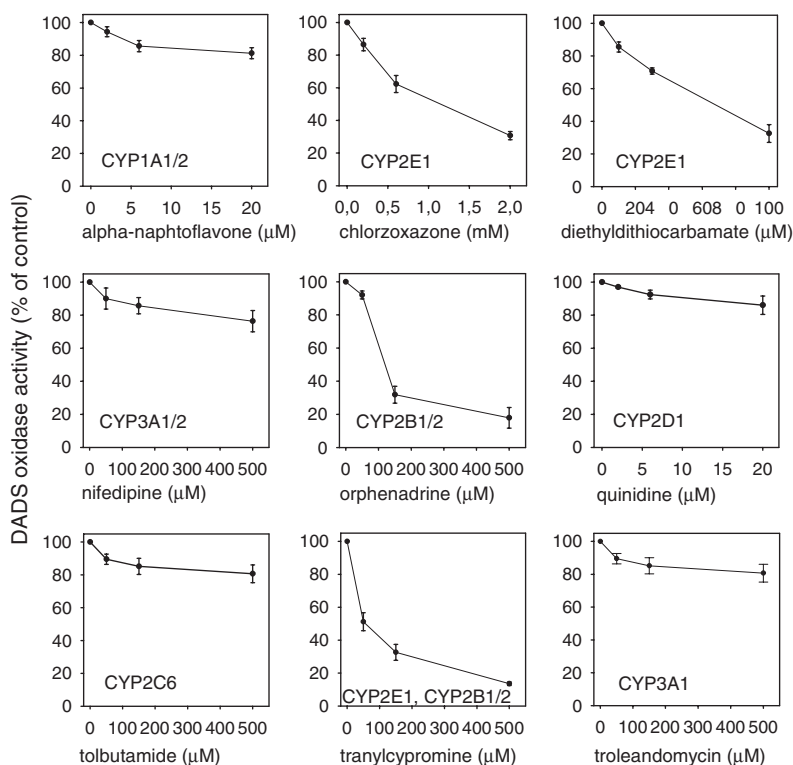


Figure 3. Inhibition of DADS oxidation by chemical CYP inhibitors in rat liver microsomes. Values are the percentage of control (without inhibitor) expressed as means $\pm$ SE ($n = 4$). The inhibited CYP isoforms are specified for each inhibitor.

(clofibrate) did not produce any significant changes in DADSO formation (figure 4). These results of inhibition and induction of CYP enzymes suggest that DADS metabolism by CYP in rats is mainly due to CYP2B and to some extent, CYP2E1.

It was also investigated if AM and DADSO could be oxidized by microsomal enzymes. These potential oxidations were not observed whatever the conditions of incubation or detection (data not shown).

To explain the formation of AMSO and AMSO₂ detected *in vivo* (Germain *et al.* 2002), AMS was incubated in the presence of microsomes and NADPH. Even after 60 min of incubation, the amounts of AMSO and AMSO₂ detected were very low (data not shown) and, hence, kinetics parameters could not be accurately determined.

Conjugation of sulphur compounds in rat

The involvement of the UDP-glucuronyl transferases was analysed in the presence of [¹⁴C]-UDP-glucuronic acid, liver microsomes and DADS, DADSO or AM as substrates. No glucuronide conjugates were detected whatever the incubation conditions or HPLC analysis systems (data not shown).

To determine how AMS was formed, a methylation of AM to AMS was first hypothesized. However, incubation in the presence of [³H-CH₃]-S-adenosyl-methionine produced no methylation of AM with liver cytosol. Positive controls

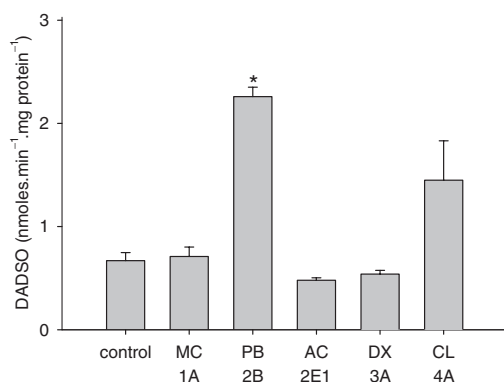


Figure 4. Effect of CYP inducers on DADSO formation by rat liver microsomes. *Differs significantly (Dunnnett's test) from the control's corresponding value ($p \leq 0.05$). The induced CYP isoform is indicated under each inducer. Results are means $\pm$ SE ($n = 4$). AC, acetone; CL, clofibrate; DX, dexamethasone; MC, 3-methylcholanthrene; PB, phenobarbital.

were performed with phenylthiol and luteolin. Some methyltransferases are known to be microsomal enzymes and hence the methylation of AM was also tested in the presence of microsomes. No formation of AMS was observed. The stability of AMS was checked in the various incubation media or during the steps preceding the HPLC injection. In the last assay, DADS or DADSO have been also tested with any success as substrates in the previous reaction media with microsomal or cytosolic enzymes.

The involvement of GST was first assessed in the presence of [³H]-GSH, liver cytosol, with DADS, DADSO or AM as substrates. When DADS was used as the substrate, two new metabolites were obtained only in the presence of cytosol. They corresponded to AM and another metabolite, which after mass spectrometry analysis was in agreement with the structure of AGS. In the MS/MS experiment, this ion with m/z of 380.0 was fragmented and gave a major peak with m/z of 251.0, corresponding to AGS without the glutamic residue. The formation of AGS fitted the Michaelis-Menten equation with an apparent K_m of 9.03 ± 2.6 mM and an apparent V_{max} of 0.25 ± 0.04 nmol min⁻¹ mg⁻¹ protein (means $\pm$ SE of four samples). When AM was assayed in the GST reaction, no product was detected. By contrast, when DADSO was incubated with GSH, a spontaneous AGS formation occurred in the absence of cytosolic proteins, suggesting a non-enzymatic reaction between DADSO and GSH.

Contribution of phase I and II enzymes to sulphur compound metabolism in human

Experiments similar to those made in rats were performed with human enzymes. In the presence of incubation medium for glutathione conjugation, DADS was metabolized to AM and AGS. Figure 5 shows the HPLC radiochromatography obtained after such an incubation. The formation of AGS from DADS by liver cytosol fitted the Michaelis-Menten equation. The apparent K_m was 1.48 ± 0.08 mM and the apparent V_{max} was 0.08 ± 0.00 nmol min⁻¹ mg⁻¹ protein (means $\pm$ SE of four samples). No glucuronide conjugates of DADS, DADSO or AM were detected whatever the incubation conditions or HPLC analysis systems (data not shown).

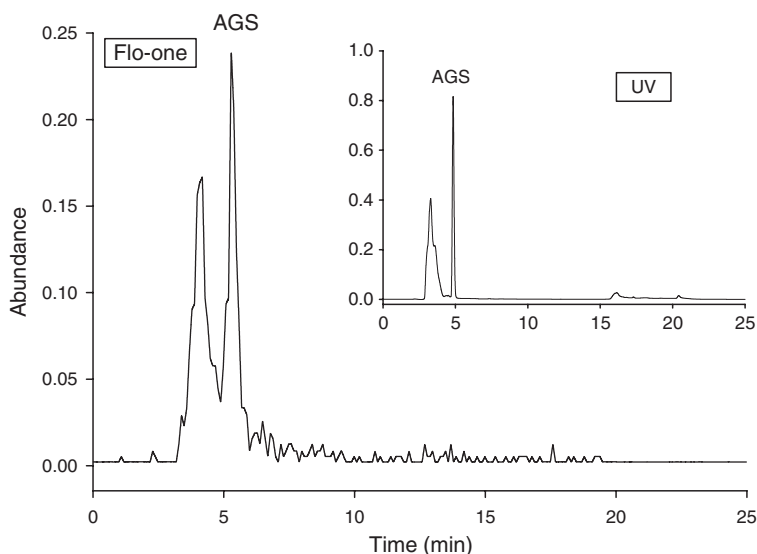


Figure 5. HPLC profile of an incubation of DADS with human cytosol and radiolabelled GSH, obtained with a Flo-One detector associated with a UV detector at 254 nm.

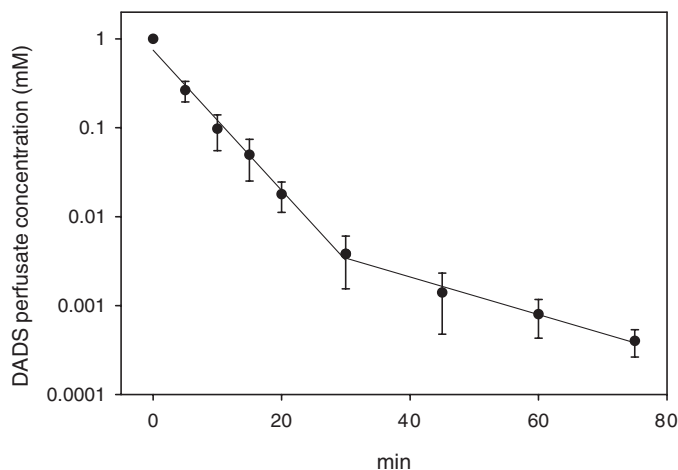


Figure 6. DADS concentration in the perfusate following addition of 1 mM DADS to the reservoir of an isolated rat perfused liver. Each datum point is the mean of four experiments.

No metabolites were determined when AM, DADS or DADSO was incubated with cytosolic and microsomal enzymes in the presence of *S*-adenosyl-methionine. On the contrary, monooxygenases oxidized AMS in AMSO and AMSO₂ (data not shown).

Ex vivo metabolism of DADS in the isolated perfused rat liver

During perfusion in a recirculating system, DADS was monitored by HPLC and its concentration was determined over 75 min, after which DADS was no longer detectable. Figure 6 shows the semilogarithmic plots of DADS disappearance versus time and the deduced pharmacokinetic parameters are presented in table 1. DADS disappearance exhibits a biphasic pattern suggesting

Table 1. Pharmacokinetical parameters of DADS in the isolated perfused rat liver.

k_{el} (min^{-1})	0.13 ± 0.03
$\text{AUC}_{0-\infty}$ (min mmol l^{-1})	4.77 ± 0.80
Half-life, β (min)	6.09 ± 1.26
Clearance (ml min^{-1})	34.22 ± 5.6
Volume of distribution (ml)	311.9 ± 0.08

Results are the means of four replicate experiments $\pm$ SE.

DADS was perfused in the liver at a concentration of 1 mM, as described in Materials and methods.

its extensive tissue distribution, consistent with the calculated distribution volume (312 ml). The elimination half-life of DADS was about 6 min. In order to assess the metabolites appearing during the experiment, the perfusate was analysed by HPLC using conditions to detect either organosulphur compounds or the conjugates. As far as organosulphur compounds were concerned, a metabolite, identified as AM by comparison with the spectrum and retention time of the standard, was detected within the first minutes of perfusion. It slowly disappeared until 60 min suggesting its transformation to another product. DADSO was not detected at any time during the experiment. In the conjugate elution conditions, the continuous increasing production of AGS was observed.

After perfusion, livers, perfusates and collected bile contents were analysed by either HPLC (conjugates) or GC-MS (sulphur compounds). The metabolites were identified by both methods through direct comparisons to synthesized standards. AGS was the only conjugate detected by HPLC and was found in both the liver and the perfusate. On the contrary, many organosulphur compounds were detected (figure 7). Their relative partition between the three compartments is shown in figure 8. Only DADS and AM were detected in the bile. In the liver, AM and DADS were identified in trace amounts, and AMS, AMSO and AMSO₂ in larger quantities. In the perfusate, all organosulfur compounds were also recovered at very low concentrations compared with liver levels.

Discussion

After the *in vivo* administration of DADS to rats, AM, AMS, AMSO and AMSO₂ have been previously detected in various tissues (Germain *et al.* 2002). To investigate further the enzymes producing these metabolites, *in vitro* studies were first undertaken in rat and human subcellular fractions. In the presence of rat microsomes, DADS was a good substrate for phase I reactions, leading to DADSO formation. This latter compound did not seem to be subject to further oxidation and both CYPs and FMOs appeared to be involved in this reaction. Two complementary approaches using selective chemical inhibitors or CYP induction implied that CYP2B1/2 was responsible for DADS oxidation with a contribution by CYP2E1. The phase I metabolism of DADS is similar between rats and human with regard to the formed metabolites, whereas the kinetic parameters and the enzymes involved are different. The intrinsic clearance ($V_{\max}/K_m$) was $0.54 \mu\text{l min}^{-1} \text{mg}^{-1}$ protein for rat, whereas it reached a maximum of $30.32 \mu\text{l min}^{-1} \text{mg}^{-1}$ protein for human enzymes (Teyssier *et al.* 1999). In humans, the

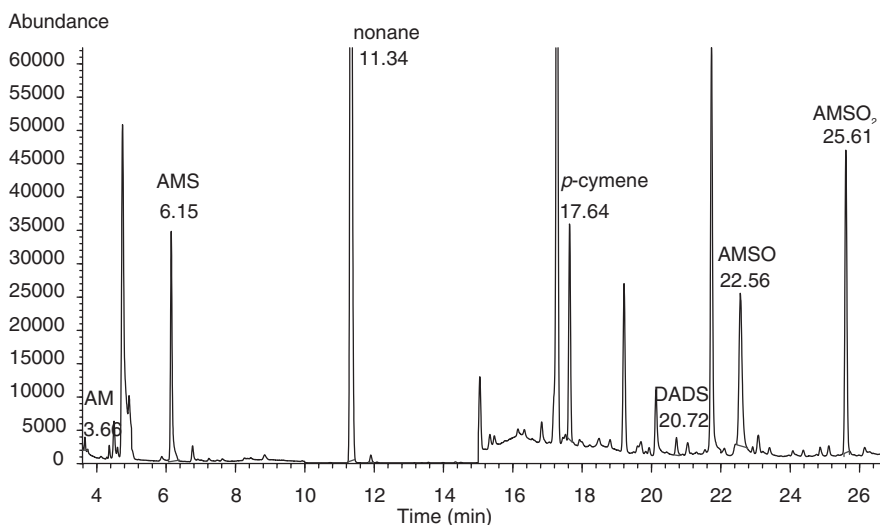


Figure 7. Total GC-MS ionic current chromatogram of an extract of rat liver. The liver had been previously perfused by 1 mM DADS in a recirculating system for 90 min.

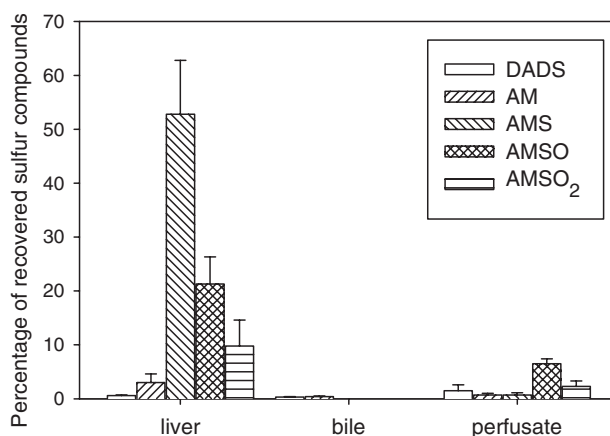


Figure 8. Relative proportions (%) of sulphur compounds in different compartments of an isolated rat perfused liver. The sulphur compounds in the liver, bile and perfusate were extracted at the end of the perfusion experiment and analysed by GC-MS. A total of 100% represents the sum of normalized areas of all detected products in the three compartments. Results are means $\pm$ SE ($n = 4$).

major enzyme involved is CYP2E1, which represents 7% of total hepatic CYP (Shimada *et al.* 1994). In rats, CYP2B1/2 is the main enzyme involved and represents 37.7% of total hepatic CYP (Waxman *et al.* 1985). Taking into account the percentage of each CYP and the observed intrinsic clearance between the two animal species, human CYP2E1 is apparently much more efficient in catalysing the oxidation of DADS to DADSO. In the same way, FMOs seemed to be more active in rats' DADS oxidation than in humans. In adult rat liver, the two major forms are FMO1 and FMO3, whereas FMO1 is almost absent in adult human liver (Yeung *et al.* 2000, Lattard *et al.* 2002). This suggests that FMO1 is more efficient than FMO3 in this oxidation. Microsomal monooxygenases also catalysed the transformation of AMS into AMSO and AMSO₂, although at very low rates in

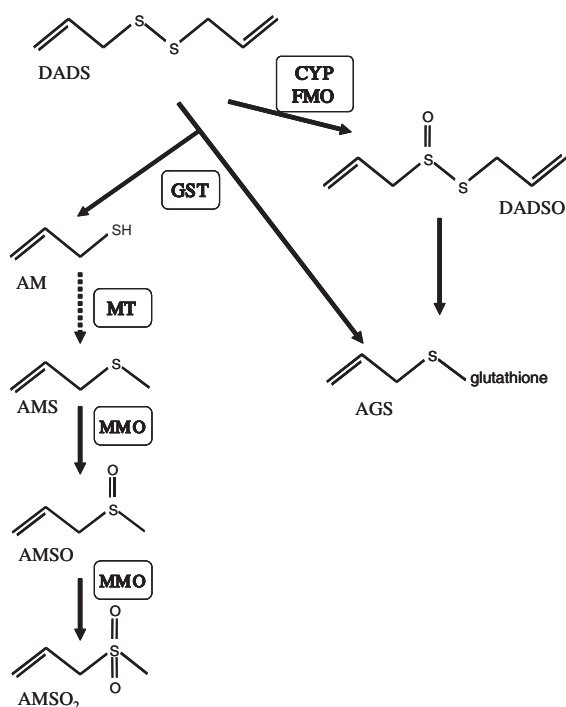


Figure 9. Proposed biotransformation pathways of DADS in rat and man. The dotted arrow represents a hypothetical route. MMO, microsomal monooxygenase; MT, methyl transferase.

both species. By analogy, Nnane and Damani (1999, 2002) reported that the rate of oxidation of ethylmethyl sulphide and diphenyl sulphide to sulphones was <0.1%.

Phase II reactions were carried out in the presence of many potential substrates such as DADS, DADSO and AM. Only enzymatic glutathione conjugation of DADS was observed, producing AGS and AM. A likely reaction pathway to explain the formation of AM is glutathione conjugation of DADS, and the subsequent release of half of the molecule corresponding to AM. AGS was also produced through a non-enzymatic reaction between DADSO and GSH, a phenomenon previously described (Miron *et al.* 2000). This indicates that AM is not the intermediate in the formation of a conjugate from DADSO.

Based on these observations, a scheme is proposed for the metabolism of DADS in rats and humans (figure 9). This scheme is in agreement with the general metabolism of xenobiotics containing sulphur compounds (Damani 1987). Generally, *S*-oxidation is predominant, explained by the readily accessible lone pair of electrons on the divalent sulphur of most sulphur compounds. Damani proposed that *S*-methylation of thiol groups is frequent and is followed by *S*-oxygenation to water-soluble sulfoxides and, in some case, sulphones. This probably represents the most important detoxification pathway for foreign thiols as well as for thiols generated *in vivo* from cystein conjugates. However, the authors could not show *in vitro* formation of AMS by methylation in the presence of either cytosolic and microsomal protein extracts. Nevertheless, this reaction is proposed to be the link between AM and AMS, which is further oxidized to AMSO and AMSO₂. Sulfoxides and sulphones are hydrophilic and generally chemically stable and are therefore the ultimate metabolites detected in the urine of animals

treated with sulphide compounds such as DADS (Germain *et al.* 2002) and DPDS (data not shown). In this proposed scheme, DADS has two possible routes of biotransformation: oxidation to DADSO or conjugation producing AM plus AGS.

The isolated perfused rat liver is a system that largely preserves the integrity of the organ. The enzymes are present in the same proportions as the whole organism and can compete for the transformation of DADS. In such a system, the sulphur compounds formed from DADS were AM, AMS, AMSO and AMSO₂, in addition to AGS from conjugation. DADSO that appeared to be an important metabolite of DADS *in vitro* was not found at any time in the perfused liver and among the *in vivo* metabolites of DADS after *in vivo* administration (Germain *et al.* 2002). Two possibilities could explain its absence *in vivo* and *ex vivo*: (1) this compound could only be formed *in vitro* in the total absence of glutathione; and (2) the rate of formation of DADSO is lower than its rate of transformation into AGS thus interfering with its detection. In agreement with this last hypothesis, DADSO has been described to disappear rapidly from the circulation after intravenous injection (Lawson and Wang 1993). The metabolites identified in the isolated perfused liver herein are in accordance with previous reports: AM was observed in the isolated perfused liver (Egen-Schwind *et al.* 1992). Surprisingly the latter authors did not identify other metabolites. AM and AMS have previously been identified as metabolites of DADS in rat hepatocytes (Sheen *et al.* 1999). Except for AGS, the panel of metabolites presented here is identical to those recovered in liver of rat receiving an oral DADS administration (Germain *et al.* 2002). It is important to draw attention that this is the first time AGS is described as metabolite of DADS. Unfortunately, this was not confirmed in the present *in vivo* study as our analytical conditions were then unsuitable to detect conjugates.

Pharmacokinetic parameters are available for the sulphur metabolites of DADS but the rapid disappearance of DADS from the circulation prevented an accurate determination of its kinetics (Germain *et al.* 2002). DADS pharmacokinetic parameters were determined using the isolated rat liver and indicated a rapid biotransformation of DADS during liver perfusion, mainly into AM and AGS as shown by the direct analysis of the perfusate. The large DADS distribution volume suggested substantial tissue retention, confirmed by the organosulphur compound composition of liver tissue, bile and perfusate. The route of elimination of AM, AMS, AMSO and AMSO₂ can be predicted from the analysis of the fluids (perfusate and bile) leaving the perfused liver. DADS and AM were eliminated by the bile and the perfusate whereas AMSO and AMSO₂ were eliminated only by the perfusate. AMS was detected only as trace in the perfusate and totally absent in the bile. The comparison of the proportion of AMS stored in the liver or eliminated through the perfusate highlighted the low elimination of this compound through the fluids indicating that metabolism was the only clearance mechanism for this compound in this system. Nevertheless, AMS metabolism was low as it accumulates in the liver. In addition, a metabolic elimination should also occur for AM, which was not eliminated by the fluids. The conjugate AGS was detected in the perfusate. Surprisingly, AGS was never found in the biliary samples during the present studies, although its polar structure and molecular weight would favour it.

In conclusion, DADS is extensively metabolized in the rat and human liver. The same metabolites were formed in both species. DADSO is formed by CYPs

and FMOs. Conjugation with glutathione takes place with DADS and DADSO to produce AGS. In the case of DADS, this conjugation produces also a thiol, AM, which could be methylated to AMS and then is oxidized by monooxygenases to AMSO and AMSO₂. These metabolites, except DADSO, are observed in the rat liver perfused with DADS. Garlic is intensively used in folk medicine and DADS and DADSO are often described as important active compounds of garlic (Le Bon and Siess 2000, Milner 2001). With regard to their rapid metabolism by the liver, it is suggested that the beneficial effects of garlic are not only mediated by these sulphur compounds. Among the potential active compounds, AGS has been reported to exhibit high antioxidant properties (Rabinkov *et al.* 2000), whereas nothing has been reported on the health properties of some metabolites such as AMSO and AMSO₂.

Acknowledgements

The authors thank Professor Jacques Auger for giving the DADSO, and Etienne Sémon, Christian Ginies and Marlène Steib for technical assistance. Research was carried out under the framework of the EU Garlic & Health Project (QLK1-CT-1999-00498).

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Identification of the human cytochrome P450s responsible for the *in vitro* metabolism of a leukotriene B4 receptor antagonist, CP-195,543

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Received 7 July 2003

1. The major human cytochrome P450 (CYP) form(s) responsible for the metabolism of CP-195,543, a potent leukotriene B4 antagonist, were investigated.

2. Incubation of CP-195,543 with human liver microsomes resulted in the formation of three major metabolites, M1–3. M1 and M2 were diastereoisomers and formed by oxidation on the benzylic position. M3 was formed by aromatic oxidation of the benzyl group attached to the 3-position of the benzopyran ring.

3. The results from experiments with recombinant CYPs, correlation studies and inhibition studies with form-selective inhibitors and a CYP3A antibody strongly suggest that the CYP3A4 plays a major role in the metabolism of CP-195,543. Recombinant CYP3A5 did not metabolize CP-195,543.

4. The apparent K_m and V_{max} for the formation of M1–3 in human liver microsomes were determined as $36\ \mu\text{M}$ and $4.1\ \text{pmol min}^{-1}\ \text{pmol}^{-1}$ P450, $44\ \mu\text{M}$ and $10\ \text{pmol min}^{-1}\ \text{pmol}^{-1}$ P450, and $34\ \mu\text{M}$ and $2.0\ \text{pmol min}^{-1}\ \text{pmol}^{-1}$ P450, respectively. The average *in vitro* intrinsic clearance for M2 was the highest both in human liver microsomes and recombinant CYP3A4 compared with M1 and M3. Intrinsic clearance for M2 in human liver microsomes and recombinant CYP3A4 was 0.231 and $0.736\ \text{ml min}^{-1}\ \text{pmol}^{-1}$ P450, respectively. The intrinsic clearances for M1 and M3 in human liver microsomes and CYP3A4 were 0.114 and 0.060 and 0.197 and 0.088 $\text{ml min}^{-1}\ \text{pmol}^{-1}$ P450, respectively. This suggests that benzylic oxidation is the predominant phase I metabolic pathway of CP-195,543 in man.

Introduction

Leukotriene B4 (LTB4), a potent neutrophil-activating agent (Ford-Hutchinson *et al.* 1980), is generated by the 5-lipoxygenase pathway of arachidonic acid metabolism (Showell *et al.* 1998). Elevated levels of LTB4 have been associated with tissue inflammation in chronic disease states such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and asthma (Crooks and Stockley 1998). In addition, inflammatory cell recruitment and activation has been implicated in shock, trauma, stroke, and intestinal and myocardial injury (Carlos and Harlan 1994). Current therapies for various chronic inflammation diseases include non-steroidal anti-inflammatory drugs, corticosteroids, sulfasalazine, methotrexate and immunosuppressive drugs, which potentially have long-term safety and efficacy concerns. CP-195,543, (+)-2-(3-benzyl-4-hydroxy-chroman-7-yl)-4-trifluoromethyl-benzoic acid (figure 1) is an antagonist

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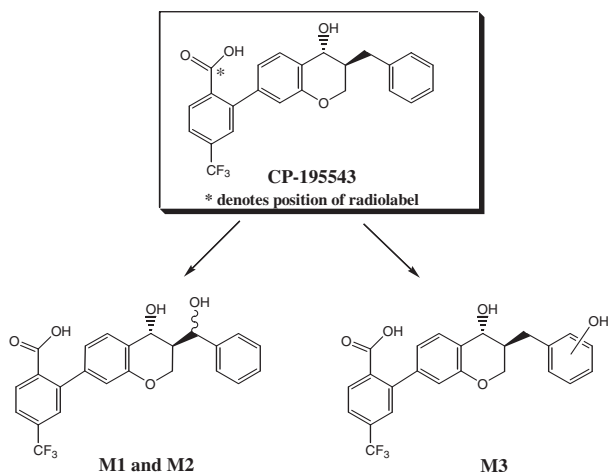


Figure 1. Structure of CP-195,543 and its proposed metabolic pathways in human liver microsomes.

of LTB₄ and offers a new selective and novel approach to the development of anti-inflammatory drugs.

Previous studies in rats and monkeys have shown that CP-195,543 is extensively metabolized with a very little unchanged drug excreted in urine or bile (Dalvie and O'Donnell 1999). The major routes of CP-195,543 elimination in preclinical species are glucuronidation of the unchanged drug and oxidative metabolism, presumably by CYPs. As found in rats and monkeys, the primary oxidative metabolites of CP-195,543 in human liver microsomes are due to oxidation at the benzylic group and the 3-position of the benzopyran ring to form metabolites M1–3 (figure 1).

Large interindividual variability in the expression of CYP enzymes and the related differences in the metabolic clearance may be caused by genetic differences or differences in exposure to xenobiotics, which may induce or inhibit CYPs (Guengerich 2000). Therefore, knowledge of the CYPs involved in the metabolism of new chemical entities is very important in predicting drug interactions and explaining interindividual variability. The purpose of the present study was to identify and characterize the human liver CYPs involved in the metabolism of CP-195,543, and to elucidate the kinetics of the formation of major oxidative metabolites in human liver microsomes.

Material and methods

Chemicals and materials

CP-195,543 (> 99.8% chemical purity) and ¹⁴C-CP-195,543, labelled at the carbon of the carboxylic acid moiety (figure 1), were synthesized at Pfizer Global Research & Development (Groton, CT, USA). The radiolabelled material had a specific activity of 8.19 mCi mmol⁻¹ and showed a radiochemical purity of > 98% on high-performance liquid chromatography (HPLC). Furaflavone and ketoconazole were purchased from Research Diagnostics, Inc. (Flanders, NJ, USA). (*S*)-mephenytoin and sulfaphenazole were from Gentest, Inc. (Woburn, MA, USA). Isocitric dehydrogenase, isocitric acid, NADP⁺, quinidine and coumarin were from Sigma-Aldrich Co. (St Louis, MO, USA). Testosterone was from Steraloids (Wilton, NH, USA). Tru-Count Scintillation cocktail was from INUS (Tampa, FL, USA). Other reagents and solvents used were of HPLC grade and from VWR (S. Plainfield, NJ, USA). Inhibitory monoclonal antibodies against specific human CYPs were from Panvera (Madison, WI, USA).

Human liver samples were from organ donors (both male and female between 18 and 65 years of age) and purchased from Tissue Transformation Technology (Edison, NJ, USA) on approval from the vendor's ethics committee. Liver and microsomes samples were stored at -70°C until used. Human liver microsomes were prepared and characterized for CYPs at Pfizer Global Research & Development using standard ultracentrifugation procedures (Prakash *et al.* 2000). Each microsomal preparation was characterized for five major drug-metabolizing CYPs: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. The specific enzymatic activities of CYP in microsomes were determined by phenacetin *O*-deethylase for CYP1A2 (Butler *et al.* 1989), tolbutamide hydroxylase for CYP2C9 (Miners *et al.* 1988), (*S*)-mephenytoin hydroxylase for CYP2C19 (Meier *et al.* 1985), bufuralol 1'-hydroxylase for CYP2D6 (Kronbach *et al.* 1987), and testosterone 6 β -hydroxylase for CYP3A4 (Sonderfan *et al.* 1987).

Microsomes prepared from human B-lymphoblastoid cells, containing cDNA-expressed CYP1A2, were from Gentest Corporation (Woburn, MA, USA). Recombinant CYPs expressed in insect cells (CYP2C9, CYP2C19, CYP2D6, and CYP3A4 and CYP3A5) were provided by the Molecular Sciences Department, Pfizer Global Research & Development. The activities of expressed CYP1A2 (7-ethoxyresorufin deethylation), CYP2C9 (tolbutamide hydroxylation), CYP2C19 (*S*-mephenytoin hydroxylation), CYP2D6 (bufuralol 1'-hydroxylation), and CYP3A4 (testosterone 6 β -hydroxylation) were 0.1, 1.8, 0.11, 0.91, and 9.0 nmol min $^{-1}$ mg $^{-1}$ microsomal protein, respectively. Microsomal protein was assayed using BCA assay kit (Pierce, Rockford, IL, USA) and its CYP contents were determined by the method of Omura and Sato (1964).

In vitro incubations

Metabolites of CP-195,543 in human liver microsomal incubations. Before use, microsomes were thawed on ice. The incubation mixture (final volume of 1 ml) contained human liver microsomes (0.5 μM CYP), CP-195,543 or ^{14}C -CP-195,543, 100 mM potassium phosphate buffer (pH 7.4) and an NADPH-generating system (11 mM MgCl_2 , 0.54 mM NADP^+ , 6.2 mM DL-isocitric acid, and 0.5 U ml $^{-1}$ isocitric dehydrogenase). The reactions were initiated by addition of 100 μl NADPH-regenerating system after a 3-min pre-incubation period at 37°C . Reactions were incubated at 37°C for 30 min and were terminated using 1.0 ml cold acetonitrile.

Kinetic studies. Before kinetics studies, linearity of formation of metabolites with respect to incubation time and microsomal protein content was confirmed. Enzyme kinetics were performed using pooled human liver microsomes (22.1 pmol P450 per incubation) or recombinant CYP3A4 (30 pmol P450) in triplicate at 37°C for 15 min at a substrate concentration of 1.0, 5.0, 10, 50, 100 and 200 μM . The reaction mixture in a final volume of 1.0 ml was incubated at 37°C for 15 min. Assays with recombinant CYPs were performed at 10 μM CP-195,543 for 15 min.

Inhibition studies

Inhibition studies were performed with each of the following inhibitors: furafylline (30 μM), coumarin (50 μM), quinidine (1 μM), (*S*)-mephenytoin (50 μM), sulfaphenazole (1 μM) and ketoconazole (1 μM). Inhibitors were dissolved in methanol, and concentrated stocks were used to minimize concentration of organic solvent in the incubation to 0.5%. Control samples were subject to the equivalent amount of organic solvent without inhibitors. Inhibitory antibody incubations were performed based on manufacturers recommendations per lot analysis to achieve >80% inhibition for marker substrates. The total amount of each antibody used in each incubation (ratio mg total Ig mg $^{-1}$ microsomal total protein, lot number) was: anti-CYP1A1 0.177 mg (0.629, lot number 15988), anti-CYP1A2 0.265 mg (0.494, 16432), anti-CYP2A6 0.0986 mg (0.186, 18124), anti-CYP2B6 0.264 mg (1.23, 15989), anti-CYP2C8/9/18/19 0.071 mg (0.0392, 15990), anti-CYP2D6 0.277 mg (0.235, 15897), anti-CYP2E1 0.068 mg (0.0333, 15991), and anti-CYP3A4/5 0.318 mg (0.373, 15992). Each antibody was shown to inhibit >85% metabolism of CYP-specific probe substrates with human liver microsomes and does not cross-react with other human CYPs.

Correlation studies

Formation rates of metabolites were also measured using microsomes from 10 different individual human liver microsomes at a substrate concentration of 10 μM as described above. Formation rates were correlated with activities of CYP-selective probe substrates to assess the possible involvement of individual forms.

Sample preparation for analysis

Samples were added to 100 μl of 5 $\mu\text{g ml}^{-1}$ internal standard (CP-293,243, a 2,2-dimethyl analogue of CP-195,543) dissolved in acetonitrile. All samples were centrifuged at 2100 *g* for 10 min. The supernatant was transferred and evaporated to dryness using nitrogen gas at 40°C . Samples were reconstituted in 25:75 acetonitrile:ammonium formate (20 mM, pH 6.8) before LC/MS analysis.

HPLC analysis of the metabolite profiles

The HPLC system consisted of a Hewlett Packard 1100 gradient pump, degasser and autosampler (Wilmington, DE, USA). Chromatography was carried out on a reversed phase Zorbax RX-C8 column (4.6×150 mm, 5μ ; Hewlett-Packard) at a flow rate of 1.0 ml min^{-1} with a dual-solvent system consisting of ammonium formate (50 mM, pH 6.8; solvent A) and acetonitrile (solvent B). The following HPLC gradient method was used: 25 to 35% B in 2.5 min, 35 to 45% B in 4.5 min, maintained at 45% B for another 4.5 min, followed by a 4.5-min re-equilibration to 25% B. M1–3 eluted at 5.0, 5.3 and 6.0 min, respectively. CP-195,543 and the internal standard eluted at 7.6 and 8.8 min under these HPLC conditions. Radioactivity was monitored using a flow through β -radioactivity detector (β -Ram, INUS, Riviera Beach, FL, USA), equipped with a $500\text{-}\mu\text{l}$ flow cell and a 2.5 ml min^{-1} flow rate of scintillation cocktail.

Characterization of metabolites

Metabolites were characterized using a PE-Sciex API 2000 HPLC/MS/MS system (Thornhill, Ontario, Canada) with the same HPLC gradient system as described above. The effluent from the HPLC column was split and approximately $50 \mu\text{l min}^{-1}$ was introduced into the atmospheric pressure ionization (API) interface. The remaining effluent was directed to the flow cell of the β -RAM detector. The β -RAM response was recorded in real time by the mass spectrometer that provided simultaneous detection of radioactivity (RAD) and mass spectrometry data. The mass spectrometer was operated in both positive- and negative-ion mode and the ion-spray interface was operated at ± 4400 V and the collision-induced dissociation (CID) studies were performed using nitrogen gas at a collision energy of 35–45 eV and collision gas thickness of about 2.5×10^{14} molecules cm^{-2} .

For quantitation, the compounds were detected in a negative-ion mode using single ion monitoring of m/z 443 for the metabolites, m/z 427 for CP-195,543, and m/z 455 for the internal standard. The lower limit of quantification was about 10 pmol and the coefficient of variation was $< 10\%$. Standard curves of CP-195,543 were used for quantitation of M1–3 because the authentic standards of metabolites were not available.

Data analysis

LC/MS data were quantitated using Turboquan 1.0 (PE Sciex, Thornhill, Ontario, Canada) and expressed as a ratio of the analyte peak area/internal standard peak area. Incubations containing ^{14}C -CP-195,543 were analysed to provide a conversion factor of metabolite peak areas to velocity. Data from radiochromatograms were expressed as the calculated per cent radioactivity of the analyte peak/total radioactivity in the chromatogram. Apparent K_m and $V_{\max}$ for human liver microsomes and human recombinant enzymes were determined using the Pharmacodynamic model of WinNonlin Pro non-linear regression software v.2.1 (Pharsite Corp, Palo Alto, CA, USA). Inhibition data were calculated as a percentage of positive control samples without inhibitors.

Results

In vitro incubations

The HPLC metabolite profile of the incubation of CP-195,543 with pooled human liver microsomes in the presence of NADPH shows the formation of three main oxidative metabolites, M1–3 (figure 2). M1 and M2 were diastereoisomeric products formed by oxidation on the benzylic position. M3 was formed by oxidation on the phenyl ring of the benzylic moiety (figure 1). These structures were confirmed by comparing the HPLC retention times and CID product ion spectra with those of metabolites identified previously in rats and monkeys (Dalvie and O'Donnell 1999). Metabolite formation was NADPH dependent and largely abolished in the presence of 1-aminobenzotriazole (ABT, 5 mM) indicating that CYP was responsible (data not shown; Ortiz de Montellano *et al.* 1995).

Kinetics of the formation of metabolites

For kinetics purposes, linearity studies for the formation of metabolites with respect to incubation time and microsomal protein content were performed. Under these incubation conditions, rates of formation for each metabolite are consistent with Michaelis–Menten kinetics over the substrate concentration range 1–200 μM .

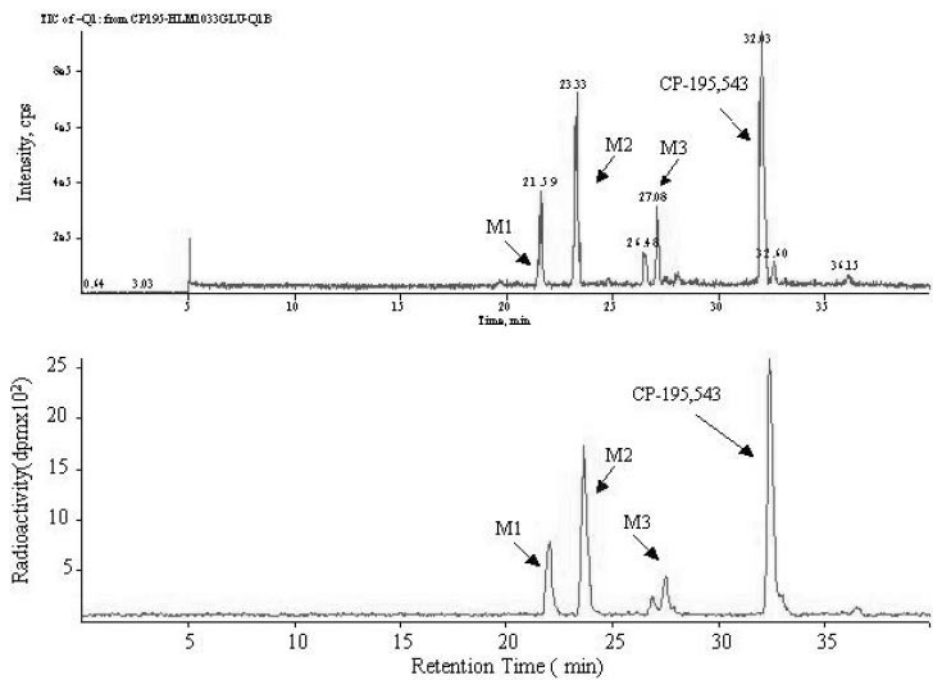


Figure 2. Total ion chromatogram (top) and HPLC-radiochromatogram of metabolites of CP-195,543 in human liver microsomes.

Table 1. Kinetic parameters for the formation of M1–3 in human liver and recombinant CYP3A4 microsomes.

Enzyme source	Metabolites	K_m (μM)	$V_{\max}$ $\text{pmol min}^{-1} \text{pmol}^{-1} \text{P450}$	Cl_{int}^a $\text{ml min}^{-1} \text{pmol}^{-1} \text{P450}$
Human liver	M1	36.2 ± 6.1	4.1 ± 0.3	0.114
	M2	44.2 ± 7.5	10.2 ± 0.4	0.231
	M3	33.5 ± 7.2	2.0 ± 0.22	0.060
Recombinant CYP3A4	M1	43.6 ± 6.4	8.6 ± 1.3	0.197
	M2	29.2 ± 3.8	21.5 ± 2.9	0.736
	M3	36.4 ± 3.1	3.2 ± 0.4	0.088

Enzyme kinetics were performed using human liver microsomes (HLMix-12; 85 μg or 22.1 pmol P450 per incubation) or recombinant CYP3A4 (30 pmol P450) in triplicate at 37°C for 15 min at 1.0, 5.0, 10, 50, 100 and 200 μM CP-195,543 at a final volume of 1.0 ml.

^a Cl_{int}' is calculated as the mean of $V_{\max}$ divided by K_m .

Data are the mean $\pm$ SEM of three determinations.

At concentrations $>200 \mu\text{M}$, the velocity of metabolite generation decreased, which suggested possible substrate inhibition. The apparent kinetic parameters [K_m , $V_{\max}$, and Cl_{int} ($V_{\max}/K_m$)] derived from human liver microsomes are shown in table 1. In human liver microsomes, the K_m and $V_{\max}$ for M1–3 formation were 36 μM and 4.1 $\text{pmol min}^{-1} \text{pmol}^{-1} \text{P450}$, 44 μM and 10 $\text{pmol min}^{-1} \text{pmol}^{-1} \text{P450}$, and 34 μM and 2.0 $\text{pmol min}^{-1} \text{pmol}^{-1} \text{P450}$, respectively (table 1). The average *in vitro* intrinsic clearance ($V_{\max}/K_m$) for M2 was two- and fourfold higher than for M1 and M3, respectively.

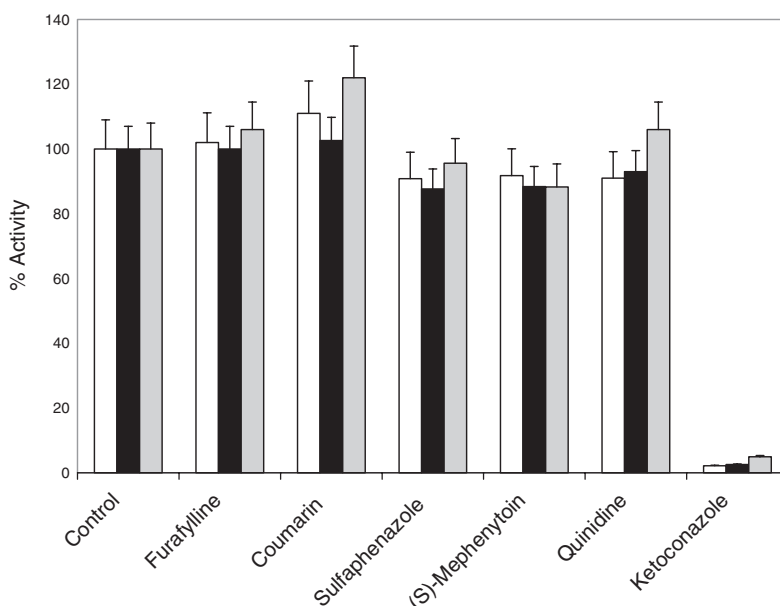


Figure 3. Inhibition of M1 (white bar), M2 (black bar) and M3 (grey bar) by isoform-selective chemical inhibitors in human liver microsomes (22 pmol P450) at 10 μ M CP-195,543 and an incubation time of 15 min. Inhibitor concentrations were furafylline at 30 μ M, coumarin at 50 μ M, quinidine at 1 μ M, (S)-mephenytoin at 50 μ M, sulfaphenazole at 1 μ M and ketoconazole at 1 μ M. Data are the mean $\pm$ SEM of three determinations. Absolute values for 100% controls were 3.24, 8.32 and 1.55 pmol min⁻¹ pmol⁻¹ P450 for M1–3, respectively.

Inhibition studies

To characterize the potential contribution of CYPs to CP-195,543 metabolism, CP-195,543 was incubated with or without CYP-selective inhibitors. Inhibition of metabolite formation by each inhibitor is summarized in figure 3. Ketoconazole at 1 μ M significantly inhibited the formation of all the three metabolites. Furafylline (30 μ M), coumarin (50 μ M), quinidine (1 μ M), (S)-mephenytoin (50 μ M) and sulfaphenazole (1 μ M) did not inhibit the formation of these metabolites (figure 3).

Similarly, with inhibitory CYP antibodies, of all the antibodies tested, anti-CYP3A4/5 was the only antibody that significantly inhibited the formation of all the three metabolites (figure 4).

Correlation studies

For correlation studies, the formation rates of each metabolite at 10 μ M CP-195,543 were determined in human liver microsomes derived from 10 individual donors. The formation rate of each metabolite correlated well with the activity of CYP3A ($r^2 = 0.71$ for M1, 0.80 for M2 and 0.86 for M3), determined by testosterone 6 β -hydroxylase activity (table 2). No significant correlations were found between the formation of CP-195,543 metabolites and the other CYP-specific substrates.

Metabolism by recombinant CYPs

To evaluate further the involvement of each of the major CYPs, recombinant human CYPs were incubated with 10 μ M CP-195,543 (figure 5). Recombinant CYP3A4 generated all three metabolites, M1–3, at the highest rate of formation

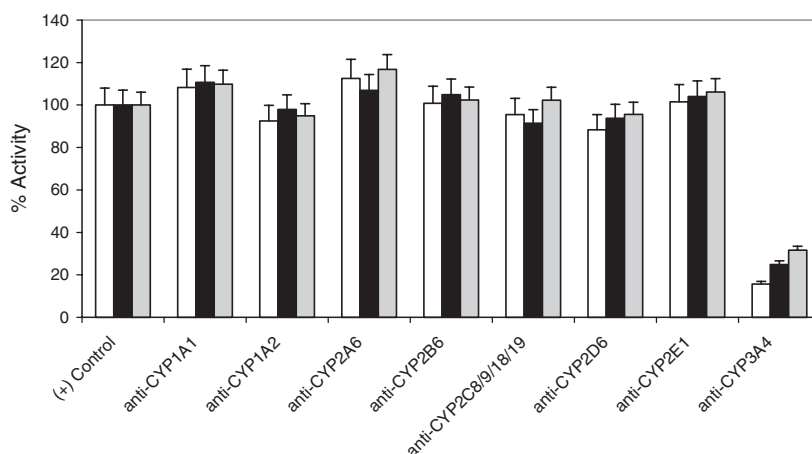


Figure 4. Inhibition of M1 (white bar), M2 (black bar) and M3 (grey bar) by inhibitory monoclonal P450 antibodies in human liver microsomes (22 pmol) at 10 μ M CP-195,543. The total amount of each antibody used in each incubation was: anti-CYP1A1 at 0.177 mg, anti-CYP1A2 at 0.265 mg, anti-CYP2A6 at 0.0986 mg, anti-CYP2B6 at 0.264 mg, anti-CYP2C8/9/18/19 at 0.071 mg, anti-CYP2D6 at 0.277 mg, anti-CYP2E1 at 0.068 mg, and anti-CYP3A4. Data are the mean $\pm$ SEM of three determinations. Absolute values for 100% controls were 3.24, 8.32 and 1.55 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 for M1–3, respectively.

Table 2. Correlation of various CYP-selective monooxygenase activities with M1–3 in human liver microsomes.

CYP	Activity	Correlation coefficient (r^2)		
		M1	M2	M3
CYP1A2	phenacetin <i>O</i> -deethylase	0.0045	0.090	0.0032
CYP2C9	tolbutamide hydroxylase	0.19	0.31	0.22
CYP2C19	(<i>S</i>)-mephenytoin hydroxylase	0.05	0.21	0.16
CYP2D6	bufuralol 1'-hydroxylase	0.31	0.26	0.18
CYP3A	testosterone 6 β -hydroxylase	0.71	0.80	0.86

Correlation studies were performed using human liver microsomes in triplicate at 37°C for 15 min at 10 μ M CP-195,543 at a final volume of 1.0 ml.

than any other CYP tested (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A5). M2 additionally was formed to a minor extent by CYP2C9 at 4% of the rate of CYP3A4. As the enzyme activities of the recombinant CYPs were selected to reflect activities typical of a human liver microsomal preparation (see the Materials and methods), it can be concluded that the other CYPs do not contribute significantly to CP-195,543 metabolism. The kinetic parameters for the formation of M1–3 by recombinant CYP3A4 are shown in table 1. The K_m and V_{max} for recombinant CYP3A4 for M1–3 were 44 μ M and 8.6 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450, 29 μ M and 22 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450, and 36 μ M and 3.2 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450, respectively.

Discussion

The present study reports the *in vitro* metabolism of CP-195,543 by human CYPs to three main oxidative metabolites, namely M1–3. M1 and M2 are diastereoisomers and formed by oxidation on the benzylic position. M3 is formed

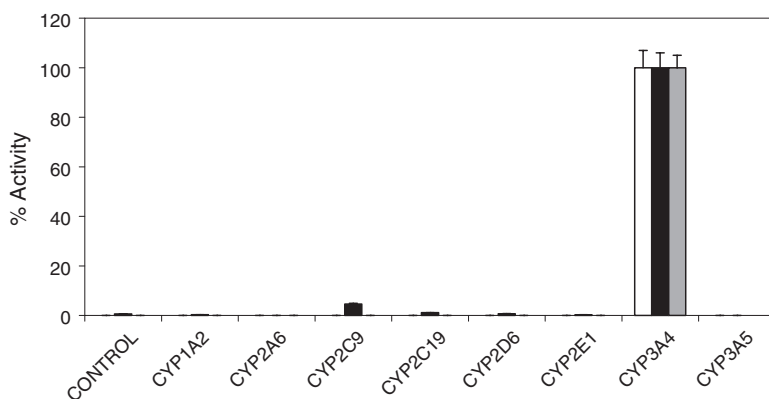


Figure 5. Recombinant human CYP450 (30 pmol) tested for the formation of M1 (white bar), M2 (black bar) and M3 (grey bar) at 10 μ M CP-195,543 and 15 min incubation. Data are the mean $\pm$ SEM of three determinations. Absolute values for 100% controls were 8.6, 22 and 3.2 pmol min⁻¹ pmol⁻¹ P450 for M1–3, respectively.

by aromatic oxidation of the benzyl group attached to the 3-position of the benzopyran ring (figure 1). These metabolites were identical to those excreted *in vivo* in rat and dog (Dalvie and O'Donnell 1999). The kinetic parameters for the formation of these metabolites show very similar K_m values of around 30 μ M. However, the V_{max} of M2 was 2.4- and 7-fold higher than M1 and M3, respectively. The apparent intrinsic clearance for M2 in CYP3A4 is 3.7- and 8.4-fold higher than M1 and M3, respectively. This suggests that *in vivo* in humans, M2 would be the major oxidative metabolic pathway for CP-195,543 elimination.

Kinetic analysis of the formation these metabolites produced a monophasic Lineweaver–Burk plot suggesting that a single CYP was responsible for their formation. Based on correlation studies, CYP-selective chemical inhibitors, monoclonal antibody and metabolism by recombinant human CYPs, it has been shown that CYP3A4 is the major form responsible for the metabolism of CP-195,543 to oxidative M1–3. Inhibition and correlation studies were performed at low substrate concentration (10 μ M), which were consistent with predicted *in vivo* plasma concentration in humans. This suggests that in humans, CYP3A4 will be the major form for metabolism and elimination of CP-195,543. Based on correlation studies, significant correlation ($r^2 > 0.70$) was found between microsomal activity for formation of each metabolite and activity of testosterone 6 β -hydroxylase in liver microsomes derived from 10 donors. Additionally, no significant correlation was found between microsomal activities of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 for marker substrates and metabolite formation for M1–3 of CP-195,543. Ketoconazole (1 μ M) incubated with CP-195,543 in microsomes inhibited the formation of these metabolites > 90%, again suggesting involvement of CYP3A4 in metabolite production. Monoclonal anti-CYP3A4/5 antibody inhibited up to 80% of the formation of M1–3 in microsomes. Finally, the formation of these metabolites was catalysed by recombinant CYP3A4 microsomes. Other recombinant CYPs demonstrated low or no metabolism of CP-195,543. These data demonstrate that CYP3A4 is the major CYP responsible for the metabolism of M1–3 of CP-195,543. Based on these findings and expected

plasma drug concentrations *in vivo*, it is predicted that the major route of human *in vivo* Phase I metabolism will be CYP3A4 mediated.

The human CYP3A family, accounting for about 50% of the total P450 in human liver is clinically very important because it has been shown to metabolize a large number of drugs (Wrighton *et al.* 1996). The human CYP3A subfamily includes CYP3A4, CYP3A5, CYP3A7 (Li *et al.* 1995), and CYP3A43 (Domanski *et al.* 2001) as subfamily members. CYP3A4 is the major human liver CYP3A enzyme, whereas CYP3A5 is present in only about 10–30% of the human liver and CYP3A7 is only present in the foetal liver. The highest concentration of transcript expression of the recently discovered CYP3A43 is in the prostate, whereas the hepatic mRNA concentration is only 0.2–5% that of CYP3A4 (Gellner *et al.* 2001). Most of the CYP3A4 substrates are also metabolized by CYP3A5 and CYP3A7 (Williams *et al.* 2002). In the current study, CP-195, 543 was found to be a substrate of only CYP3A4 but not CYP3A5. Since CYP3A4 exhibits highly variable expression (>10-fold) in both the liver and intestinal wall (Lin *et al.* 1999), this may result in extensive first-pass metabolism of CP-195,543. Changes in abundance of CYP3A4 by induction or inhibition may result in variability in the levels observed in humans (Lin and Lu 1998, Thummel and Wilkinson 1998). However, the relative contribution of this oxidative pathway has not yet determined in humans. It is possible that the conjugation pathway plays a larger role in clearance of the drug and so the possible variability of CYP3A4 may not be significant. In addition, the two metabolic pathways may work in tandem to decrease the likelihood that elevation of drug concentrations would occur in circumstances where there is a low abundance or inhibition of CYP3A4 due to disease state or drug–drug interactions in patients.

In conclusion, the involvement of the CYP3A4 in the metabolism of CP-195,543 has been demonstrated by means of four different *in vitro* approaches. Clinical studies to determine definitive radiolabel biotransformation and drug–drug interactions need to be performed to assess the clinically relevant metabolic interactions of CP-195,543 with any of the known CYP3A4 inhibitory drugs.

Acknowledgements

The authors thank Dr Keith McCarthy for synthesizing radiolabelled CP-195,543, Mr Larry Cohen for technical assistance and Dr Larry Tremaine for critical evaluation of the manuscript.

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Inhibition of rat liver sulfotransferases SULT1A1 and SULT2A1 and glucuronosyltransferase by dietary flavonoids

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Received 3 March 2003

1. Dietary flavonoids including kaempferol, quercetin, genistein and daidzein were tested for their ability to alter the conjugation of oestradiol (E_2) via rat liver sulfotransferases and glucuronosyltransferase.

2. All four flavonoids inhibited the sulfonation of E_2 via phenol sulfotransferase, SULT1A1 with IC_{50} s ranging from 0.29 to 4.61 μ M. Sulfonation of dehydroisoandrosterone (DHEA) via hydroxysteroid sulfotransferase, SULT2A1, was inhibited by higher amounts of the flavonoids (IC_{50} s ranging from 34 to 116 μ M).

3. All flavonoids inhibited the formation of E_2 - β -glucuronides (at carbon atoms 3 and 17) with IC_{50} s ranging from 43 to 260 μ M. Glucuronidation of 4-methylumbelliferone (4-MU) was inhibited by high amounts of the flavonoids (IC_{50} s ranging from 860 to 1550 μ M).

4. Hydrolysis of sulfonated oestrogens via arylsulfatase-c (ARSC) or 4-methylumbelliferone β -glucuronidate (MUG) were not inhibited by the flavonoids.

5. It is concluded that SULT1A1 but not SULT2A1 or glucuronosyltransferase is highly sensitive to inhibition by dietary flavonoids. The potency of the inhibition for SULT1A1 (quercetin > kaempferol > genistein > daidzein) suggests a dependency on the number and position of hydroxyl radicals in the flavonoid molecule.

Introduction

Interest in the use of flavonoids in preventing and treating breast cancer has been stimulated by a number of epidemiological correlations and laboratory studies indicating a low incidence of this disease in human populations with a high dietary intake of these plant natural products (Wang *et al.* 1998, Setchell and Cassidy 1999). For example, women from Asia, Africa, and South and Central America who come from populations consuming high dietary amounts of soy products and yellow fruits, which are rich in isoflavones, have a relatively low frequency of breast cancer compared with their contemporaries living in Western Europe and the USA (Wu *et al.* 1998). Quercetin and kaempferol are the most common flavonoids found in foods including the citrus family, apples, onions, tea leaves and many vegetables (de Vries *et al.* 1998, Morand *et al.* 1998, Denis *et al.* 1999). There are many laboratory studies in animals (So *et al.* 1996, Fritz *et al.* 1998, Lamartiniere *et al.* 1998) and cell culture systems (So *et al.* 1997) that are in accord with epidemiological studies indicating that isoflavones are beneficial in reducing the occurrence not just of mammary tumours and other human cancers. Multiple mechanisms have

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been proposed for the beneficial effects of isoflavones in human health including oestrogenic and anti-oestrogenic effects, induction of cancer cell proliferation, inhibition of tyrosine kinase and DNA topoisomerase, suppression of angiogenesis and antioxidant effects (Setchell and Cassidy 1999).

The close structural similarity between many flavonoids and mammalian oestrogens suggest that the oestrogenic and anti-oestrogenic effects of flavonoids might be of prime importance to their beneficial effects in breast cancer. However, relatively little attention has been given to the actions of flavonoids on the metabolism of endogenous oestrogens, which is complex and involves a number of oxidative and conjugative pathways including glucuronidation and sulfonation (Zhu and Conney 1998).

Sulfonation of steroid hormones via cytosolic sulfotransferases is a major route of steroid hormone metabolism in rodents as well as humans. Moreover, the rat continues to be widely used as an animal model when studying the influence of various xenobiotics, including dietary constituents, on the initiation and progression of hormone-dependent cancers (e.g. Harvell *et al.* 2001, Mesía-Vela *et al.* 2002). Earlier studies of the influence of flavonoids on recombinant sulfotransferase activities were carried out primarily on the human isoforms (Eaton *et al.* 1996, Otake *et al.* 2000), which differ significantly in structure from rat isoforms. Accordingly, we evaluated the capacity of different dietary flavonoids to disrupt pathways of oestrogen sulfonation and glucuronidation. The actions of genistein, daidzein, kaempferol and quercetin on the sulfonation of E_2 by recombinant phenol sulfotransferases (SULT1A1) and hydroxysteroid (SULT2A1) cloned from rat liver were determined. These actions on recombinant sulfotransferases were compared with the effects of the flavonoids on sulfotransferases, glucuronosyltransferases and arylsulfatase present in rat liver cytosol and microsomes.

Materials and methods

Reagents

Oestradiol, dehydroepiandrosterone (DHEA), quercetin (3,3',4',5,7-pentahydroxyflavone), kaempferol (3,4',5,7-tetrahydroxyflavone), β -glucuronidase (EC 3.2.1.3.1), 4-methylumbelliferone, 4-methylumbelliferyl glucuronide, uridine diphosphate glucuronic acid (UDPGA), saccharic acid 1,4-lactone and Tris-base were obtained from Sigma Chemical Co. (St Louis, MO, USA). Genistein (4',5,7-trihydroxyisoflavone) was purchased from Alexis Biochemicals (San Diego, CA, USA). Daidzein (7,4'-dihydroxyisoflavone) was purchased from ICC-Indofine Chemical Co., Inc. (Belle Mead, NJ, USA). Oestradiol (3- and 17 β -) glucuronides were purchased from Steraloids, Inc. (Wilton, NH, USA). ^{35} S-PAPS was from New England Nuclear (Boston, MA, USA). PAPS (>99% pure) was purchased from H. Glatt and R. Landseidel (German Institute of Nutrition, Potsdam, Germany). Expression vector pET17b and *Escherichia coli* strain BL21(DE3)pLysS were from Novagen (Madison, WI, USA). All other chemicals were of the highest grade from standard sources.

Animals and preparation of rat hepatic subcellular fractions

Male Sprague-Dawley rats, weighing 250 g, were purchased from Taconic (Germantown, NY, USA) and housed individually in an AAALAC-accredited barrier facility under controlled temperature, humidity and lighting conditions. Animals were fed a standard rat chow diet (PMI 5012, LabDiet®, PMI Nutrition International, LLC, Brentwood, MO, USA) *ad libitum*. Experiments involving animals were carried out in compliance with guidelines specified in the Guide to the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, 1996). Rats expired under deep surgical anaesthesia (75 mg sodium pentobarbital kg^{-1}) while livers were removed and homogenized in 4 vols ice-cold 20 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged at 10 000g for 20 min at 4°C. Microsomes were obtained by centrifugation of the 10 000g supernatant fraction at 100 000g for 60 min at 4°C (Thomas *et al.* 1983). The resulting microsomal fraction was washed and resuspended in phosphate buffer, pH 7.4, frozen immediately and stored at -80°C until used for assays.

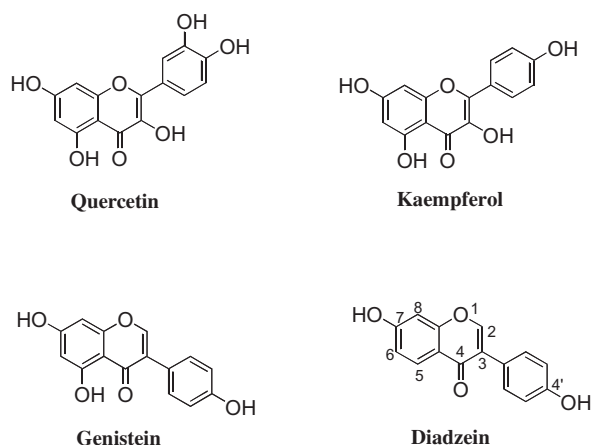


Figure 1. Chemical structures of flavonoids.

Aliquots of cytosols and microsomes were diluted and assayed for protein using bicinchoninic acid in a 96-well format according to recommendations of Pierce Biotechnology (Rockford, IL, USA). Duplicate standards of bovine serum albumin (0–60 µg) were incubated in parallel to construct standard curves.

Expression of recombinant enzymes

A full-length cDNA clone encoding rat liver SULT1A1 was isolated from a rat liver library prepared in λZAP (Stratagene, La Jolla, CA, USA) using the polymerase chain reaction (PCR). Primer design was based on a published sequence for phenol sulfotransferase SULT1A1 (accession no. L19998). The rat SULT1A1 cDNA was subcloned into pET17-b for expression in *E. coli* BL21(DE3)pLysS. The rat cDNA for SULT2A1 was derived from a library prepared in λGT11 (Glatt *et al.* 2000) and subcloned into the bacterial expression vector pKK233-2 and transformed into *E. coli* BL21(DE3)-pLysS. Expression and preparation of *E. coli* cell-free extracts containing recombinant SULT enzymes were performed as described (Dajani *et al.* 1999).

Sulfotransferase assays

Enzyme activities were determined using PAP³⁵S according to a modification of the method described by Foldes and Meek (1973). Briefly, bacterial cell-free extracts (8–24 µg protein ml⁻¹) or liver cytosol (240 µg protein ml⁻¹) were incubated with either 0.025 mM E₂ (substrate for SULT1A1) or 0.025 mM DHEA (substrate for SULT2A1), 0.01 mM ³⁵S-PAPS (0.01 µCi/tube), 20 mM MgCl₂ with or without the flavonoids (0–0.3 mM) in a final volume of 0.1 ml 20 mM Tris-HCl buffer (pH 7.4) for 20 min at 37°C. The reaction was stopped by adding 0.2 ml 0.1 M barium acetate. Protein, unreacted PAPS and free sulfate were precipitated by the sequential addition of 0.1 M barium hydroxide (0.2 ml) and 0.1 M zinc sulfate (0.2 ml). Radioactivity was measured in 0.6 ml of the supernatant, following centrifugation at 15 600g (14 000 rpm) for 2 min in an Eppendorf centrifuge (Model No. 54156).

Initial experiments using ³⁵SPAPS in assays of cytosolic SULTs indicated the sulfonation of unknown substance(s) led to high blanks. Consequently, further experiments with tritiated E₂ were carried out in experiments measuring cytosolic SULTs. Sulfonation of E₂ by SULTs from rat liver cytosol was performed using [³H]-E₂ (0.02 µCi/tube) as substrate, 0.01 mM PAPS and 4 mM MgCl₂ in 0.2 ml 0.05 M Tris-HCl buffer, pH 7.4. Reactions were stopped by addition of 0.2 ml ice-cold 0.25 M Tris-HCl, pH 8.7. Free and sulfated steroid were separated by a single extraction with 3 ml chloroform, followed by centrifugation. Aliquots (0.2 ml) of the aqueous phase were counted for radioactivity (Kauffman *et al.* 1998). All incubations were performed in triplicate. Coefficients of variation between replicate samples were <3%. Minimal detectable concentrations of metabolite were approximately 50 nM. Control incubates (without substrate) were performed for each inhibitor concentration.

Sulfatase assay

Oestrone sulfate (E₁-3) sulfatase activity was determined by measuring the formation of [6,7-³H]-E₁ from [6,7-³H]-E₁-3-sulfate as described (Kauffman *et al.* 1998). The incubation mixture consisted of hepatic microsomes (6 µg protein ml⁻¹) and 0.01 mM [6,7-³H] E₁-3-sulfate (0.2 µCi/tube) in 0.15 ml 20 mM Tris-HCl buffer, pH 7.4. Incubations were carried out at 37°C for 20 min. Reactions were

stopped by addition of 0.2 ml ice cold water and tubes placed immediately on ice. The $[6,7-^3\text{H}]\text{-E}_1$ formed was extracted with 2 ml ethyl acetate. Aliquots (0.2 ml) of the organic supernatants were removed for measurement of radioactivity (Kauffman *et al.* 1994). Blank values were determined for each individual assay by omitting hepatic microsomes from incubation mixtures.

UDP-glucuronosyltransferase assay

4-Methylumbelliferone (4-MU) and E_2 were used as substrates. The glucuronidation of E_2 was measured in 0.15 ml 50 mM Tris-HCl buffer, pH 8.7, containing: 1.0 mg microsomal protein, 2 mM UDPGA, 5 mM MgCl_2 and 0.1 mM E_2 (Zhu *et al.* 1998). The reaction was initiated by addition of UDPGA. Incubations were carried out at 37°C for 15 min and reactions arrested by addition of 0.05 ml ice-cold acetonitrile. The reaction mixture was then vortexed and centrifuged at 5750g (5000 rpm) for 5 min in an Eppendorf centrifuge. Blank values, obtained from incubations in the absence of UDPGA, were determined for each individual assay and subtracted. Aliquots (0.1 ml) of the supernatants were used for the determination of E_2 and E_2 -glucuronides by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu SCL-10A system controller with a Shimadzu SIL-10A auto-injector, 2 LC-10AD pumps, SPD-10A UV-VIS detector set at 280 nm and an Eclipse XDB C18 (4.6×150 mm) column (MACMOD Analytical, Chadds Ford, PA, USA). The solvent system consisted of solvents A: 0.1% acetic acid, and B: (20:80) methanol/acetonitrile with 0.1% acetic acid. E_2 -glucuronides were eluted using a 30-min linear gradient from 25 to 90% of solvent B. The elution of E_2 and its glucuronides were detected by corresponding metabolites agreed exactly with corresponding UV absorbing peaks at 280 nm. Metabolite quantification was based on the area of absorbance for each metabolite determined with pure standards.

Assays employing 4-MU as a substrate for glucuronosyltransferase were carried out at 37°C in 1 ml 0.05 M Tris-HCl buffer, pH 8.7, containing 2 mM UDPGA, 0.5 mM 4-MU, freshly made 1 mM saccharic acid 1,4 lactone (to inhibit β -glucuronidase) and 1 mg microsomal protein. At various intervals ranging up to 1 h, 0.1-ml aliquots were withdrawn and added to 0.2 ml ice-cold 50 mM sodium acetate, pH 5.0, to stop reactions. Unreacted umbelliferone was removed by three successive extractions with 0.8 ml toluene:butanol (3:1 v/v) (Conway *et al.* 1988). An aliquot of the aqueous fraction (0.01 ml) was added to 0.05 ml sodium acetate buffer, pH 5, containing β -glucuronidase (1000 U ml^{-1}) and incubated for 1 h at 37°C to hydrolyse 4-methylumbelliferyl glucuronide (4-MUG), which was measured fluorimetrically as detailed below.

β -Glucuronidase assay

4-MUG (0.5 mM) served as substrate for β -glucuronidase (200 U ml^{-1}). Incubation was performed with or without flavonoids (0.001–1 mM) in 0.1 ml 50 mM buffer acetate at 37°C for 1 h. The reaction was stopped by addition of 1 ml 0.02 M sodium carbonate buffer, pH 10. The liberated umbelliferone was measured fluorometrically (366 $\rightarrow$ 470 nm) (El Mouelhi and Kauffman 1986).

Data analysis

The equation $V_i/V_o = 1/(1 + [I]/\text{IC}_{50})$, where V_i is the total activity and V_o is the remaining activity, was fitted to data to calculate the IC_{50} s using the program KaleidaGraph 3.0 for Windows (Synergy Software, Reading, PA, USA). All experiments were performed in triplicate. Inhibitor constants (K_i) were obtained by least-squares non-linear regression analyses for the best fit of data of measured rates using software for the Macintosh commuter (Enzyme Kinetics, Trinity Software, Version 1.1, Campton, NH, USA).

Results

Effect of flavonoids on sulfotransferase activities

All the flavonoids tested inhibited E_2 sulfonation by recombinant SULT1A1 with IC_{50} s $< 5 \mu\text{M}$ (table 1). This inhibition was a non-competitive type (figure 2) with K_i s of 0.1, 0.4, 1.6 and $0.7 \mu\text{M}$ for quercetin, kaempferol, genistein and diadzein, respectively. Sulfonation of E_2 by sulfotransferases present in rat liver cytosol was also inhibited with IC_{50} s ranging from 0.8 to $2.2 \mu\text{M}$ (table 1). Flavonoids tested also inhibited DHEA sulfonation by SULT2A1; however, IC_{50} s were appreciably higher than those noted with SULT1A1 or rat liver cytosol ($> 30 \mu\text{M}$) (table 1). Since SULT 2A1 showed considerable sexual dimorphism in rats (Sharp *et al.* 1993), inhibition of this isoform may be more pronounced *in vivo* in

male rats that have approximately 15-fold lower amounts of this isoform in liver compared with amounts present in livers of female rats.

Flavones and isoflavones differed in their capacities to serve as substrates for the recombinant SULT1A1 and SULT2A1. The two flavonones, quercetin and kaempferol, were not substrates for either recombinant SULT1A1 or SULT2A1 or rat cytosolic sulfotransferases when tested at concentrations ranging up

Table 1. Inhibition of recombinant and cytosolic rat liver sulfotransferases by dietary flavonoids.

Flavonoid	SULT1A1	IC ₅₀ (μM) SULT2A1	Cytosol
Flavones			
Quercetin	0.29 ± 0.06	64 ± 13	1.2 ± 0.4
Kaempferol	0.41 ± 0.05	34 ± 11	1.7 ± 0.4
Isoflavones			
Genistein	1.1 ± 0.06	106 ± 18	0.8 ± 0.5
Daidzein	4.6 ± 0.45	116 ± 13	2.2 ± 0.4

IC₅₀s were calculated as described in the materials and methods and represent the amount (μM) of flavonoid required to inhibit 50% of maximum activity. Assays were performed using 25 μM E₂ as substrate for SULT1A1 and rat liver cytosol, and 25 μM DHEA as substrate for SULT2A1 using at least six different concentrations of the flavonoids in triplicate.

Values are means ± SEM.

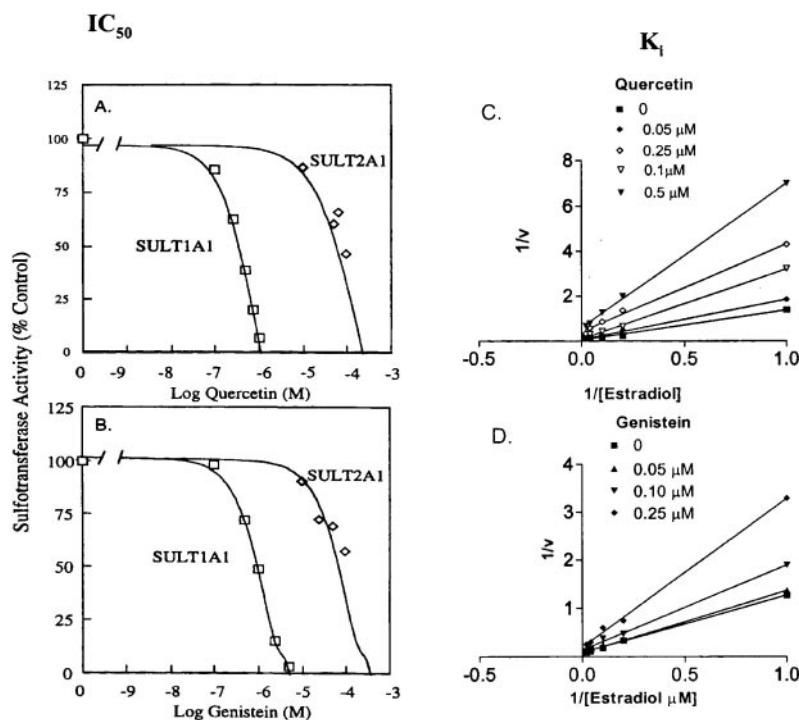


Figure 2. Effects of quercetin and genistein on recombinant SULT1A1 and SULT2A1 activities. (A, B) Comparison of the inhibitory potency (IC₅₀s) of SULT1A1 and SULT2A1 by quercetin (A) and genistein (B). (C, D) show Lineweaver-Burk plots from which K_i s were derived for quercetin (C) and genistein (D). Assays were performed as detailed in the materials and methods using 25 μM E₂ (for SULT1A1) and 25 μM DHEA (for SULT2A1). K_i s were determined using at least six concentrations of E₂ ranging from 0 to 30 μM.

to 0.3 mM. In contrast, the isoflavones G ($K_m=0.63\text{ }\mu\text{M}$) and diadzein ($K_m=0.58\text{ }\mu\text{M}$) were substrates for recombinant SULT2A1 but not for SULT1A1. Similarly, genistein ($K_m=17\text{ }\mu\text{M}$, $V_{\text{max}}=12\text{ nmol mg}^{-1}\text{ protein min}^{-1}$) and diadzein ($K_m=1.4\text{ }\mu\text{M}$, $V_{\text{max}}=1.3\text{ nmol mg}^{-1}\text{ protein min}^{-1}$) were substrates for rat liver cytosolic sulfotransferases. The relatively high affinity of isoflavones for SULT2A1 raises the possibility that sulfonation via this isoform is a determinant of their biological activity *in vivo*.

Effect of flavonoids on UDP-glucuronosyltransferase activity

E₂-metabolism by liver microsomal UGTs formed two metabolites: E₂-3 β -glucuronide (E₂-3 β G) and E₂-17 β -glucuronide (E₂-17 β G). All the flavonoids inhibited the formation of both E₂-3 β G and E₂-17 β G by rat liver microsomes. IC₅₀s for inhibition of E₂-3 β G were 43 and 51 μM for K and G, respectively, and 132 and 188 for Q and D, respectively. IC₅₀s for inhibition of E₂-17 β G were slightly higher, ranging from 106 to 260 μM for the four flavonoids (table 2).

Quercetin, kaempferol and genistein also inhibited the glucuronidation of 4-MU by liver microsomes; however, much higher amounts were required (IC₅₀s = 1130, 1550 and 860 μM , respectively). Diadzein did not shown any effects on 4-MU glucuronidation when tested up to 2 mM (table 3).

Table 2. Inhibition of E₂ glucuronidation by microsomes rat liver UGTs by dietary flavonoids.

Flavonoid	E ₂ -glucuronides (IC ₅₀ , μM)	
	E ₂ -3 β -glucuronide	E ₂ -17 β -glucuronide
Flavones		
Quercetin	132 $\pm$ 43	151 $\pm$ 49
Kaempferol	43 $\pm$ 4	106 $\pm$ 10
Isoflavones		
Genistein	51 $\pm$ 5	261 $\pm$ 20
Daidzein	188 $\pm$ 56	236 $\pm$ 41

IC₅₀s were calculated as described in the materials and methods and are the amount (μM) of flavonoid required to inhibit 50% of maximum activity. Assays were performed using 0.1 mM E₂ as substrate for rat microsomal UGTs and at least six different concentrations of the flavonoids in triplicate.

Values are means $\pm$ SEM.

Table 3. Inhibition of 4-MU glucuronidation by microsomes rat liver UGTs by dietary flavonoids.

Flavonoid	4-methylumbelliferyl glucuronide formed IC ₅₀ (μM)
Flavones	
Quercetin	1130 $\pm$ 170
Kaempferol	1550 $\pm$ 150
Isoflavones	
Genistein	860 $\pm$ 180
Daidzein	NA

IC₅₀s were calculated as described in materials and methods and determined and were the amount (μM) of flavonoid required to inhibit 50% of maximum activity. Assays were performed using 0.5 mM 4-MU as substrate for rat microsomal UGTs and at least six different concentrations of the flavonoids in triplicate.

NA, no activity when tested up to 2 mM. Values are means $\pm$ SEM.

Effect of flavonoids on sulfatase and β -glucuronidase activities

None of the flavonoids tested inhibited the desulfonation of [^3H]-oestrone sulfate by rat liver microsomes when tested up to 0.3 mM. The kinetic parameters for the substrate [^3H]-oestrone sulfate by liver microsomes were: $K_m = 7.1 \mu\text{M}$ and $V_{\max} = 1.1 \text{ nmol mg}^{-1} \text{ min}^{-1}$. Similarly, the hydrolysis of 0.5 mM 4-MUG via β -glucuronidase was not inhibited by any of the compounds tested (data not shown).

Discussion and conclusions

Cytosolic sulfotransferases are important enzymes involved in phase II metabolism of a variety of xenobiotics and important endogenous compounds including steroids, thyroid hormones and catecholamines (Coughtrie *et al.* 1998, Kester *et al.* 2000). These enzymes transfer a sulfonyl moiety from the cofactor 3'-phospho-adenosine-5'-phosphosulfate to hydroxyl, amino, sulfhydryl and N-oxide groups of their substrates (Glatt *et al.* 2000). Sulfonation is generally considered a detoxification process and the ultimate terminal enzymatic step in the bioactivation of procarcinogens, promutagens and drugs (Kauffman *et al.* 1994). Furthermore, their activity can be reversed through the hydrolytic activity of the arylsulfatases. Thus, initial experiments were directed at characterizing the sulfonation of E_2 and DHEA, and the interaction of various isoflavones with these reactions by rat recombinant and cytosolic sulfotransferases. Effects of the flavonoids on sulfotransferases were compared with their actions on UGTs, β -glucuronidase and sulfatase activities.

Results presented above show that dietary flavonoids are potent inhibitors of phenolsulfotransferases, SULT1A1. The potency of the inhibition noted with quercetin > kaempferol > genistein > diadzein suggests that inhibition of SULT1A1 by flavonoids is related to the number and possibly the position of hydroxyl groups in their molecule (figure 1). The potency of inhibition of SULT1A1 decreases four to 16 times when there is a bulky substituent in the position 3 as in genistein and diadzein that also have fewer hydroxyl groups than quercetin and kaempferol. In contrast to inhibition of SULT1A1, inhibition of SULT2A1 by flavonoids did not show any correlation with the number or position of hydroxyl groups in their molecules and they are at least 100 times less potent compared to their inhibition of SULT1A1. Inhibition of cytosolic sulfotransferases by the four flavonoids did not follow the same pattern of inhibition observed with the recombinant enzymes. The reason for this is not clear but is likely related to the presence of multiple isoforms of SULTs as well as other binding proteins present in rat liver cytosol.

Glucuronidation of E_2 , another important pathway of detoxification was also reduced by flavonoids. Kaempferol ($\text{IC}_{50} = 43 \mu\text{M}$) and genistein ($\text{IC}_{50} = 51 \mu\text{M}$) were the most potent inhibitors of formation of E_2 -3 β G. The formation of E_2 -17 β G was also inhibited by the flavonoids; however, IC_{50} s were three times greater than those noted for inhibition of E_2 -3 β glucuronide formation. In contrast to patterns of inhibition observed with recombinant SULT1A1, no correlation with number or position of hydroxyl groups in the molecule is seen for the inhibition of glucuronosyltransferase(s) present in microsomes. A bulky substituent at position 3 seems to reduce the ability of isoflavonoids (genistein and diadzein) to reduce formation of E_2 -17 β G. It is unknown which isoform of UGT glucuronidates positions 3 and 17

of E₂. In humans, E₂-3βG is formed by UGT1A1 (Senafi *et al.* 1994, Fisher *et al.* 2000) whereas E₂-17βG is formed by several enzymes including UGT2B (Gall *et al.* 1997, Williams *et al.* 2002).

Overall, the effects of the flavonoids on SULTs are comparatively more potent than the effects observed on glucuronosyltransferase(s) or the hydrolytic enzymes, sulfatase and β-glucuronidase. Quercetin, genistein and diadzein have been shown previously to inhibit competitively human liver P-PST (IC₅₀ about 0.1 μM) (Walle *et al.* 1995, Eaton *et al.* 1996) and human platelet PST with K_is ranging from 0.1 to 0.3 μM (Ghazali and Waring 1999). Thus, the potent non-competitive inhibition of SULT1A1 by dietary flavonoids may be potentially beneficial since sulfotransferases that metabolize E₂ also catalyse the bioactivation of the cooked food mutagens and procarcinogen N-hydroxy-2 amino-1 methyl-6-phenylimidazo-[4,5-b] pyridine and its subsequent binding to DNA (Lewis *et al.* 1998). Furthermore, SULT1A1 has been implicated in the bioactivation of certain procarcinogens including heterocyclic amines and polycyclic aromatic hydrocarbons (Miller 1994, Glatt *et al.* 1997, Nowell *et al.* 2000). In addition, the expression of SULT1A1 that is not expressed in normal human breast is present in significant amounts in neoplastic cells, suggesting that the gene encoding this protein is activated at some stage of the neoplastic transformation (Sharp *et al.* 1994, Pasqualini and Chetrite 1999). High levels of oestrogen sulfate found in breast tumour cells are associated with high expression of E₂-sulfotransferase (Qian *et al.* 1998, Otake *et al.* 2000).

The relatively low concentrations of flavonoids found to inhibit SULT1A1 *in vitro* are relevant to concentrations that may occur in rat liver and other target tissues containing this isoform of sulfotransferase. The total content of flavonoids in standard rat chow ranges from 500 to 1000 ppm based on technical information from various suppliers. In a study of the influence of genistein on chemically induced mammary tumours in rats (Fritz *et al.* 1998), it was found that addition of 250 ppm (0.025%) of genistein to a purified diet devoid of phytoestrogens resulted in serum concentrations of total genistein ranging from 0.5 to 2 μM in lactating rats and their off spring. This isoflavone was concentrated in milk of the dams where it reached a concentration of about 4 μM. These values for concentrations of genistein noted *in vivo* in rats fed known amounts of genistein are close to IC₅₀s reported above for inhibition of SULT1A1 by the flavonoids tested. Thus, inhibitory concentrations of flavonoids reported above for SULT1A1 may approximate concentrations encountered in tissues under physiological conditions. Further studies are clearly warranted to determine whether the activities of sulfotransferases are altered pharmacologically *in vivo* by plant flavonoids, and whether this action alters the course of mammary tumours.

Acknowledgements

Work was supported by NHIS Grant No. ES 05052.

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***S*-oxidation of *S*-methyl-esonarimod by flavin-containing monooxygenases in human liver microsomes**

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Received 2 May 2003

1. Studies using human liver microsomes and recombinant human cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) were performed to identify the enzymes responsible for the metabolism of *S*-methyl-esonarimod (M2), an active metabolite of esonarimod (KE-298, a novel antirheumatic drug).

2. *S*-oxidative activities of M2 significantly correlated with those of methyl *p*-tolyl sulfide, a specific substrate of FMOs, as tested using 10 different human liver microsomes ($r^2 = 0.539$, $p < 0.05$). Thermal treatment of microsomes reduced the *S*-oxidative activity in the absence of the NADPH-generating system at 45°C for 5 min. However, methimazole, a known competitive substrate of FMOs, was a weak inhibitor of the *S*-oxidation in liver microsomes.

3. Recombinant human FMO1 and FMO5 produced M3 in greater quantities than recombinant human FMO3. The *S*-oxidation of M2 by recombinant human FMO5 was not appreciably inhibited in the presence of methimazole. In contrast, methimazole was effective in suppressing the catalytic activity of recombinant human FMO1 and FMO3.

4. The apparent K_m ($K_{m \text{ app}}$) for the *S*-oxidation of M2 in human recombinant FMO5 (2.71 μM) was similar to that obtained using human liver microsomes (2.43 μM).

5. The present results suggest that the *S*-oxidation of *S*-methyl esonarimod reflects FMO5 activity in the human liver because the recombinant FMO5 data match well with the human liver microsomal experiments.

Introduction

Esonarimod (KE-298) [($\pm$)-2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid], a novel antirheumatic drug, suppresses arthritis in various animal models by inhibiting the production of inflammatory cytokines (Kameo *et al.* 1988, Sakane *et al.* 1997, Takahashi *et al.* 1998).

In a previous study, esonarimod was rapidly and almost completely absorbed, was well distributed throughout the body and was mainly eliminated in the urine following oral administration to rats (Yoshida *et al.* 1996). In the plasma, unchanged esonarimod accounts for only a small amount of the drug and the main components were active metabolites (figure 1), deacetyl-esonarimod (M1) and *S*-methyl-esonarimod (M2) (Yoshida *et al.* 1996). M1, which is a primary metabolite, undergoes further methylation to form M2. M2 is deactivated by *S*-oxidation and 4-hydroxylation and excreted as M2 sulfoxide (M3) and 4-hydroxy-M2 (M4) into the urine, respectively (Yamaguchi *et al.* 2001).

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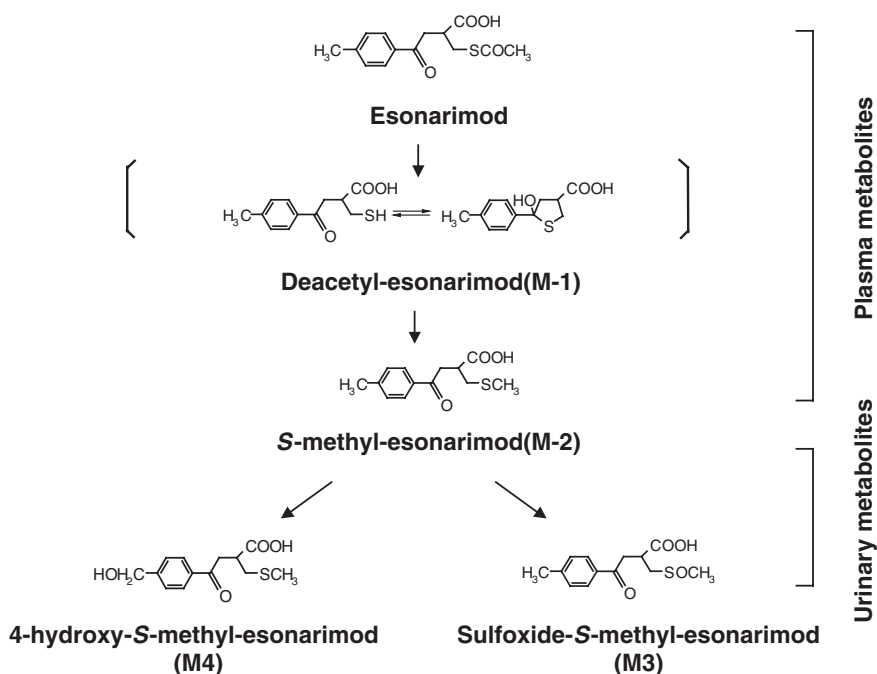


Figure 1. Metabolic pathways of esonarimod.

Cytochrome P450 (CYP, EC 1.14.14.1) and flavin-containing monooxygenase (FMO, EC 1.14.13.8) are the two main microsomal enzyme systems involved in the oxidation of xenobiotics (Damani and Nnane 1996). Of the many classes of drug-metabolizing enzymes, the CYP superfamily is the major enzyme system often involved in the rate-limiting step of drug biotransformation processes. An estimated 750 P450s have been sequenced, of which 55 are human isoforms (Venkatakrishnan *et al.* 2001). FMOs are microsomal enzymes that catalyse the NADPH- and O_2 -dependent oxidation of many nitrogen-, sulfur-, selenium- and phosphorus-containing compounds (Ziegler 1993). However, investigations of FMO have lagged behind investigations related to CYP. There are five known FMO isoforms, designated FMO1–5 (Lawton *et al.* 1994). The predominant FMO isoform in the adult human liver is FMO3. Based on data from various studies, it has been suggested that FMO3 makes the greatest contribution to FMO-mediated drug metabolism in adult human livers (Sadeque *et al.* 1993, Lomri and Cashman 1995, Phillips *et al.* 1995, Overby *et al.* 1997). FMO5 is a secondary FMO isoform in the human liver (Overby *et al.* 1997). FMO1 is the major FMO enzyme in the human foetal liver (Yeung *et al.* 2000), but it is hardly expressed in the adult human liver. FMO4 mRNA is present to a low degree in foetal and adult human livers (Dolphin *et al.* 1996). However, evidence for protein expression has apparently not been reported.

In vitro experiments with human liver microsomes and different CYPs and FMOs expressed in baculovirus-infected insect cells have indicated a role for CYP2C9 in the 4-hydroxylation of M2. In addition, it has already been found by the present authors that FMO is involved in M2 *S*-oxidation in rat liver microsomes (data not shown). The objective of the present study is to clarify the roles of

FMOs in human liver microsomes and, in particular, the importance of FMO5 in the S-oxidation of M2 *in vitro*.

Material and methods

Chemicals

S-methyl-esonarimod (M2) (($\pm$)-4-(4-methylphenyl)-2-methyl-thiomethyl-4-oxobutanoic acid, chemical purity > 99%) and sulfoxide -M2(($\pm$)-4-(4-methyl-phenyl)-2-methylsulfinylmethyl-4-oxobutanoic acid, chemical purity > 96%) were synthesized by Taisho Pharmaceutical Co., Ltd (Tokyo, Japan). NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Both methimazole and N-benzylimidazole were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Thiourea was purchased from Wako Pure Chemical Industries, Inc. (Osaka, Japan). Benzydamine was purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were of the highest quality commercially available.

Human liver microsomes and recombinant systems specifically expressing human CYP and FMO enzymes

Pooled microsomes from 15 human livers were purchased from Xeno Tech, LLC (Kansas City, KS, USA). Individual microsomes from 10 different human livers (coded HG3, HG6, HG23, HG30, HG42, HG43, HG56, HG66, HG70 and HG89) were purchased from GENTEST (Woburn, MA, USA). Recombinant systems from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9-Arg, 2C9-Cys, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 4A11 and FMO1, FMO3 and FMO5 were purchased from GENTEST and used according to the supplier's instructions. The microsomal preparations were stored at -80°C until used.

Incubation conditions with human liver microsomes

S-oxidation of M2 to form M3 was studied *in vitro* by incubation of M2 with human liver microsomes. The standard incubation conditions were selected based on results of preliminary experiments varying the concentrations of substrate and the microsomal proteins. The formation of M3 was a linear function of protein concentration ($0.05\text{--}0.15\text{ mg protein ml}^{-1}$) and incubation time (for at least 60 min). A typical incubation mixtures of 500 μl containing 50 μg microsomal protein in 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl₂ and an NADPH-generating system (1 mM NADP⁺, 10 mM glucose 6-phosphate, 2 IU ml⁻¹ glucose 6-phosphate dehydrogenase) were pre-incubated for 10 min at 37°C followed by initiation of the reaction with 10 μM M2 (complete system). The reaction was continued with constant shaking at 37°C.

The following FMO substrates were examined for their inhibitory effect on the metabolism of M2 by human liver microsomes: N-benzylimidazole, methimazole and thiourea. In a thermal inactivation study, thermal treatment microsomes were prepared by pre-incubating microsomes for 5 min at 45°C in the presence or absence of an NADPH-generating system. The remaining part of the procedure was performed as described above for the samples and a control activity of M2 S-oxidation was determined in the absence of any inhibitors and pre-incubations in the complete system. Benzydamine (100 μM) was used as a positive control substrate in these studies.

Blank reactions with zero incubation time were run in parallel to correct for any non-enzymatic S-oxidation. Microsomes were incubated with M2 for 15 min at 37°C. The reaction was terminated by adding 3 vols ethanol. The denatured protein was removed by centrifugation for 2 min at 9000g, and the supernatant was evaporated to dryness under conditions of reduced pressure. The residue was dissolved in methanol/water (1:1) and subjected to high-performance liquid chromatography (HPLC) analysis.

Incubation conditions with recombinant human CYP and FMO isoforms

Recombinant CYP (50 pmol) or FMO (100 μg protein) were incubated with M2 and the NADPH-generating system (1 mg ml⁻¹ NADP⁺, 1 mg ml⁻¹ glucose 6-phosphate, 0.4 IU ml⁻¹ glucose 6-phosphate dehydrogenase, 0.67 mg ml⁻¹ MgCl₂ 6H₂O) at 37°C for 30 min (complete system), respectively. Sample treatment and kinetic analysis were carried out in a similar fashion of incubation conditions with human liver microsomes. The inhibition study of recombinant human FMOs was performed by incubated methimazole with M2. Control incubations were also carried out in the absence of methimazole in the complete system.

HPLC assay

The metabolites of M2 were determined using HPLC. An HPLC system consisting of a model LC-6A pump, model SIL-10A autosampler, model CTO-6A oven and a model SPD-10A UV detector, chromatopac C-R7A (Shimadzu Co., Kyoto, Japan) was used. An analytical column TSKgel ODS-120A (4.6 $\times$ 150 mm, Tosoh Co., Tokyo, Japan) was used. The mobile phase consisted of 0.1 M

ammonium acetate (pH 4.2) and methanol (4:1 v/v) delivered at 1.0 ml min^{-1} . Column temperature was maintained at 40°C and detection was at 252 nm. The retention time for M3 was 17.6 min. However, each enantiomer of M3 was not further separated on HPLC. The assay system in the present paper satisfies the criteria of validation by Shah *et al.* (1992). The coefficient of variance (CV) of M3 in a lower limit of quantification ($0.05 \text{ }\mu\text{M}$) was $< 14.4\%$.

Correlation study

Correlation between marker enzyme activities (CYP and FMO) and the *S*-oxidation activities of M2 were studied using 10 different human liver microsomes. The specific catalytic activities of each CYP isoform or FMO for those microsomes were obtained from data sheets from GENTEST. Assays for the *S*-oxidation activities of M2 were done for those microsomes under the optimal conditions described above.

Kinetic analysis

Kinetic studies were achieved using pooled human microsomes and recombinant systems expressing human FMO5 in baculovirus-infected insect cells. To determine the kinetic parameters, M2 concentrations ranging from 1 to $100 \text{ }\mu\text{M}$ were used. Eadie–Hofstee plots were initially constructed to determine if the formation of metabolite was mono- or biphasic. The kinetic parameters estimates (apparent K_m and $V_{\max}$) were then calculated from an apparently linear portion in Eadie–Hofstee plots using a non-linear regression analysis. Assays for M2 *S*-oxidation were achieved using pooled human liver microsomes under the optimal conditions described above. Formation of M3 in human liver microsomes was consistent with single-enzyme Michaelis–Menten kinetics based on Eadie–Hofstee plots.

Incubation conditions with recombinant human FMO5 were carried out similar to those used for human liver microsomes. The standard incubation condition using recombinant human FMO5 had been initially determined in a preliminary study. The formation of M3 was a linear function of protein concentration ($0.05\text{--}0.15 \text{ mg protein ml}^{-1}$) and incubation time (for at least 45 min). M3 formation in recombinant FMO5 was consistent with a two-binding site model with a Michaelis–Menten component and a non-saturable low-affinity component, which was approximated by a linear function (equation 1):

$$V = V_{\max}S/(K_m + S) + PS, \quad (1)$$

where parameter P is an estimate of the intrinsic clearance of the low-affinity component.

Statistics

Results are given as means and standard deviation (SD). The significance of differences was evaluated using ANOVA with the SAS/STAT package v.6.1 (SAS Institute, Cary, NC, USA). Comparisons between the *S*-oxidation activity of control and inhibition treatments were assessed using Dunnett's multiple comparison test. The square of correlation coefficients was determined by least-squares linear regression analysis. The significance level for all tests was $p < 0.05$.

Results

Chemical inhibition study in human liver microsomes

The effects of *N*-benzylimidazole (1 mM), methimazole (1 mM) and thiourea (1 mM) on *S*-oxidation of M2 (M3) at $10 \text{ }\mu\text{M}$ in human liver microsomes are shown in figure 2. Screening of FMO substrates showed a statistically significant inhibition of M2 *S*-oxidation to $63.2 \pm 16.5\%$ and $67.8 \pm 5.0\%$ of control activity (no inhibition treatment) by methimazole and thiourea, respectively. Minimal inhibition was observed with *N*-benzylimidazole, an overall inhibitor of CYP ($80.6 \pm 8.7\%$ of control activity). Although the formation of benzydamine *N*-oxide from benzydamine ($100 \text{ }\mu\text{M}$) mediated by FMOs was fully inhibited in human liver microsomes by adding either methimazole or thiourea to the incubation (not detectable), neither methimazole nor thiourea gave complete inhibition of M3 formation.

Thermal inactivation study in human liver microsomes

Results of thermal treatment of microsomes are shown in figure 3. Thermal treatment in the presence or absence of the NADPH-generating system at 45°C

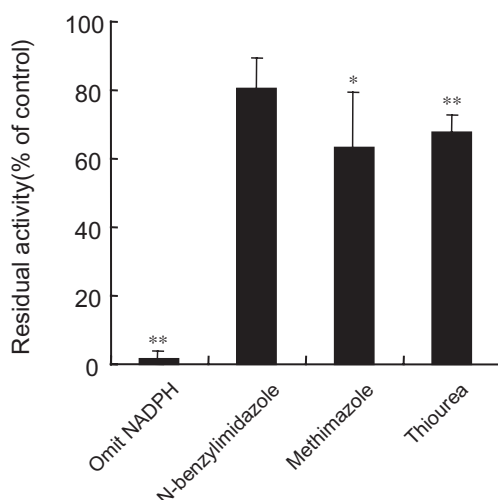


Figure 2. Effect of various treatments on M2 *S*-oxidation by human liver microsomes. Residual activities are a percentage of control (complete system) activity. The complete system contained 50 µg microsomal protein in 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl₂ and NADPH-generating system. M2 (10 µM) was incubated for 15 min at 37°C as described in the Material and methods. Results are the mean ± SD of triplicate experiments. **p* < 0.05, ***p* < 0.01.

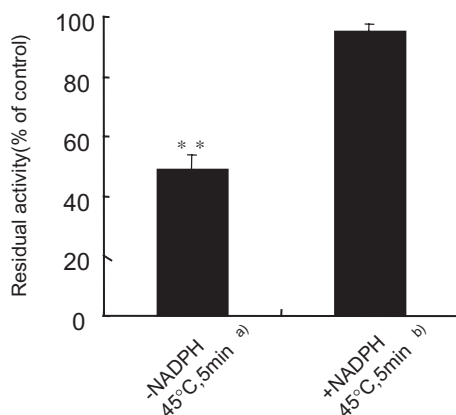


Figure 3. Effect of thermal treatment of human liver microsomes on M2 *S*-oxidation. Residual activities are a percentage of control (complete system) activity. The complete system contained 50 µg microsomal protein in 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl₂ and NADPH-generating system. M2 (10 µM) was incubated for 15 min at 37°C as described in the Material and methods. (a) Microsomes were heated at 45°C for 5 min in the absence of an NADPH-generating system; (b) microsomes were heated at 45°C for 5 min in the presence of an NADPH-generating system. Results are the mean ± SD of triplicate experiments. ***p* < 0.01.

for 5 min, inhibited by $95.1 \pm 2.4\%$ and $49.7 \pm 4.7\%$ of control activity, respectively.

Correlation study

The formation of M3 in 10 different human liver microsomes was determined. *S*-oxidation activities ranged from 213–440 pmol min⁻¹ mg⁻¹ protein in

Table 1. Correlation of *S*-oxidation with marker enzyme activities of CYPs or FMO in microsomes from 10 different human livers.^a

CYP isoforms or FMO	Metabolic reaction of substrate	Correlation coefficient (r^2) <i>S</i> -oxidation ^b
1A2	phenacetin <i>O</i> -deethylation	0.085
2A6	coumarin 7-hydroxylation	0.182
2B6	(<i>S</i>)-, mephenytoin <i>N</i> -demethylation	0.086
2C8	paclitaxel 6 α -hydroxylation	0.125
2C9	diclofenac 4'-hydroxylation	0.014
2C19	(<i>S</i>)-mephenytoin 4'-demethylation	0.105
2D6	bufuralol 1'-hydroxylation	0.120
2E1	chlorzoxazone 6-hydroxylation	0.029
3A4	testosterone 6 β -hydroxylation	0.236
4A	lauric acid 12-hydroxylation	0.000
FMO	methyl <i>p</i> -tolyl sulfide oxidation ^c	0.539*

^aMarker enzyme activities of individual CYPs and FMO were provided from GENTEST.

^bStatistical significant of correlation is denoted by * $p < 0.05$.

^cAssay was conducted at 0.8 mg ml⁻¹ protein in 0.05 M glycine buffer (pH 9.5) with an NADPH generating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate and 0.4 IU ml⁻¹ glucose 6-phosphate dehydrogenase), 3.3 mM MgCl₂, 1.2 mM diethylenetriaminepentaacetic acid, 0.5 mg ml⁻¹ Triton X-100 and incubated for 10 min.

individual human liver microsomes. Correlations between *S*-oxidation activities of M2 and marker enzyme activities (CYPs and FMOs) using 10 different human liver microsome preparations were examined (table 1). The *S*-oxidation activity of M2 showed only a significant correlation ($r^2 = 0.539$, $p < 0.05$) with methyl *p*-tolyl sulfide *S*-oxidation activity as a probe for FMO activity. However, there were no significant correlations ($r^2 = 0-0.236$) between the *S*-oxidation activity of M2 and any other metabolic activity of probe substrates.

Recombinant CYP and FMO isoforms study

Recombinant CYP and FMO isoforms expressed in baculovirus-infected insect cells were used to assess the potential for these enzymes to metabolize M2 (figures 4 and 5). No CYP isoforms exhibited any appreciable metabolic activities (< 10 pmol/30 min/pmol P450). In contrast, recombinant FMO1 had the highest metabolic activity, forming M2 sulfoxide (M3) (28.25 nmol/30 min mg⁻¹ protein). Recombinant FMO5 also had high metabolic activity forming M3 (5.45 nmol/30 min mg⁻¹ protein). However, recombinant FMO3 did not appreciably catalyse the M3 formation (0.77 nmol/30 min mg⁻¹ protein).

Inhibition study with recombinant human FMO isoforms

The effect of methimazole (1 mM) on *S*-oxidation of M2 (10 μ M) with recombinant human FMO isoforms are shown in figure 6. Methimazole almost completely inhibited ($\geq 97\%$) *S*-oxidation activity to 0–3% of the control (no inhibition treatment) in both human recombinant FMO1 and FMO3, but inhibited the *S*-oxidation activity to only 87% of the control with human recombinant FMO5. These results obtained from the inhibition studies with recombinant FMO1 or FMO3 were dissimilar to those with human liver microsomes.

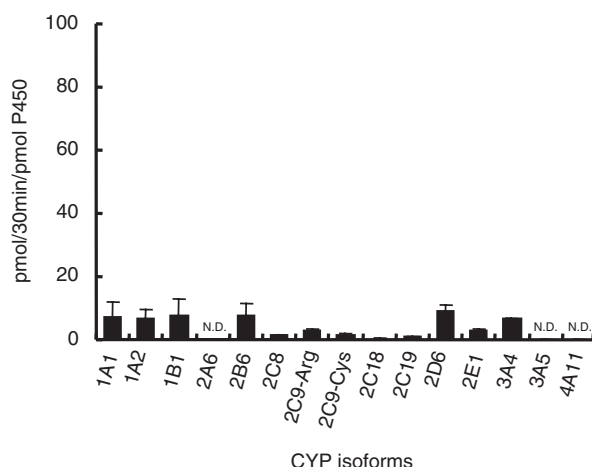


Figure 4. S-oxidation of M2 by recombinant human CYP isoforms. M2 concentration, 10 μ M; CYP contents, 50 pmol P450 ml^{-1} ; incubation time, 30 min. Results are the mean $\pm$ SD of triplicate experiments. N.D. < 0.05 μ M.

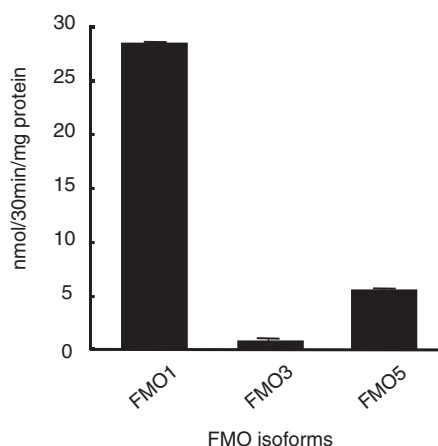


Figure 5. S-oxidation of M2 by recombinant human FMO isoforms. M2 concentration, 10 μ M; microsomal protein concentration, 0.2 mg ml^{-1} ; incubation time, 30 min. Results are the mean $\pm$ SD of triplicate experiments.

Kinetic studies

The enzyme kinetics of M2 S-oxidation by human liver microsomes and recombinant human FMO5 was then determined (figures 7 and 8). Using pooled human liver microsomes, a monophasic model described the kinetic formation of M2 S-oxide, with $K_{m \text{ app}} = 2.43 \mu\text{M}$ and $V_{\text{max app}} = 0.58 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$. The high-affinity component for M2 S-oxidation by expressed FMO5 had a $K_{m \text{ app}} = 2.71 \mu\text{M}$ and $V_{\text{max app}} = 0.37 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$. The high-affinity component was estimated to account for more than 95% of the intrinsic clearance. The $K_{m \text{ app}}$ for M2 S-oxidation in human liver microsomes was comparable with that obtained from the high affinity component in recombinant human FMO5.

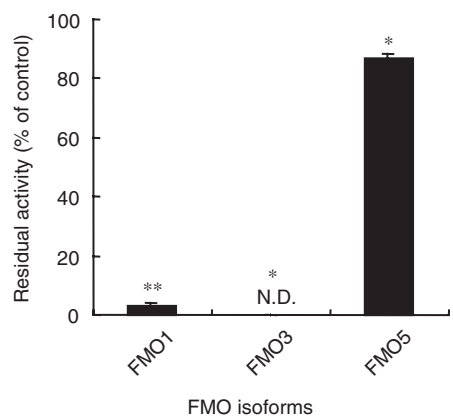


Figure 6. Effect of methimazole on *S*-oxidation of M2 by recombinant human FMO isoforms. M2 concentration, 10 μ M; microsomal protein concentration, 0.1 mg ml⁻¹; incubation time, 15 min. Results are the mean $\pm$ SD of triplicate experiments. N.D. < 0.05 μ M; * p < 0.05, ** p < 0.01.

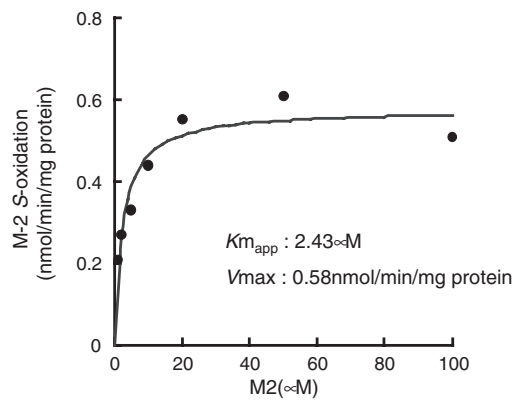


Figure 7. Michaelis–Menten kinetics of M2 *S*-oxidation as a function of M2 concentration in human liver microsomes. Each point is the mean of three experiments.

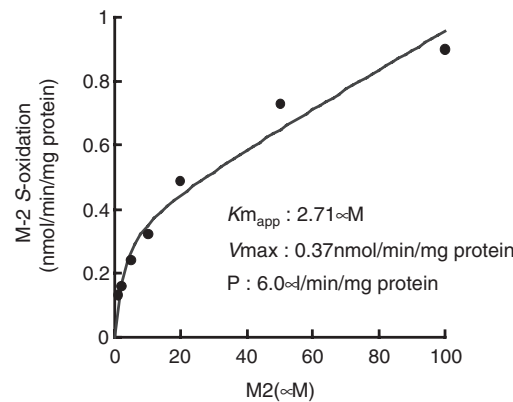


Figure 8. Michaelis–Menten kinetics of M2 *S*-oxidation as a function of M2 concentration in recombinant human FMO5. Each point is the mean of three experiments.

Discussion

The present studies have identified a primary role of FMO5 for the *S*-oxidation of M2 in human liver. *N*-benzylimidazole only weakly inhibited the *S*-oxidation of M2 in human liver microsomes. In addition, no significant correlation was noted between M2 *S*-oxidation and P450 substrate oxidation. However, the *S*-oxidation activity of M2 correlated significantly with the *S*-oxidation of methyl *p*-tolyl sulfide, a marker enzyme activity of FMO. The *S*-oxidation activity was inhibited in human liver microsomes after thermal treatment in the presence of the NADPH-generating system at 45°C for 5 min, although neither methimazole nor thiourea fully inhibited the *S*-oxidation activity in human liver microsomes. The present studies therefore suggest the participation of FMO in the *S*-oxidation of M2.

The results of Lang *et al.* (2000) demonstrated that benzydamine was metabolized to its *N*-oxide metabolite most efficiently by recombinant human FMO1 and FMO3. In the present experiments, the rate of benzydamine *N*-oxidation was fully inhibited in human liver microsomes by either adding methimazole or thiourea to the incubation. The human microsomal enzyme data using benzydamine did not match well with those using M2, which found poor congruence between benzydamine *N*-oxygenase activity and M2 *S*-sulfoxide activity in human liver microsomes. Recombinant human FMO1 and FMO5 catalyse the *S*-oxidation of M2 with high efficiency. In contrast, little formation of *S*-oxide from M2 was seen with recombinant human FMO3, a predominant FMO isoform in the adult human liver. Methimazole extensively inhibited *S*-oxidation activity by recombinant human FMO1 and FMO3, but little inhibition of the activity was confirmed using recombinant human FMO5 ($87.0 \pm 1.6\%$ of control activity). When inhibition experiments on *S*-oxidation of M2 were carried out using human liver microsomes ($63.2 \pm 16.7\%$ of control activity), the findings were almost comparable with those using recombinant FMO5. Overby *et al.* (1997) reported that FMO5 is marginally involved in the metabolism of methimazole, in agreement with the present study.

Methyl *p*-tolyl sulfide oxidase activities in recombinant FMO1, FMO3 and FMO5 were 16 900, 30 200 and 1200 pmol min⁻¹ mg⁻¹ protein, respectively (data from GENTEST). As to why the correlation coefficient between M2 *S*-oxidation and methyl *p*-tolyl sulfide *S*-oxidation is weak in human liver microsomes, FMO5 seems not to be fully responsible for methyl *p*-tolyl sulfide *S*-oxidation while M2 *S*-oxidation is mainly by FMO5. Some of the data related to M2 *S*-oxidation in the present study did not correspond to what would be expected for an enzyme. A possible explanation for this discrepancy may be due to the difference in involvement of each FMO isoform in the metabolism of other drugs that can potentially be metabolized by FMO systems.

The *S*-oxidation activity with human recombinant FMO1 was the highest among the three FMO isoforms. However, it has been reported that FMO1 is expressed only at low levels in human liver (Cashman 1995). Therefore, no appreciable *S*-oxidation activity of M2 by FMO1 would appear in the human liver. However, FMO1 may be involved in the *S*-oxidation of M2 in the human kidney (*in vivo*), where FMO1 is a predominant isoform. Although M2 *S*-oxidation is not an FMO5 isoform-specific reaction, consideration of the tissue distributions of human FMO1 and FMO5 supports the expectation that the

metabolic capacity of hepatic FMO5 would be a much greater contributor to form the *S*-oxide *in vivo* than extrahepatic FMO1.

It has been reported that the major FMO enzymes in human liver are FMO3 and FMO5 (Overby *et al.* 1997), with FMO3 content consistently greater than of FMO5, and the ratio of FMO3:FMO5 varied from 2:1 to 10:1 (Sadeque *et al.* 1993). Despite FMO5 being one of the major FMO enzymes in the human liver, there is less known about FMO5 than any other FMO isoforms. No specific substrate for FMO5 has been reported and information on the substrate specificity of FMO5 in the human liver is limited.

Experiments were also undertaken to determine if M2 is oxidized by FMO5 in the human liver, and the $K_{m\text{ app}}$ for M2 *S*-oxidation was compared between human liver and recombinant human FMO5. A monophasic *S*-oxidation formation kinetic value was observed for M2 in the human liver; hence, it was suggested that one enzyme is involved in M2 *S*-oxidation. It is of interest that formation of M3 from M2 in recombinant FMO5 was consistent with a process described by a biphasic model. This has been described for biotransformation of CYP substrates such as naproxen and phenytoin (Korzekwa *et al.* 1998, Giancarlo *et al.* 2001). In the present study, the $K_{m\text{ app}}$ obtained from the high-affinity component in recombinant human FMO5 was similar to that seen for human liver microsomes, and accordingly the possibility that FMO5 is involved in *S*-oxidations is very likely.

The present studies suggest that the biotransformation of M2 *S*-oxidation in human liver microsomes is mediated by FMOs and one of the most probable major contributor is FMO5.

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‘Open access’ generic method for continuous determination of major human CYP450 probe substrates/metabolites and its application in drug metabolism studies

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Received 17 July 2003

1. An ‘open access’ generic high-performance liquid chromatography method was developed for different combination sets each containing specific cytochrome P450 probe substrate and the corresponding metabolite. Method development, optimization and validation were carried out with the following combinations: phenacetin + paracetamol + internal standard (IS, celecoxib), bufuralol + hydroxybufuralol + IS, testosterone + 6 β -hydroxytestosterone + IS, chlorzoxazone + 6-hydroxychlorzoxazone + IS, coumarin + 7-hydroxycoumarin + IS, tolbutamide + hydroxytolbutamide + IS, and diazepam + desmethyldiazepam + IS.

2. The assay procedure involved a simple one-step liquid/liquid extraction followed by reverse phase chromatography (Inertsil ODS 3V column) employing a ternary gradient system and the eluate was monitored by a photodiode array/fluorescence detector. The standard curve for each compound, in the concentration range 0.1–10 $\mu\text{g ml}^{-1}$, in various sets was linear ($r^2 > 0.99$) and absolute recoveries of all analytes were $> 90\%$. The lower limit of quantification was 0.1 $\mu\text{g ml}^{-1}$. The intraday precision and accuracy in the measurements of quality control were $< 15\%$ relative standard deviation and $< 15\%$ deviation from nominal values, respectively.

3. Each combination set was tested with individual chemical inhibitors (furafylline, quinidine, ketoconazole, disulfiram, diethyldithiocarbamate, sulphaphenazole and tranylcypromine) and all analytes were well resolved. Overall, the assay is simple, uses conventional instrumentation and provides a scope to analyse all cytochrome P450 combination sets continuously. The application of the method in the cytochrome P450 liability screen of novel compounds is also presented.

Introduction

Recent clinical experiences with several compounds have highlighted the importance of conducting metabolism-mediated drug–drug interactions in the evaluation of new drugs at an early stage of the discovery process. Potent drug–drug interactions can result in serious side-effects as evidenced by literature data (Herings *et al.* 1993, Honig *et al.* 1993, Flockhart *et al.* 2000, Michalets and Williams 2000). Consequently, preclinical (*in vitro*) and some clinical (*in vivo*) interaction studies are now important components of the drug candidate selection process (Honig *et al.* 1993, Peck *et al.* 1993, Ball *et al.* 1997, Ko *et al.* 1997). Most of the drug–drug interactions that have been clinically observed are due to either inhibition or induction of cytochrome P450 (CYP) enzymes participating in the

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metabolism of the drug by the co-administered drug or vice versa. With the increased understanding of the role of human CYP enzymes and availability of individual recombinant CYP enzymes, *in vitro* hepatic CYP inhibition screens are now being routinely used in the drug evaluation process.

Major human CYP enzymes that are particularly important in this context are CYP3A4, 2D6, 2C9, 1A2, 2C19, 2A6 and 2C8. One of the routinely used methods to assess the activities of these enzymes is by measuring the specific metabolite formation when the CYP enzyme is incubated with its corresponding substrate. The most challenging part in these studies is the bioanalytical methodology to measure simultaneously the substrates and metabolites from the incubation matrix. Although high throughput methodologies (Crespi *et al.* 1997, Ayrton *et al.* 1998, Moody *et al.* 1999) employing hyphenated techniques such as fast gradient LC-MS/MS, ion source Fourier-transform mass spectrometer, etc. are available for this purpose (Li *et al.* 1999, Scott *et al.* 1999), until today no single high-performance liquid chromatography (HPLC) method has been reported to analyse simultaneously and continuously all substrates and metabolites. The structural diversity of CYP probe substrates (figure 1) currently in use has led to a number of different HPLC methods for the analysis of substrate(s) and metabolite(s) involved for each major CYP enzyme (Chauret *et al.* 1997, Ayrton *et al.* 1998, Hickman *et al.* 1998, Busby *et al.* 1999, Easterbrook *et al.* 2001). In general, due to the diversity of the HPLC methods reported, the requirement of different skills is mandatory for the proper execution of this challenging task. Also, analysis entails reconfiguring HPLC systems to accommodate the change of detectors or the alteration of mobile phase composition or the switching of column(s) for analysing each combination set (substrate/metabolite). Hence, the available HPLC methods may not be suitable for continuous analysis of all combination sets in sequence. In this context, there is an urgent need of a generic 'open access' assay capable of analysing major CYP probe substrates that would simplify analysis, increase relative throughput and allow for easy handling of the system without the need for a fulltime dedicated analyst. In addition, such an assay would benefit immensely by employment of a 'universal' system with adequate sensitivity, adoptability and selectivity.

A generic 'open access', new, simple HPLC method is presented for the simultaneous determination of probe CYP substrate(s) and their metabolite(s) from the protein matrix. This method has been successfully employed in measuring the functional activities of CYP enzymes while evaluating the inhibitory potential of new chemical entities. Moreover, the present method offers the advantage of the use of conventional instrumentation, ease of handling and allows continuous analysis of different combination sets in a single method.

Materials and methods

Chemicals, enzymes and reagents

Phenacetin, paracetamol, diazepam, tolbutamide, testosterone, coumarin, 7-hydroxycoumarin, carbamazepine, rifampicin, metoprolol, diethyldithiocarbamate, tranlycypromine, orphenadrine, glizalazide, diclofenac, resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, 4-methylpyrazole, quinidine and dimethylsulfoxide were obtained from Sigma Chemical Co. (St Louis, MO, USA). Disulfiram was procured from Aldrich Chemical Co. (Milwaukee, WI, USA). Bufuralol, hydroxybufuralol, chlorzoxazone, 6-hydroxychlorzoxazone, ketoconazole, 6 β -hydroxytestosterone, furafylline and sulphaphenazole were procured from Ultra Fine Chemicals (Manchester, UK). Celecoxib, dextrophan,

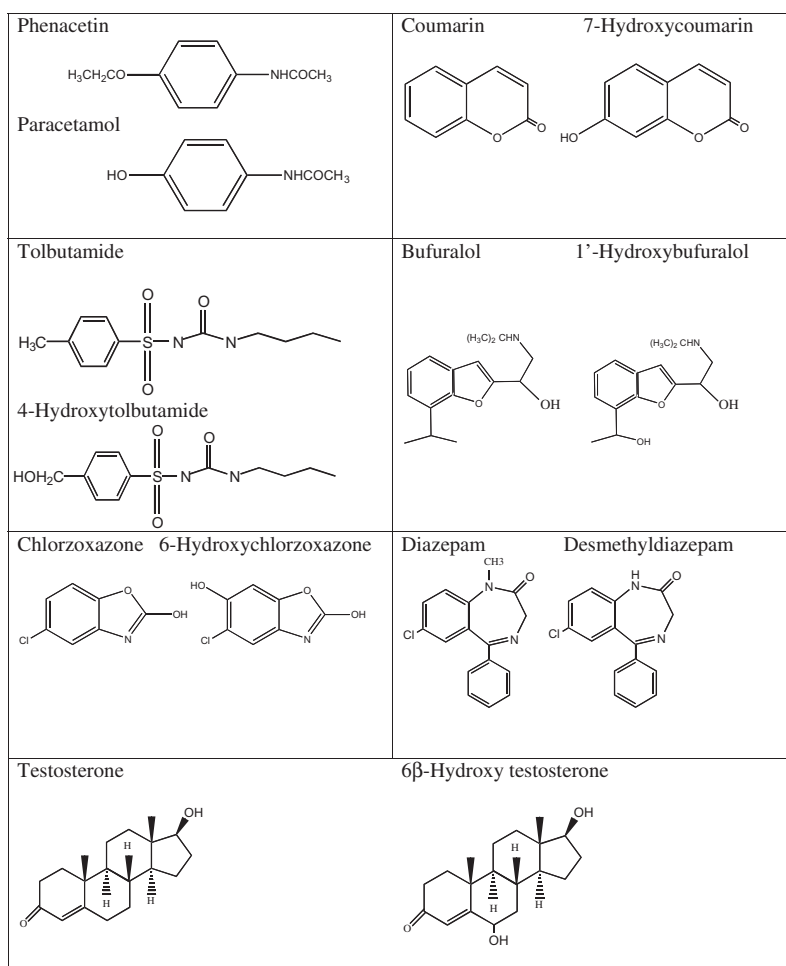


Figure 1. Structural diversity of the probe substrates/metabolites used.

desmethyldiazepam, 4-hydroxytolbutamide, hydroxycelecoxib and dehydronifedipine were synthesized by the Chemistry Group, Discovery Research, Dr Reddy's Laboratories Ltd (DRL, Hyderabad, India) and were characterized using chromatographic and spectral techniques by the Analytical Group, Discovery Research, DRL. Dextromethorphan, nifedipine, omeprazole and fluoxetine were procured from Bulk Actives (DRL, Hyderabad). All chemicals, standards and reagents used had purities > 99%. Methanol, acetonitrile, ethyl acetate (all HPLC grade), potassium dihydrogen orthophosphate and ammonium acetate (analytical grade) were obtained from E. Merck India Ltd (Mumbai, India). Ethylene diamine tetraacetic acid disodium salt (EDTA) and phosphoric acid (analytical grade) were obtained from Qualigens (Mumbai, India). All aqueous solutions including the buffer for the HPLC mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. Reduced NADPH+H⁺ was procured from SRL Ltd (Mumbai, India). Human liver microsomes (protein concentration 20 mg ml⁻¹) and human lymphoblast expressed CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 were procured from Gentest Corporation (Woburn, MA, USA).

Standard solutions

Primary stock solutions (1 mg ml⁻¹) of probe substrates (drugs) and metabolite reference standards were prepared in methanol or dimethylsulfoxide depending on the probe substrate and were stored at 4°C until use. Internal standard (IS, celecoxib) stock solution (1 mg ml⁻¹) was prepared in methanol. All secondary stock solutions at various concentrations were freshly prepared in methanol, as and when required for the purpose of chromatographic optimization, preparation of calibration, quality control (QC) samples and testing the application of the method.

Chromatographic conditions

The HPLC system consisted of a Waters 2695 Alliance separation module attached with a Waters® 2996 photodiode array (PDA) detector. A C18 Inertsil® ODS 3V column (4.6 × 250 mm, 5 µm, GL Sciences, Inc., Tokyo, Japan) was used for the analysis. The ternary mobile phase system consisted of reservoir A (0.01 M ammonium acetate buffer, pH 5.0:acetonitrile; 90:10), reservoir B (0.01 M ammonium acetate buffer, pH 5.0:acetonitrile; 5:95) and reservoir C (0.01 M ammonium acetate buffer, pH 5.0:methanol; 5:95) were run as per the gradient program (table 1) with a total flow rate of 1.0 ml min⁻¹ through the column to elute the analytes. The eluate was monitored by the PDA detector (scan range 200–400 nm) and data integration was carried out by Millennium³² software (v.4). Chromatograms were extracted from PDA data depending upon the analyte spectra to obtain the quantitative information. For the quantitation of dextrophan, dextromethorphan, resorufin, 7-ethoxyresorufin, 7-pentoxeresorufin, bufuralol and hydroxybufuralol, a fluorescence detector was connected in series with PDA detector.

Calibration standards

Linearity and limit of quantitation. The calibration standards were prepared by spiking the analyte working solutions (substrate and metabolite) into an incubation mixture (containing human liver microsome protein 0.5 mg ml⁻¹) to give concentrations of 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 5.0 and 10 µg ml⁻¹ and to each of these samples 1 µg IS was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 4.0 ml ethyl acetate, the contents were vortexed for 2 min; followed by centrifugation for 10 min at 3000 rpm on a tabletop centrifuge (Remi Instruments). The clear organic layer (3.2 ml) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (N-evap, Organomation, Berlin, MA, USA). The residue was reconstituted in 200 µl mobile phase (A:B, 1:1) and 100 µl of this solution were injected into the HPLC. The lower limit of quantitation was defined as the lowest concentration at which the measure of precision (i.e. relative standard deviation, RSD) and accuracy (i.e. per cent deviations from the nominal concentration) was less than 20%. Calibration samples over the entire curve range were run in triplicate for each combination set.

Accuracy, precision and recovery. Quality control (QC) samples for determination of precision and accuracy were prepared by appropriately spiking into incubation mixture (containing human liver microsome protein 0.5 mg ml⁻¹) at known concentrations of substrate(s)/metabolite(s) (0.2, 0.4, 1 and 5 µg ml⁻¹). At each QC level, four replicates were processed and analysed to calculate the RSD and per cent relative deviation from nominal concentrations. Analytes of representative combination sets at the spiked QC levels were directly analysed in the reaction mixture (in the absence of protein matrix). These neat samples were used as absolute recovery standards for calculating the extraction recoveries.

Application in in vitro metabolism studies: assessment of CYP inhibitory potential of new drug versus standard chemical inhibitors

Incubations were performed with each representative CYP substrate (table 2) with lymphoblast expressed specific human CYP enzymes. The incubation mixture contained 430 µl 100 mM potassium phosphate buffer (pH 7.4), 40 µl protein (0.8 mg) and 5 µl of either combination of substrate, substrate + inhibitor, substrate + test compound (in case of test), or methanol (in case of blank). The reaction was initiated by adding 25 µl NADPH + H⁺ (2 mM) after pre-incubation at 37°C for 1 min. The final volume of incubation mixture was kept at 500 µl. After a predetermined incubation time (time varies from substrate to substrate), the reaction was terminated by the addition of 4.0 ml ethyl acetate. IS (1 µg) was added and contents were vortex mixed and centrifuged at 3000 rpm for 10 min.

Table 1. Gradient-time program for the HPLC method with a ternary mobile phase system.

Time (min)	Flow rate (ml min ⁻¹)	A (%)	B (%)	C (%)
0	1.0	100	–	–
2	1.0	90	–	10
9	1.0	50	20	30
12	1.0	50	25	25
25	1.0	30	50	20
28	1.0	10	85	5
35	1.0	10	85	5
36	1.0	100	–	–
40	1.0	100	–	–

A: 0.01 M ammonium acetate (pH 5.0):acetonitrile = 90:10; B: 0.01 M ammonium acetate (pH 5.0):acetonitrile = 5:95; C: 0.01 M ammonium acetate (pH 5.0):methanol = 5:95.

Table 2. Combination sets for various CYP enzymes and chromatographic details.

No.	CYP enzyme	Combination sets substrate/metabolite/inhibitor	HPLC parameters	
			RT (min)	Detection UV/Fl (Ex/Em)
1	1A2	Phenacetin	14	UV @ 245 nm
		Paracetamol	7	UV @ 245 nm
		Furafylline	12	UV @ 272 nm
2	2A6	Coumarin	14	UV @ 276 nm
		7-Hydroxycoumarin	12	UV @ 324 nm
		Diethyldithiocarbamate	23	UV @ 242 nm
3	2C9	Tolbutamide	19	UV @ 229 nm
		Hydroxytolbutamide	12	UV @ 229 nm
		Sulphaphenazole	13	UV @ 265 nm
4	2C19	Diazepam	26	UV @ 230 nm
		Desmethyldiazepam	23	UV @ 230 nm
		Tranlycypromine	8	UV @ 264 nm
5	2D6	Bufuralol	15	UV @ 246 nm; Fl: 280/300 nm
		Hydroxybufuralol	10	UV @ 246 nm; Fl: 280/300 nm
		Quinidine	12	UV @ 280 nm; Fl: 280/300 nm
6	2E1	Chlorzoxazone	16	UV @ 281 nm
		6-Hydroxychlorzoxazone	11	UV @ 297 nm
		Disulfiram	31	UV @ 230 nm
7	3A4	Testosterone	23	UV @ 244 nm
		6 β -Hydroxytestosterone	15	UV @ 239 nm
		Ketoconazole	29	UV @ 294 nm

UV, ultraviolet; RT, retention time; Fl, fluorescence; Ex, excitation; Em, emission.

The clear organic layer (3.2 ml) was drawn and evaporated to dryness under nitrogen gas at 40°C. The residue was reconstituted in 200 μ l mobile phase (A:B, 1:1) and 100 μ l of this solution were injected onto HPLC. The metabolite formed was measured with all substrates based on the reference standard comparisons.

For the assessment of inhibitory effects of test compounds, incubations were carried out in a similar manner as mentioned above in the presence of probe substrates (at its K_m) for their respective enzymes (namely CYP1A2, 2A6, 2C9, 2D6, 2E1 and 3A4) and at concentrations ranging from 1 to 100 μ M for the novel compounds. At the end of incubation, sample(s) were extracted and analysed for the per cent reduction in formation of metabolite(s) in presence of test compounds.

Miscellaneous metabolism standards in in vitro metabolism studies

To elucidate the broader application and suitability of the present method, about 10 different standard drugs, which are known to be probe substrates/inhibitors, were additionally tested in the present method. Retention times and UV/fluorescence maxima of substrates and inhibitors as well as for the chosen novel compounds to screen for CYP450 enzyme drug interaction are given in table 3.

Results

Specificity and chromatography

Figure 2 shows typical chromatograms of blank and various combination sets. Chromatograms clearly indicate that peaks are well resolved, corresponding to each of the analyte(s) present in the combination set and quite amenable to run the different combination sets continuously without having to change either the column or the associated HPLC conditions. Extracted blank and various combination sets from the protein matrix indicated that chromatograms from blank samples did not show any endogenous peaks at the retention time of analytes from all combination sets. Chromatograms for all practical purposes were extracted either by PDA (based on UV absorption maximum) or by fluorescence detection in each of the combination set. Retention time and UV/fluorescence maxima of substrates, metabolites and

Table 3. Chromatographic properties of metabolism standards.

S. no	Probe substrate/metabolite	HPLC parameters	
		RT (min)	Detection UV/Fl (Ex/Em)
1	Dextromethorphan	14	UV @ 280 nm, Fl: 280/300 nm
2	Dextrorphan	10	UV @ 280 nm, Fl: 280/300 nm
3	Carbamazepine	17.5	UV @ 284 nm
4	Metoprolol	10	UV @ 223 nm
5	Omeprazole	17	UV @ 300 nm
6	Rifampicin	22	UV @ 237 nm
7	Nifedipine	22.5	UV @ 237 nm
8	Diclofenac	22	UV @ 281 nm
9	Orphenadrine	15.9	UV @ 264 nm
10	Gliclazide	22	UV @ 230 nm
11	Fluoxetine	19	UV @ 228 nm
12	Celecoxib*	30	UV @ 250 nm
13	Resorufin	13	UV @ 221 nm, Fl: 525/580 nm
14	7-Pentoxoresorufin	33	UV @ 221 nm, Fl: 525/580 nm
15	7-Ethoxoresorufin	24	UV @ 221 nm, Fl: 525/580 nm
16	DMPK-1	15	UV @ 250 nm
17	DMPK-2	15.6	UV @ 252 nm
18	DMPK-3	33.9	UV @ 278 nm
19	DMPK-4	27.9	UV @ 222 nm

*Used as an internal standard. UV, ultraviolet; Fl, fluorescence; Ex, excitation; Em, emission; DMPK-1–4 are compounds currently under development in the authors' laboratories.

inhibitors are given in table 2. HPLC profiles for each combination set clearly indicated the specificity in the measurement of analyte(s).

Versatility, suitability and robustness of the method were checked with various other C₁₈ columns from various manufacturers, i.e. Kromasil C18 (250 × 4.6 mm, 5 µm, Hichrom, Theale, Berkshire, UK) and Supelcosil LC-318 (250 × 4.6 mm, 5 µm, SUPELCO, Bellefonte, PA, USA) by running four replicates of each combination set under identical HPLC conditions. It was found that chromatographic resolution, selectivity and sensitivity were similar with all the columns (data not shown).

Calibration curves for analytes

Calibration samples, across the concentration range 0.1–10.0 µg ml⁻¹, were run in triplicate for all combination sets for constructing the calibration curve. The linearity relationship was established between peak area ratios of the analyte (separately for the probe substrate and metabolite) to IS on the y-axis against analyte concentration on the x-axis and coefficients of their relationship were obtained by regression analysis. Coefficients of regression for all analytes are given in table 4. Representative chromatograms of the calibration samples at 0.1 µg ml⁻¹ (i.e. LLOQ) for all combination sets extracted at the wavelength maxima of metabolites are shown in figure 3. Calibration curve and detection capabilities indicated that the reported procedure was quite amenable to carry out metabolism studies and both substrate and metabolite concentrations could be detectable even below the *K_m* of the respective reactions (Yuan *et al.* 2002).

Accuracy, precision and recovery

Recoveries calculated based on the absolute standards were > 90% with all probe substrates and metabolites at QC levels and IS at 10 µg ml⁻¹. Accuracy and

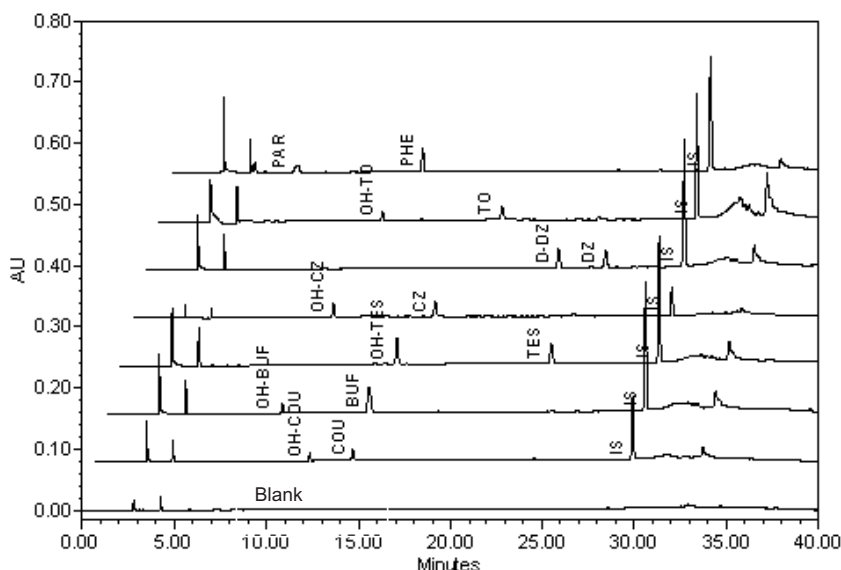


Figure 3. Overlay chromatograms of a combination set of CYP probe substrate, metabolite and internal standard spiked in an incubation mixture at LLOQ along with a blank incubation mixture processed without any standards. BUF, bufuralol; OH-BUF, 1-hydroxybufuralol; IS, internal standard; PAR, paracetamol; PHE, phenacetin; TO, tolbutamide; OH-TO, hydroxy-tolbutamide; DZ, diazepam; D-DZ, desmethyldiazepam; CZ, chlorzoxazone; OH-CZ, hydroxy-chlorzoxazone; TES, testosterone; OH-TES, 6 β -hydroxytestosterone; COU, coumarin; OH-COU, 7-hydroxycoumarin.

precision (intraday) in the measurement of all analytes of each combination set against the nominal values were within the acceptable limits, i.e. RSD was less than $\pm 15\%$ and the coefficient of variation (CV) was $<15\%$. Intraday assay precision and accuracy data for various analytes in all combination sets are shown in table 5.

Application of the method in metabolism studies: CYP inhibitory screens

Incubated samples of lymphoblast-expressed CYP enzymes with representative CYP probe substrates alone and in the presence of standard chemical inhibitors could be successfully and continuously analysed with good resolution of substrate, metabolite(s), inhibitor and IS. Subsequently the method was applied in the CYP inhibitory screen of a variety of new compounds having varied chemical structures. Suitability and resolution were first evaluated by running an extracted sample from microsomal incubation of the new drug in the present method in order to ensure that the retention time of a new drug and its metabolite(s) did not interfere with that of the probe substrate(s)/metabolite(s) and/or inhibitors. The inhibitory effects of these test compounds on probe substrate metabolism are shown in table 6.

Discussion

Currently, it has become customary to perform *in vitro* human CYP drug metabolism studies to evaluate the potential for any clinically relevant drug–drug

Table 5. Assay performance data: intraday precision and accuracy.

Probe substrate/metabolite Nominal concentration ($\mu\text{g ml}^{-1}$)	Intra-day precision (%CV)				Accuracy (%)			
	0.2	0.4	1.0	5.0	0.2	0.4	1.0	5.0
Phenacetin	1.2	2.7	2.3	1.2	116.4	104.6	93.9	100.2
Paracetamol	1.1	2.7	1.0	1.5	113.4	103.0	95.4	100.1
Tolbutamide (TO)	2.4	6.1	0.3	2.8	113.5	101.2	96.8	100.1
4-Hydroxy TO	6.2	2.2	1.1	3.0	105.9	101.6	97.9	100.1
Diazepam (DZ)	9.4	2.9	4.9	1.3	104.5	94.8	107.9	100.0
Desmethyl DZ	5.6	3.3	1.3	1.5	87.0	91.4	107.1	99.8
Dextromethorphan	1.9	4.1	0.9	4.9	101.0	109.8	84.9	102.0
Dextrorphan	1.1	2.5	2.2	1.0	116.4	104.6	93.9	100.2
Chlorzoxazone (CH)	5.1	2.6	1.8	0.5	82.6	90.4	108.3	99.7
6-Hydroxy CH	8.5	7.1	1.0	2.0	100.9	85.7	106.4	99.8
Testosterone (TES)	2.1	4.5	0.5	4.4	101.0	109.8	84.9	102.0
6 β -Hydroxy TES	5.9	5.2	2.9	4.8	83.0	110.9	84.4	100.2
Coumarin (COU)	8.8	3.9	16.9	0.3	113.7	114.8	114.5	99.5
7-Hydroxy COU	4.7	1.5	0.5	3.4	109.9	101.4	97.0	100.1
Bufuralol (BUF)	3.1	2.9	3.0	2.8	113.6	94.6	95.2	101.7
Hydroxy BUF	3.3	4.3	3.0	4.5	112.0	106.2	90.7	91.5

interactions. Such studies are warranted from a pharmaceutical industry point of view to ensure the selection of an ideal drug candidate when it enters the early drug development phase. From a regulatory point of view, such *in vitro* metabolism studies are needed to plan appropriate clinical drug–drug interaction studies to judge the level of clinical interaction for labelling purposes (i.e. appropriate dosage adjustment and/or contraindication). In this context, as emphasized recently, it is important to validate the appropriateness of the probe substrates and experimental conditions (Yuan *et al.* 2002). Additionally, the experimental protocol needs to be carefully designed while carrying out the *in vitro* metabolism studies (Yuan *et al.* 2002).

As compared with the kind of *in vitro* metabolism work performed 5–10 years ago in the pharmaceutical industry, the number of CYP enzymes being evaluated and the number of probe substrates/metabolites being studied have increased substantially. In order to address the current situation, it has become essential to devise innovative analytical capabilities that would not only increase the throughput, but also keep the method simple enough for universal applicability. When all the reported HPLC methods were carefully examined for the analysis of various probe substrates (Chauret *et al.* 1997, Hickman *et al.* 1998, Busby *et al.* 1999, Easterbrook *et al.* 2001), in addition to our own experiences in our laboratories, it was found that most of the probe substrates/metabolites resolved well on the C18 column except for the few like phenacetin, dextromethorphan and their respective metabolites that required either C5 or C11 columns for an effective resolution. One of the key reasons for this observation may be attributable to the wide polarity difference between the probe substrates and their metabolites. It is believed that this particular problem of differential polarities may be difficult to be accommodated within a reasonable run time on a C18 column. Therefore, attention was turned to altering the mobile phase contents and composition to enable molecules of varied polarities to be well resolved on a C18 column. While optimizing the conditions, it was found that reservoir C of the ternary mobile phase was very

Table 6. Inhibitory profile of investigative compounds against representative substrates in human lymphoblast cDNA expressed CYP enzymes.

Test compound	Concentration (μM)	CYP 1A2	CYP 2A6	CYP 3A4	CYP 2C19	CYP 2C9	CYP 2D6	CYP 2E1
		7-Ethoxycoumarin (10 μM)	Coumarin (50 μM)	Testosterone (50 μM)	Diazepam (50 μM)	Tolbutamide (200 μM)	Dextromethorphan (20 M)	Chlorzoxazone (50 μM)
DMPK-1	1	NI	NI	NI	NI	14%	NI	NI
	3	NI	NI	NI	NI	16%	NI	NI
	10	NI	NI	NI	NI	20%	NI	NI
	30	NI	NI	NI	NI	25%	NI	NI
DMPK-2	100	NI	NI	NI	NI	59%	NI	NI
	1	NI	NI	NI	NI	NI	NI	NI
	3	NI	NI	NI	NI	NI	NI	NI
	10	NI	NI	NI	NI	NI	NI	NI
DMPK-3	30	NI	NI	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI	NI	NI
	1	NI	NI	NI	NI	NI	NI	NI
	3	NI	NI	NI	NI	NI	NI	NI
DMPK-4	10	NI	NI	NI	NI	NI	NI	NI
	30	NI	NI	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	NI	NI	NI
	1	NI	NI	NI	NI	NI	NI	NI
	3	NI	NI	NI	NI	NI	NI	NI
	10	NI	NI	NI	NI	NI	NI	NI
	30	NI	NI	NI	NI	NI	NI	NI
	100	NI	NI	NI	NI	40%	28%	NI
						75%	60%	NI

NI, no inhibition.

crucial in obtaining the desired resolution between analytes of varying polarities. The present strategy was put to test by using a difficult combination set comprising of phenacetin + paracetamol as the starting point. It was found that the present ternary mobile phase system adequately separated the two compounds. Later, the ease of separation of the chemical inhibitor (furafylline) and IS along with phenacetin and paracetamol was verified. All four analytes were well resolved in the chromatography and amenable for quantitation. To the best of the authors' knowledge, this is the first paper on the simultaneous measurement of this combination set (phenacetin + paracetamol + furafylline + celecoxib) on a C18 column. After the separation issue of the most difficult combination set along with its inhibitor had been tackled, attention was turned to other probe substrate combination sets along with respective chemical inhibitor(s). It was found that the chosen conditions worked well (figure 2). It should be noted that celecoxib eluted as the last peak in all combination sets, and the peak symmetry was not compromised. Moreover, celecoxib exhibits reasonable UV absorption at all required wavelengths and adequate fluorescing properties, which further supported the rationale in selecting this compound as IS. Based on previous experience, the present authors have handled celecoxib as an IS for quantitative determination of other compounds and their metabolites, which worked out well, even though celecoxib was the last eluting peak (Rao *et al.* 2003).

Similar to the practice followed in the pharmaceutical industry, the present authors have a general classification system of potent ($IC_{50} \leq 1 \mu M$), moderate ($IC_{50} 1-10 \mu M$) and weak ($IC_{50} > 10 \mu M$) CYP enzyme inhibitors. However, based on the therapeutic doses and expected peak concentrations in man, even a compound classified as a weak enzyme inhibitor may have the potential to cause a clinical interaction. As is evident from table 6, the distribution of compounds tested in the inhibitory CYP screens using the newly developed method is confined to the panel representing either weak inhibitory potential or no inhibitory potential.

While this system was being put in place for continuous use, the present authors were aware that industry-wise practice had been to use different probe substrates to study the *in vitro* CYP-based drug interactions. In principle, although such practices ensures diversity, it may not necessarily allow comparisons to understand and/or predict potential clinical impact by evaluating data either from the published literature, product labels (i.e. package inserts), or submitted new drug applications. Moving forward, one would like to introduce additional probe substrates for the same CYP enzyme not only to validate the original findings, but also unequivocally to extrapolate the findings to more probe substrates for the given CYP enzyme. Some of next term goals are to evaluate and validate midazolam 1'-hydroxylation activity for CYP3A4 and *S*-mephenytoin 4'-hydroxylation activity for CYP2C19. These new systems when developed and established will be permanently incorporated in the present authors' *in vitro* drug metabolism screen tools. Another area that the authors are probing is to introduce a different scheme to measure CYP2B6 activity. Based on the present literature, it appears that *S*-mephenytoin N-demethylation activity may be considered to represent CYP2B6. A recent report states the involvement of CYP2B6 in the mediation of *in vitro* hydroxylation of bupropion and having a high degree of correlation with *S*-mephenytoin N-demthylation activity (Hesse *et al.* 2000). A variety of antidepressants such as paroxetine, fluvoxamine, sertraline, fluoxetine,

norfluoxetine, nefazodone and venlafaxine have been found to have a tendency to inhibit to a mild-to-moderate extent, the hydroxylation pathway of bupropion (Hesse *et al.* 2000).

Overall, the developed analytical procedure employs the same column, an optimized ternary composition of mobile phase, and a compatible UV detection system for probe substrates/metabolites/inhibitors. It was found that this generic method was simple and convenient and has led to the successful assessment of CYP inhibitory potential of new drugs in the authors' laboratories. The authors believe their new system is cost effective, easily operable, and can further be accommodated to manage additional probe substrates.

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Pharmacokinetics and metabolism of NO-1886, a lipoprotein lipase-promoting agent, in cynomolgus monkey

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Received 7 July 2003

1. The study was conducted to investigate the pharmacokinetics and metabolism of NO-1886 (diethyl 4-[(4-bromo-2-cyanophenyl) carbamoyl] benzylphosphonate) in cynomolgus monkeys.

2. After single intravenous administration of NO-1886 at a dose of 3 mg kg^{-1} , the total clearance (CL_{tot}), area under the plasma concentration–time curve (AUC_{0-t}), half-life ($t_{1/2}$), and volume of distribution (V_d) in cynomolgus monkeys were $531 \text{ ml h}^{-1} \text{ kg}^{-1}$, $5.63 \mu\text{g h ml}^{-1}$, 0.96 h and 679 ml kg^{-1} , respectively. The AUC_{0-t} for oral administration of NO-1886 (3 mg kg^{-1}) was $4.23 \mu\text{g h ml}^{-1}$ and the bioavailability was 75%.

3. M-2 (ethyl 4-[(4-bromo-2-cyanophenyl) carbamoyl] benzylphosphonate) and M-3 (4-[(diethoxy-phosphoryl methyl)] benzoic acid) were present as metabolites in plasma and urine. In faeces, M-2 was present but M-3 was not.

4. The major metabolite of NO-1886 in liver S9 or microsomes was M-2 in the presence of NADPH. On the other hand, M-3 was formed in the absence of NADPH in liver S9 or microsomes and its formation was inhibited by bis-(*p*-nitrophenyl) phosphate (BNPP) in liver S9, suggesting that the formation of M-3 was catalysed by carboxylesterase.

5. The findings suggest that the main metabolic pathway of NO-1886 in cynomolgus monkeys is the O-deethylation of NO-1886 to M-2, as in rats and humans, and that the hydrolysis of the amide bond is a minor metabolic pathway.

Introduction

A number of studies have shown a significant inverse relationship between high-density lipoprotein (HDL) cholesterol and coronary heart disease, and HDL cholesterol is now known to be a substantial protector against coronary artery atherosclerosis (Gorden and Rifkin 1989). Plasma HDL originates from three sources: the liver, small intestine and lipoprotein lipase-mediated lipolysis of chylomicrons and very-low-density lipoproteins (Eisenberg 1984). The novel compound NO-1886 was shown to increase lipoprotein lipase activity and induce an increase in HDL cholesterol levels in several studies (Tsutsumi *et al.* 1993, 1995, 1997). It has also been demonstrated that long-term administration of NO-1886 significantly inhibited the development of atherosclerosis in cholesterol-fed rats (Tsutsumi *et al.* 1993) and rabbits (Chiba *et al.* 1997).

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It was reported previously that the metabolic pathway of NO-1886 in rats involves the O-deethylation of diethyl ester phosphonate, hydrolysis of the amide bond, hydroxylation of the amino compound (M-1) produced by hydrolysis of the amide bond and sulfation following hydroxylation of M-1 (Morioka *et al.* 1996). At 24 h post-dosing in rats, NO-1886 was almost completely excreted in the urine (28%) and faeces (64%) as metabolites. The major metabolite was monoethyl phosphonate (M-2), which accounted for 70% of all metabolites in rats. Subsequently, it was found in inhibition studies using human liver S9, microsomes and recombinant human CYPs that CYP2C8 and CYP3A4 catalysed the O-deethylation of NO-1886 (Morioka *et al.* 2002).

The present study investigated the pharmacokinetics and excretion of NO-1886 in cynomolgus monkeys following single intravenous or oral administration and also examined the metabolism of NO-1886 in liver S9 and microsomes.

Materials and methods

Chemicals

NO-1886 was synthesized at Otsuka Pharmaceutical Co., Ltd (Tokushima, Japan) and had a chemical purity of 99.9%. Three metabolites of NO-1886 (M-2, M-3, M-5) were synthesized at Otsuka Pharmaceutical Factory (Tokushima, Japan) and had chemical purities > 99.0%. ^{14}C -NO-1886 (2.04 GBq mmol $^{-1}$ [4.51 MBq mg $^{-1}$]) was synthesized at Amersham International Plc (Amersham, UK). The radiochemical purity of the sample was not less than 98% by TLC. Polyethylene glycol 400, acacia, 0.5 M tetra-*n*-butyl ammonium hydroxide (TBA) solution and bis-(*p*-nitrophenyl) phosphate (BNPP) were from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and *n*-butyl *p*-hydroxybenzoate of certified grade (for use as the internal standard) was from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Sigma Chemical Co. (St Louis, MO, USA). Bond Elut CH:100 mg ml $^{-1}$ was from GL Sciences, Inc. (Tokyo, Japan). A mixed pool of male cynomolgus monkey liver S9 and microsomes was obtained from GENTEST Corp. (Woburn, MA, USA). All the compounds used were the highest grade commercially available.

Animals and procedures

Four male cynomolgus monkeys estimated to be 3 years of age and weighing about 3 kg (housed at the Wakayama Breeding Farm of Keari Co., Ltd, Wakayama, Japan) were used. During the experiment, animals were housed individually in metabolic cages at 22–26°C and a relative humidity of 45–65%. Animals were fed solid diet (PS, Oriental Yeast Co., Ltd, Tokyo, Japan) once daily and allowed free access to water. During the preliminary housing, they were trained to complete feeding in 1 h. NO-1886 was administered at 09:00 hours and the food given at 11:00 hours. The studies were approved by the Committee on the Care and Use of Laboratory Animals of Otsuka Pharmaceutical Factory, Inc., and followed the guidelines that followed the Guide for the Care and Use of Laboratory Animals (US National Research Council).

A dosing solution of NO-1886 was prepared by polyethylene glycol 400 (for intravenous dose) and aq. 5% (w/v) acacia (for oral dose) and it was stable for 1 month at 4°C. NO-1886 content was checked by HPLC before use. NO-1886 (3 mg ml $^{-1}$) dissolved in polyethylene glycol 400 was administered intravenously at a dose of 3 mg kg $^{-1}$ using a syringe. Blood samples (approximately 1 ml) were collected from the median cephalic vein before dosing and at 5, 20 min, 1, 2, 4, 6, 8 and 24 h after dosing. Urine and faeces were collected at 0–24, > 24–48 and > 48–72 h after dosing.

A suspension of NO-1886 (3 mg/5 ml) in aq. 5% (w/v) was administered to the animals at a dosing volume of 5 ml kg $^{-1}$ using a stomach tube. Blood samples (approximately 1 ml) were collected from the median cephalic vein before dosing and at 0.5, 1, 2, 3, 4, 6, 8 and 24 h after dosing. Urine and faeces were collected at 0–24, > 24–48 and > 48–72 h after dosing. Oral administration was carried out 2 weeks after the intravenous administration.

Sample preparation for HPLC (plasma)

Plasma samples were obtained by centrifuging the blood samples at 1800g for 10 min at 4°C (8700, Kubota Co., Ltd, Tokyo, Japan). A 200- μ l aliquot of the internal standard solution (5 μ g ml $^{-1}$ acetonitrile) was pipetted into a 100- μ l aliquot of plasma sample. After centrifugation at 10000g for 10 min at 4°C (HIMAC Centrifuge CR15T, Hitachi, Tokyo, Japan), the supernatant was evaporated to dryness under a gentle stream of nitrogen at 40°C, and the residue was then dissolved in 250 μ l of a

solution of acetonitrile and sodium phosphate buffer (10 mM, pH 6.4) at a ratio of 1:4 (v/v). Recovery for NO-1886, internal standard and metabolites were > 95.6%. A 100- μ l aliquot of the sample was used for HPLC.

Sample preparation for HPLC (urine)

A 200- μ l aliquot of the internal standard solution (25 μ g ml⁻¹) was transferred to a test tube containing 0.5 ml urine sample. To this mixture, a 1.5-ml aliquot of 1.0 M phosphate buffer (pH 2.0) was added, and then a 1.0-ml aliquot of each mixture was applied to the Bond Elut CH column, which had previously been washed with acetonitrile, water and 1.0 M phosphate buffer (pH 2.0). The Bond Elut CH column was then washed through with several solvents in the following order: 1.0 ml acetonitrile/50 mM phosphate buffer solution (pH 2.0, containing 5 mM TBA) (10:90 v/v), 1.0 ml acetonitrile/50 mM phosphate buffer solution (pH 2.0, containing 5 mM TBA) (15:85), 1.0 ml distilled water, 1.0 ml 25 mM phosphate buffer solution (pH 7.4), and 1.0 ml methanol/25 mM phosphate buffer solution (pH 7.4) (3:97). Finally, the bond Elut CH column was treated with 0.5 ml acetonitrile/50 mM phosphate buffer solution (pH 2.0) (70:30). Recovery for NO-1886, internal standard and metabolites was > 96.6%. A 200- μ l aliquot of the sample was used for HPLC.

Sample preparation for HPLC (faeces)

The faeces homogenates for determination were prepared by adding 3 vols water to 1 weight faeces and homogenizing the mixture with a Polytron (PT 10/35, Kinematica Co., Ltd, Tokyo, Japan). A total of 120 μ l of the internal standard (25 μ g ml⁻¹ acetonitrile) was then placed in a 10-ml screw-capped test tube and evaporated to dryness under a nitrogen stream at 40°C. About 300 mg faeces homogenate were placed into the test tube (described above) and 1.5 ml 50 mM phosphate buffer at pH 2.0 (containing 5 mM TBA) and 4 ml ethyl acetate were added. After centrifugation at 1800g for 10 min at 4°C, 2 ml organic layer were evaporated under nitrogen gas and dissolved in 300 μ l 30% (v/v) acetonitrile/50 mM phosphate buffer at pH 2.0 (containing 5 mM TBA). Recovery for NO-1886, internal standard and metabolites was > 90.3%. A 100- μ l aliquot of the sample was used for HPLC.

Protein binding

An aliquot of serum was placed in a test tube at 4°C and ¹⁴C-NO-1886 solution (0.18 MBq mg⁻¹ ml⁻¹ in acetonitrile) was then added to the tube to adjust the final concentration of NO-1886 to 10 μ g ml⁻¹. The mixture was allowed to equilibrate at 4°C, after which two 1-ml aliquots of the mixture were pipetted into tubes and incubated at 37°C for 5 min. Aliquots (0.2 ml) of the mixture were then transferred to tubes and a scintillant (ACSII, Amersham) was added for the measurement of total radioactivity concentrations. The remaining volume of each mixture was placed in an ultrafiltration apparatus (Centrifree; Nihon Millipore Ltd, Tokyo, Japan) at 1500g for 20 min at room temperature for ultrafiltration, and the radioactivity concentrations in 0.2-ml aliquots of the filtrates were measured. The adsorption percentage of NO-1886 to the ultrafiltration membrane was 11.8% at 10 μ g ml⁻¹ NO-1886 concentration.

The extent of protein binding was obtained using the following equation (Graben and Parsons 1988):

$$\text{Extent of protein binding (\%)} = 100 - [\text{radioactivity concentration in ultrafiltrate} / (1 - \text{adsorption ratio}) / (\text{total radioactivity concentration})] \times 100.$$

Metabolism of NO-1886 in liver S9 or microsomes

All incubations involving liver microsomes were carried out at a protein concentration of 0.4 mg ml⁻¹ in 100 mM potassium phosphate buffer (pH 7.4) including 0.1 mM ethylenediamine tetra-acetic acid (EDTA) and the NADPH-generating system (1.25 mM NADP⁺, 2.5 mM glucose 6-phosphate, 6 mM MgCl₂, and 0.75 unit ml⁻¹ glucose 6-phosphate dehydrogenase) at 37°C. Control incubations were carried out without liver microsomes. In the enzyme kinetics experiments, 0.9–66 μ M NO-1886 were incubated at a final volume of 0.5 ml for 10 min. The reaction was initiated by the addition of the sample solution after a 5-min pre-incubation at 37°C. After incubation, the reaction was terminated by the addition of 1.0 ml acetonitrile containing 5 μ g ml⁻¹ of the internal standard. After the reaction mixture was centrifuged at 1800g for 10 min at 4°C, the supernatant was evaporated to dryness and the residue dissolved in 250 μ l of a solution consisting of acetonitrile and sodium phosphate buffer (10 mM, pH 6.4) at a ratio of 1:4 (v/v). A 100- μ l aliquot of the sample was used for HPLC. Recovery for NO-1886, internal standard and metabolites was > 97.1%. Incubation with liver S9 was carried out at a protein concentration of 2.3 mg ml⁻¹ under the same conditions as for liver microsomes.

Inhibition studies

Incubation was carried out at 37°C for 60 min with the liver S9 (final protein concentration of 2.3 mg ml⁻¹) in 100 mM potassium phosphate buffer (pH 7.4) at a final volume of 0.5 ml including 110 μ M NO-1886 and 1 mM or 10 μ M BNPP (a carboxylesterase inhibitor).

HPLC analysis

The concentrations of NO-1886 and its metabolites were determined using an HPLC system (Tosoh, Tokyo, Japan) consisting of a computer-controlled multiple pump CCPM-II, a system controller SC-8020, UV detectors UV-8000 and UV-8010, an autoinjector AS-8010, and a column oven CO-8010. HPLC condition, calibration curve, and inter- and intraday accuracy are shown in table 1. NO-1886 and metabolites in HPLC samples were stable in the auto sampler (about 4°C) within 48 h.

Data analysis

The concentration at time 0 (C_0) was extrapolated from initial slope. The maximum concentration ($C_{\max}$) and the time to reach the maximum concentration ($T_{\max}$) were the observed values. The half-life ($t_{1/2}$) was estimated as $\ln 2/k$, where k is the slope of the terminal linear portion of the logarithmic plasma concentration–time curve. The area under the plasma concentration–time curve (AUC_{0-t}) was calculated by a trapezoidal rule and the mean residence time (MRT) up to the last detectable point was estimated according to the method of Yamaoka *et al.* (1978). Total clearance (CL_{tot}) and volume of distribution (V_d) were calculated according to the following equations:

$$CL_{\text{tot}} = D_{\text{i.v.}}/AUC_{0-t}$$

$$V_d = D_{\text{i.v.}}/C_0,$$

where $D_{\text{i.v.}}$ is the dose. Bioavailability (F) was calculated as $AUC_{\text{p.o.}}/AUC_{\text{i.v.}}$.

Results

Pharmacokinetics

The time-courses of the changes in the plasma concentrations of NO-1886 and its metabolites following single intravenous administration of NO-1886 at a dose of 3 mg kg⁻¹ are shown in figure 1, and the pharmacokinetic parameters are shown in table 2.

The $t_{1/2}$ of NO-1886 was 0.96 h. NO-1886 was completely eliminated from the plasma and its concentration was below the lower limit of determination (40 ng ml⁻¹) at 24 h after dosing. The decline in concentration of NO-1886 was monoexponential from 15 min to 8 h. The AUC_{0-t} , CL_{tot} , V_d and MRT values of NO-1886 were 5.63 µg h ml⁻¹, 531 ml h⁻¹ kg⁻¹, 679 ml kg⁻¹ and 1.27 h, respectively. Three metabolites (M-2, M-3, M-5) were identified in the plasma, with the major metabolites being M-2 and M-3. The $C_{\max}$ values for these metabolites were 0.198, 0.098 and 0.015 µg ml⁻¹, respectively, and they were also completely eliminated from the plasma at 24 h after dosing.

The changes in the plasma concentrations of NO-1886 and its three metabolites following single oral administration of NO-1886 at a dose of 3 mg kg⁻¹ are shown in figure 2, and the derived pharmacokinetic parameters of these compounds are summarized in table 3. The plasma $T_{\max}$, $C_{\max}$ and MRT values of NO-1886 were 1.0 h, 1.472 µg ml⁻¹ and 2.3 h, respectively. The $t_{1/2}$ (0.86 h) was similar to that observed after intravenous administration, and NO-1886 was not detected in the plasma at 24 h after oral administration. Bioavailability (F) at a dose of 3 mg kg⁻¹ was 75%. Two metabolites (M-2, M-3) were measurable in the plasma, with the major metabolite being M-2, as was the case for intravenous administration. On the other hand, M-5 was not detected. The $C_{\max}$ values of M-2 and M-3 were 0.180 and 0.046 µg ml⁻¹, respectively. The AUC_{0-t} for M-2 and M-3 was 0.61 and 0.14 µg h ml⁻¹, respectively. The AUC_{0-t} for M-2 after oral administration was higher than that after intravenous administration.

Table 1. HPLC conditions and validation data for NO-1886 and its metabolites.

	Sample			
	Plasma	Urine	Faeces	Liver S9 or microsomes
Column	TSKgel ODS-120A (4.6 mm i.d. × 250 mm)	TSKgel ODS-80Ts (4.6 mm i.d. × 250 mm)	TSKgel ODS-120A (4.6 mm i.d. × 250 mm)	TSKgel ODS-120A (4.6 mm i.d. × 250 mm)
Column temperature(°C)	40	43	40	42
Mobile phase	50 mM Phosphate, pH 2.15 acetonitrile (100%)	50 mM phosphate, pH 2.0 acetonitrile (100%)	50 mM phosphate, 5 mM TBA, pH 2.0 acetonitrile (100%)	50 mM phosphate, pH2.2 acetonitrile (100%)
Gradient (linear)				
Time (min)	0–10–20–32–32.1–60–60.1– 70–70.1	0–15–15.1–35–45–55–70– 70.1–80–80.1	0–25–25.1–45–60–60.1– 70–70.1	0–10–20–28–40–60–60.1– 70–70.1
B (%)	20–20–22–24–36–36–70–70–20	17–17–18–22–30–40–40–70–70–17	19–19–33–36–45–70–70–19	20–20–22–24–36–38–70–70–20
Flow rate (ml min ⁻¹)	1.2	1.2	1.0	1.2
Detection (nm)	260 (NO-1886, M2, and M3)	239 (NO-1886, M2, and M3)	260 (NO-1886 and M2)	260 (NO-1886 and M2)
Calibration curve (linear range; <i>r</i> > 0.999)	340 (M5) 40–5000 ng ml ⁻¹ (NO-1886) 60–5000 ng ml ⁻¹ (M2, M3, M5) 0.6–13.2	340 (M5) 2.0–20 µg ml ⁻¹ (NO-1886) 1.0–20 µg ml ⁻¹ (M2, M3, M5) 0.1–5.9	239 (M3) 0.15–15 µg (NO-1886 and M3) 0.3–15 µg (M2) 0.4–3.3	239 (M3) 25–5000 ng ml ⁻¹ (NO-1886 and M3) 50–5000 ng ml ⁻¹ (M2) 0.6–8.2
Inter-day precision (NO-1886 and metabolites, %)	91.0–108.2	91.6–103.7	98.1–103.5	95.2–107.1
Inter-day accuracy (NO-1886 and metabolites, %)				
Lower determination limit	NO-1886 40 ng ml ⁻¹ M2, M3 and M5 60 ng ml ⁻¹	NO-1886 2.0 µg ml ⁻¹ M2, M3 and M5 1.0 µg ml ⁻¹	NO-1886 and M3 0.15 µg M2 0.3 µg	NO-1886 and M3 25 ng ml ⁻¹ M2 50 ng ml ⁻¹

IS, internal standard.

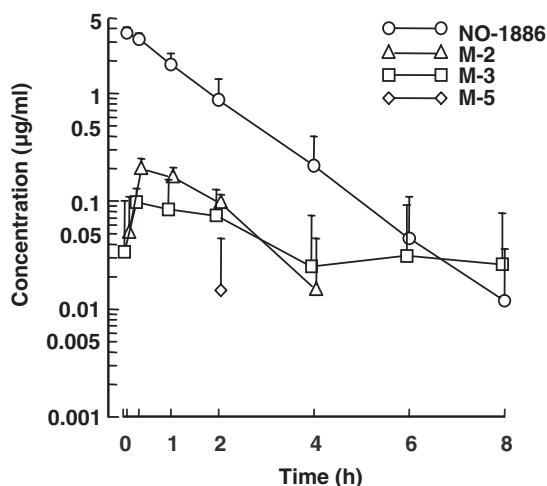


Figure 1. Plasma concentration–time profiles of NO-1886 and its metabolites in cynomolgus monkeys following single intravenous administration of NO-1886 at 3 mg kg^{-1} . Data are the mean $\pm$ SD of four animals.

Table 2. Pharmacokinetic parameters following single intravenous administration of NO-1886 to cynomolgus monkeys at a dose of 3 mg kg^{-1} .

	NO-1886	M2	M3	M5
C_0 ($\mu\text{g ml}^{-1}$)	3.90			
AUC_{0-t} ($\mu\text{g h ml}^{-1}$)	5.63	0.40	0.37	n.d.
CL_{tot} ($\text{ml h}^{-1} \text{ kg}^{-1}$)	531			
V_d (ml kg^{-1})	679			
$t_{1/2}$ (h)	0.96			
T_{max} (h)		0.33	0.33	2.0
MRT (h)	1.27			
C_{max} ($\mu\text{g ml}^{-1}$)		0.198 ± 0.051	0.098 ± 0.033	0.015 ± 0.030

AUC_{0-t} : from time 0 to the last detectable concentration.

C_{max} is the mean $\pm$ SD of four animals.

n.d., Not determined.

Excretion in urine and faeces

Table 4 shows the cumulative urinary excretion of NO-1886 and its metabolites (M-2, M-3, M-5) found in the plasma following single intravenous or oral administration of NO-1886 to cynomolgus monkeys. Of the above metabolites, M-2 showed the highest levels of urinary excretion. For intravenous administration, the maximum level of urinary excretion of M-2 was $0.10 \pm 0.09 \text{ mg kg}^{-1}$ ($3.5\% \pm 3.0\%$ of dose) up to 24 h after dosing, after which it was not detected in the urine. On the other hand, for oral administration, the maximum level of urinary excretion of M-2 was $0.19 \pm 0.10 \text{ mg kg}^{-1}$ ($6.3\% \pm 3.2\%$ of dose) up to 24 h after dosing, slightly higher than the value for intravenous administration. M-3 was the metabolite showing the second highest level of urinary excretion, with a maximum of $0.08 \pm 0.04 \text{ mg kg}^{-1}$ ($2.4\% \pm 1.2\%$ of dose) regardless of the route of administration. NO-1886 and the other metabolites were not detected in the urine samples.

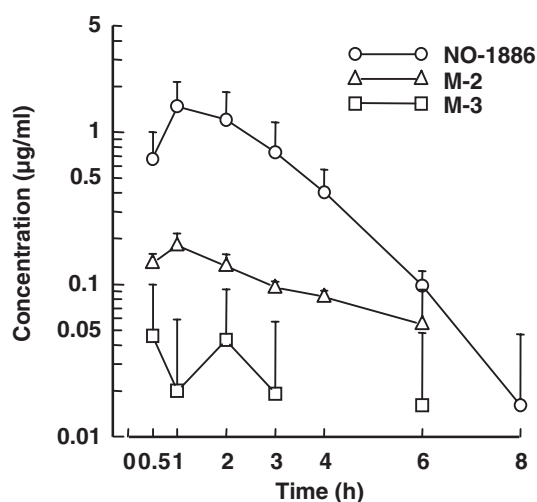


Figure 2. Plasma concentration–time profiles of NO-1886 and its metabolites in cynomolgus monkeys following single oral administration of NO-1886 at 3 mg kg^{-1} . Data are the mean $\pm$ SD of four animals.

Table 3. Pharmacokinetic parameters following a single oral administration of NO-1886 to cynomolgus monkeys at a dose of 3 mg kg^{-1} .

	NO-1886	M2	M3	M5
$T_{\max}$ (h)	1.0	1.0	0.5	n.d.
$C_{\max}$ ($\mu\text{g ml}^{-1}$)	1.472 ± 0.675	0.180 ± 0.037	0.046 ± 0.054	n.d.
AUC_{0-t} ($\mu\text{g h ml}^{-1}$)	4.21	0.61	0.14	n.d.
$t_{1/2}$ (h)	0.86			
MRT (h)	2.3			
F (%)	75			

AUC_{0-t} : from time 0 to the last detectable concentration.

$C_{\max}$ is the mean $\pm$ SD of four animals.

n.d., Not determined.

Table 5 shows the cumulative faecal excretion of NO-1886 and its metabolites (M-2, M-3) following single intravenous or oral administration of NO-1886 to cynomolgus monkeys. After the intravenous and oral administration of NO-1886, M-2 was detected in the faeces with excretions of $1.32 (43.8\% \pm 2.9\% \text{ of dose})$ and $1.25 \text{ mg kg}^{-1} (41.9\% \pm 3.2\% \text{ of dose})$, respectively, up to 72 h after dosing. On the other hand, unchanged NO-1886 and M-3 were not detected in the faeces.

Protein binding

The extent of protein binding in monkey plasma was 86.0%, which is about 7% lower than the extent in rat and human serum (93.2 and 93.3%, respectively).

Metabolism of NO-1886 in liver S9 or microsomes

The metabolism of NO-1886 was investigated in pooled cynomolgus monkey liver S9 and microsomes. Figure 3 shows an HPLC chromatogram of cynomolgus monkey liver S9 after reaction with NO-1886 for 60 min. The concentrations of metabolites in liver S9 are listed in table 6.

Table 4. Cumulative urinary excretion of NO-1886 and its metabolites in cynomolgus monkeys following single intravenous or oral administration of NO-1886 at a dose of 3 mg kg⁻¹.

Route	Time (h)	NO-1886	M2	M3	M5
i.v.	0–24	n.d.	0.10 ± 0.09	0.08 ± 0.04	n.d.
	0–48	n.d.	0.10 ± 0.09	0.08 ± 0.04	n.d.
	0–72	n.d.	0.10 ± 0.09	0.08 ± 0.04	n.d.
p.o.	0–24	n.d.	0.19 ± 0.10	0.08 ± 0.04	n.d.
	0–48	n.d.	0.19 ± 0.10	0.08 ± 0.04	n.d.
	0–72	n.d.	0.19 ± 0.10	0.08 ± 0.04	n.d.

Data are the mean ± SD of four animals.
Excretion is expressed as mg kg⁻¹ body weight.
n.d., Not detected.

Table 5. Cumulative faecal excretion of NO-1886 and its metabolites in cynomolgus monkeys following single intravenous or oral administration of NO-1886 at a dose of 3 mg/kg⁻¹.

Route	Time (h)	NO-1886	M2	M3
i.v.	0–24	n.d.	n.d.	n.d.
	0–48	n.d.	1.13 ± 0.27	n.d.
	0–72	n.d.	1.32 ± 0.08	n.d.
p.o.	0–24	n.d.	0.35 ± 0.29	n.d.
	0–48	n.d.	1.14 ± 0.14	n.d.
	0–72	n.d.	1.25 ± 0.10	n.d.

Data are expressed as mean ± SD of four animals.
Excretion is expressed as mg kg⁻¹ body weight.
n.d., Not detected.

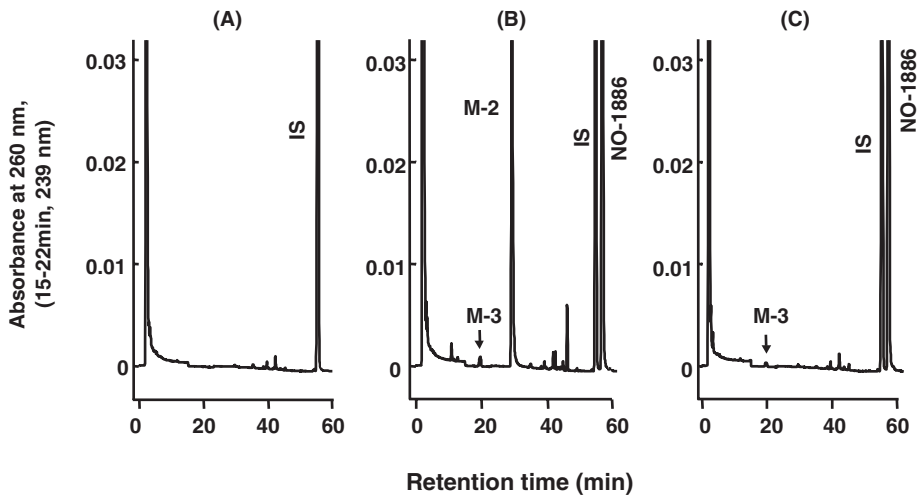


Figure 3. Representative HPLC chromatograms of metabolites of NO-1886 formed in cynomolgus monkey liver S9 with and without the NADPH-generating system. A control mixture of cynomolgus monkey liver S9 (2.3 mg ml⁻¹) was mixed with *n*-butyl *p*-hydroxybenzoic acid after incubation under the same conditions as described for the incubation of samples (A). A mixture of human liver S9 (2.3 mg ml⁻¹) was incubated with 110 µM NO-1886 for 60 min with the NADPH-generating system (B). A mixture of monkey liver S9 (2.3 mg ml⁻¹) was incubated with 110 µM NO-1886 for 60 min without the NADPH-generating system (C).

Table 6. Metabolism rates in cynomolgus monkey liver S9 and microsomes.

	Metabolism rate (pmol min ⁻¹ mg ⁻¹ protein)	
	M2	M3
S9		
NADPH (+)	103.3±0.8	2.9±0.0
NADPH (-)	n.d.	1.7±0.0
Microsomes		
NADPH (+)	493.4±15.5	11.4±0.3
NADPH (-)	n.d.	5.2±0.2

Data are the mean ± SD of triplicate determinations.

n.d.: Not detected.

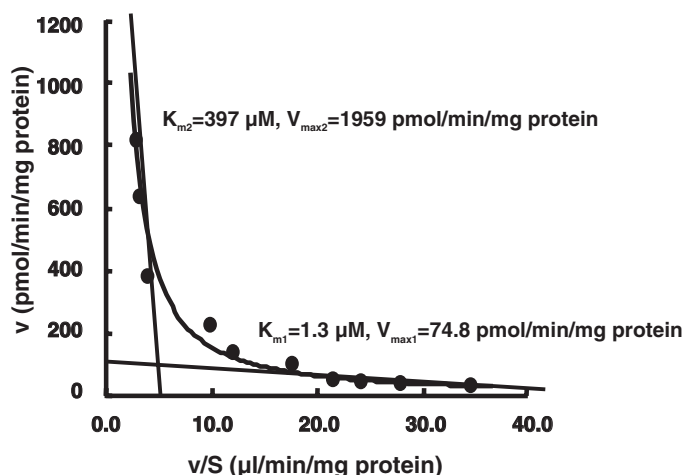


Figure 4. Eadie-Hofstee plot for NO-1886 hydrolase activity in pooled cynomolgus monkey liver microsomes. Cynomolgus monkey liver microsomes (0.4 mg ml⁻¹) were incubated with NO-1886 (0.9–166 μM) and the rates of formation of M2 were determined by HPLC. Data are the mean of duplicate determinations.

It is clear that O-deethylation of diethyl phosphonate is the major metabolic pathway of NO-1886 in the liver of cynomolgus monkeys, as in rats and humans, and that the amide bond undergoes minimal hydrolysis in cynomolgus monkey liver. The M-2 concentration per unit protein content was five times higher in microsomes than in S9, indicating that diethyl phosphonate was mainly O-deethylated in microsomes. Further, M-2 was not detected in microsomes without the NADPH-generating system (table 6), suggesting that arylesterase and other esterases are not involved in the O-deethylation of NO-1886 to M-2. Figure 4 shows an Eadie-Hofstee plot for the formation of M-2 from NO-1886 in pooled cynomolgus monkeys liver microsomes. The biphasic nature of the plot indicates the contribution of multiple enzymes in the hydrolysis reaction. The calculated values for the high-affinity component, K_{m1} and V_{max1} , were 1.3 μM and 74.8 pmol min⁻¹ mg⁻¹, respectively. For the low-affinity component, K_{m2} was 397 μM and V_{max2} was 1959 pmol min⁻¹ mg⁻¹.

In contrast to the O-deethylation of NO-1886, hydrolysis of the amide bond of NO-1886 was observed in liver S9 or microsomes with or without the NADPH-generating system. Furthermore, the M-3 concentration per unit protein content

Table 7. Inhibition of biotransformation of NO-1886 to M3 by BNPP in cynomolgus monkey liver S9.

BNPP (M)	Residual activity (%)
10^{-5}	82.5
10^{-3}	28.4

Data are percentages relative to the activity of M3 formation without BNPP ($1.60 \pm 0.02 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$, $n = 3$).

was three to four times higher in microsomes than in S9, indicating that the amido bond of NO-1886 was mainly hydrolysed in microsomes. These findings suggest that the amide bond of NO-1886 is hydrolysed by microsomal esterases.

In order to identify microsomal esterase involved in amidehydrolysis, an inhibition study was performed. Table 7 shows the inhibition of M-3 formation in liver S9 by BNPP, a carboxylesterase inhibitor. The residual activity levels of M-3 formation at BNPP concentrations of $10 \mu\text{M}$ and 1 mM were 82.5 and 28.4%, respectively, indicating that microsomal carboxylesterase catalysed amide hydrolysis of NO-1886.

Discussion

Morioka *et al.* (1996) described the metabolism of NO-1886 in rats and the present study was conducted to investigate the disposition of NO-1886 in cynomolgus monkeys.

Comparing the data obtained in monkeys with those in rats, the distribution volume and elimination rate from plasma were similar between both animals. For example, the V_d (679 ml kg^{-1}) in cynomolgus monkeys did not differ significantly from that in rats (472 ml kg^{-1}) and that was about 15 times larger than the plasma volume (44 ml kg^{-1}) in monkey (Mordenti 1986). These data suggested that NO-1886 was distributed in several tissues in cynomolgus monkeys as well as in rats. Furthermore, NO-1886 was eliminated from cynomolgus monkeys plasma as rapidly as that in rats, since the CL_{tot} and $t_{1/2}$ values in cynomolgus monkeys were similar to those in rats ($664 \text{ ml h}^{-1} \text{ kg}^{-1}$ and 0.5 h , respectively). NO-1886 was not detected in the urine or faeces following oral and intravenous administration into cynomolgus monkeys. In rats, only 1% of total excreted amount in urine and faeces was intact NO-1886. Therefore, the elimination of NO-1886 in cynomolgus monkeys and rats might be explained by the metabolism of NO-1886. The CL_{tot} ($531 \text{ ml h}^{-1} \text{ kg}^{-1}$) was small compared with liver plasma flow ($1104 \text{ ml h}^{-1} \text{ kg}^{-1}$) in monkey (Mordenti 1986), suggesting that NO-1886 was metabolized mainly in the liver.

Comparing the absorption behaviour in cynomolgus monkeys against that in rats, the C_{max} in cynomolgus monkeys was slightly higher than that in rats, in spite of similar T_{max} . The results agree with a slightly higher bioavailability in cynomolgus monkeys (75% in cynomolgus monkeys versus 63% in rats). For oral administration, the levels of excretion of M-2 in the urine and faeces up to 24 h after dosing were slightly higher than those for intravenous administration. Furthermore, the AUC_{0-t} of M-2 after oral administration was greater than that after intravenous administration. The results probably reflect the first-pass metabolism of NO-1886.

M-2, a main metabolite, was mainly excreted in the faeces in both intravenous and oral administration routes. Various types of organic anions, including glucuronides, glutathione conjugates and organic sulphates, are excreted into the bile from the liver via cMOAT/MRP2. It was thought that the excretion of M-2 from liver to bile was mediated by cMOAT/MRP2 because M-2 is an organic anion. However, in a study on the biliary excretion of M-2 in Sprague–Dawley (SD) rats and in Eisai hyperbilirubinaemic rats (EHBR) with a hereditary defect in cMOAT/MRP2 function (Hosokawa *et al.* 1992), no difference was observed in the excretion rate of M-2 into the bile between SD rats and EHBR (Y. Morioka, unpublished data). This suggests that M-2 was not excreted into the bile via cMOAT/MRP2 in the case of monkeys as well as rats.

In rats, about 10% of the administered ^{14}C -NO-1886 was excreted in the urine as M-3 (Morioka *et al.* 1996). In contrast, M-3 was excreted in the urine at 2.4% of the administered dose after intravenous or oral administration to cynomolgus monkeys. Amid bond of NO-1886 was hydrolysed in plasma and slightly hydrolysed in rat liver. On the other hand, cynomolgus monkey plasma hydrolysed only minimal NO-1886 and its liver slightly hydrolysed NO-1886 (Y. Morioka, unpublished data) (table 6). Thus, the level of excretion of M-3 in the urine was low compared with that in rats. M-5 produced by sulfation following the hydroxylation of M-1 (Morioka *et al.* 1996) was detected at very low levels in cynomolgus monkey plasma after intravenous administration and was not detected at all after oral administration. Furthermore, M-5 was excreted to only a minute extent in the urine after intravenous or oral administration. M-3 and M-1 were produced by hydrolysis of amide bond of NO-1886, and compared with the amount of M-3 excreted in urine and faeces, the amount of M-5 excreted in urine and faeces was small. Therefore, it is thought that M-1 was metabolized to more polar metabolites (except for M-5) than M-1 and then excreted in the urine or faeces.

The total amount of metabolites excreted in urine and faeces during 72 h was about 50% of dose, although NO-1886 was almost completely excreted as metabolites in rats (Morioka *et al.* 1996). Despite the similar CL_{tot} between rats and cynomolgus monkey not being explained by metabolism of NO-1886, the amount of excreted metabolites in the urine and faeces was twice as different. This discrepancy might depend on the metabolic rate. Therefore, the metabolic rate of NO-1886 was estimated in the cynomolgus monkey liver.

Incubation with NO-1886 in cynomolgus monkey liver S9 or microsomes resulted in the formation of one major metabolite (M-2) and a number of minor metabolites (figure 3 and table 6). Hence, the O-deethylation of diethyl phosphonate to monoethyl phosphonate is the major metabolic pathway of NO-1886 in cynomolgus monkey liver, with the hydrolysis of the amide bond as the minor pathway, as is the case in humans. The results of the present study suggest that the biotransformation of NO-1886 to M-2 occurs in microsomes rather than in the cytosol and that this reaction requires NADPH, in contrast to the hydrolysis of the amide bond of NO-1886. The Eadie–Hofstee plots indicated that at least two enzymes in cynomolgus monkey liver microsomes catalyse M-2 formation (figure 4). The K_{m} values of the high- and low-affinity components in cynomolgus monkeys were the same of those in humans (Morioka *et al.* 2002). On the other hand, rats shows only low-affinity component which activity is lower than that in cynomolgus monkeys (data not shown). The lower excreted amount in cynomolgus monkeys than in rats might be explained by further metabolism, such

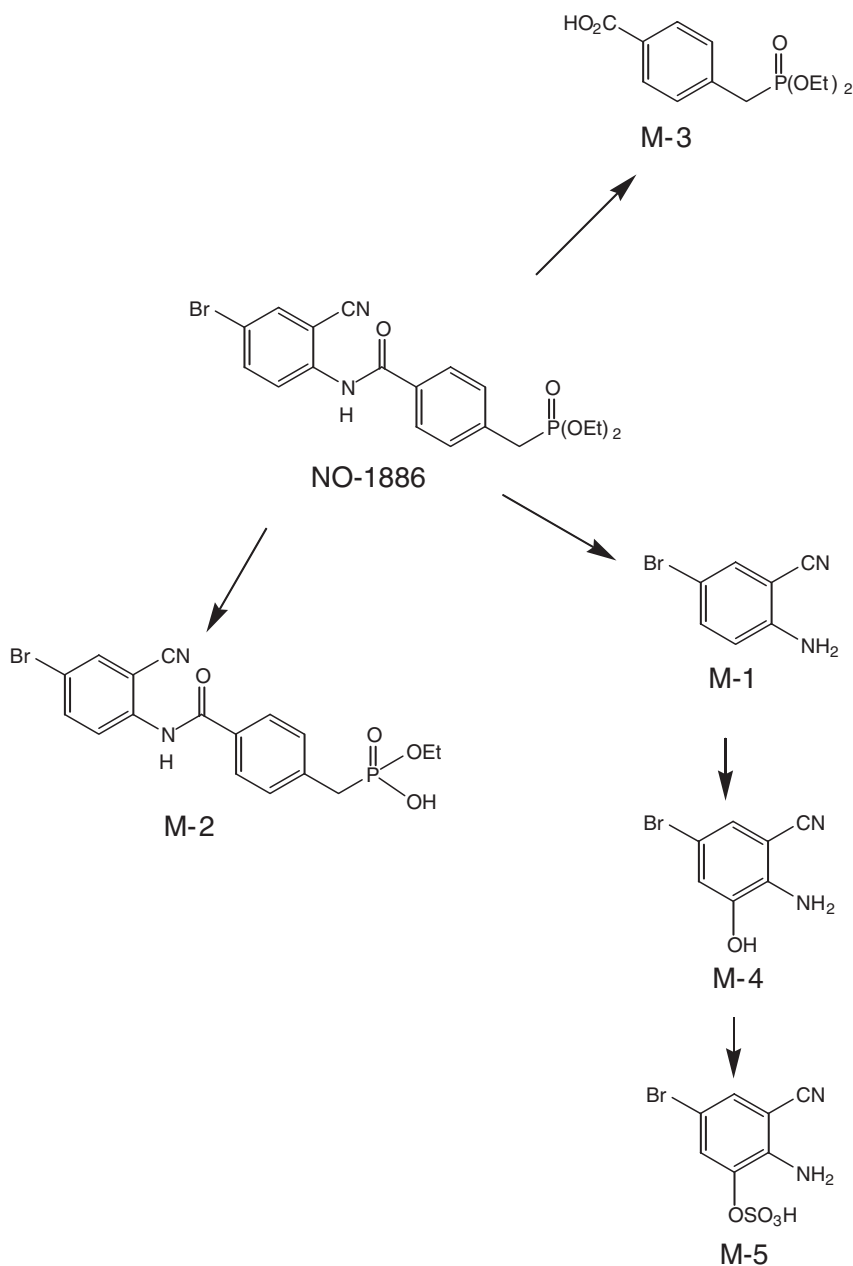


Figure 5. Proposed metabolic pathways of NO-1886 in cynomolgus monkeys. Et, ethyl.

as glucuronide and sulphate. There is also a possibility of biotransformation of M-2 and M-3 to unknown metabolites. Furthermore, it is thought that the metabolites such as M-2 and M-3 distribute into several tissues and their back diffusion from the tissues into plasma is slow. The further experiments should need to clarify the discrepancy between the metabolic rate of NO-1886 and the total excretion amount in cynomolgus monkeys.

Furthermore, it seems that the O-deethylation activity of NO-1886 in cynomolgus monkeys is higher than that in humans because the $V_{\max}$ in cynomolgus monkeys is higher than that in humans. Furthermore, the unbound fractions of NO-1886 in human plasma and cynomolgus monkey plasma were 6.7 and 14%, respectively. It is therefore thought that the metabolic clearance of NO-1886 in human liver is lower than that in cynomolgus monkeys, with delayed elimination of NO-1886 from human plasma compared with cynomolgus monkey plasma.

Esterases are capable of catalysing hydrolytic reactions of several types of bonds such as those of carboxylester, carboxamide, carboxythioester and phosphoric acid ester (Sato and Hosokawa 1998). The hydrolysis of the amide bond of NO-1886 is the second most important metabolic pathway in rats (Morioka *et al.* 1996), and it occurs mainly in rat plasma (data not shown). On the other hand, amide cleavage scarcely occurs in cynomolgus monkey plasma (data not shown). The results of an inhibition study in liver S9 using BNPP, a carboxylesterase inhibitor (Frank *et al.* 1986), showed that the biotransformation of NO-1886 to M-3 involves carboxylesterase. In addition, the M-3 concentration per unit protein content was greater in microsomes than in the S9 fraction, indicating that the amide bond was mainly hydrolysed in microsomes.

Based on the above findings, the proposed metabolic pathways of NO-1886 in cynomolgus monkeys are shown in figure 5. The main metabolic pathway of NO-1886 in cynomolgus monkeys is the O-deethylation of NO-1886 to M-2, as in rats and humans, and the hydrolysis of the amide bond is a minor metabolic pathway.

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