

Effects of renal diseases on the regulation and expression of renal and hepatic drug-metabolizing enzymes: a review

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1. The activity of drug-metabolizing enzymes (DMEs) in extrahepatic organs is highest in the kidneys. Generally, the kidneys contain most, if not all, of the DMEs found in the liver. Surprisingly, some of these DMEs show higher activity in the kidneys than in the liver.
2. Most of the renal DMEs are localized in the cortex of the kidneys, especially in the proximal tubules. DMEs are also found in the distal tubules and collecting ducts.
3. Renal diseases such as acute and chronic renal failure and renal cell carcinoma alter the regulation of both hepatic and extrahepatic phase I and II DMEs. Changes in the expression of these DMEs seem to be tissue and species specific.
4. Generally, there is significant down-regulation of most of the phase I and a few of phase II DMEs at the protein, mRNA and activity levels. Unfortunately, the mechanisms leading to the alteration in DMEs in renal diseases remain unclear, although many theories have been made.
5. The presence of some circulating factors such as cytokines, nitric oxide, parathyroid hormones and increased intracellular calcium play a role in the regulation of DMEs in renal diseases.

Introduction

Initial drug-metabolism studies in the 1960s and early 1970s focused on the role of the liver in the metabolism of exogenous and endogenous compounds. As the science of drug metabolism matured, the studies were no longer limited to the liver, but expanded to include extrahepatic organs. Drug-metabolizing enzymes (DMEs) have been identified and functionally characterized in almost every organ in the body including the kidneys, lungs, intestines, breasts, brain, skin, heart, placenta and pancreas (Kapitulnik *et al.* 1999). Contradictory to early beliefs, the kidneys share similar drug metabolism and clearance processes with the liver as they contain most, if not all, phase I and II DMEs (Ikemoto *et al.* 1992, Lohr *et al.* 1998). Furthermore, malfunction of the kidneys results in pathological and physiological alterations in every organ system of the body.

The purposes of this review are (1) to discuss the distribution and localization of DMEs in the kidneys; (2) to shed light on the effects of various renal diseases, especially renal failure (RF) and renal cell carcinoma (RCC), on the regulation and expression of renal and hepatic DMEs, (3) to explore possible mechanisms by which such changes may occur; and (4) to provide some clinical applications and examples for these changes on drug metabolism and elimination.

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Regional distribution and localization of DMEs in the kidneys

The kidney participates, along with the liver, in the processes of drug metabolism and detoxification. The distribution of the hepatic DMEs has been extensively studied (Hakkola *et al.* 1994, Morgan 1997, 2001). Unlike the liver, the heterogeneity of the kidney makes defining the regional distribution and localization of DMEs important, as there are limited data available on the distribution of renal DMEs. Therefore, defining the DMEs in the kidneys and their localization will help to understand the regulation and expression of these DMEs in renal diseases. The human kidney is composed of two main regions: the cortical region, where most of the DMEs are located, and the medullary region, which is divided into inner and outer zones (Lohr *et al.* 1998). Each kidney contains about 1 million nephrons and each nephron consists of a Bowman's capsule, a proximal tubule (PT), a Loop of Henle, a distal tubule (DT) and a collecting duct. DMEs are expressed in various amounts in the different segments of the nephron, being concentrated mainly in the cortices, and especially the PT (Hjelle *et al.* 1983, Lohr *et al.* 1998).

Renal drug-metabolism pathways

Among extrahepatic tissues, the kidney contains the highest activity of most DMEs (Guidet and Shah 1989, Leblond *et al.* 2001). Generally, all hepatic DMEs are found in the kidney but at a lower concentration than in the liver (Anders *et al.* 1980, Hjelle *et al.* 1983, Ikemoto *et al.* 1992, Lohr *et al.* 1998, Cummings *et al.* 1999, Schaaf *et al.* 2001). Certain regions of the kidney, however, such as PTs, contain DMEs that have higher activity than the corresponding enzymes in the liver. Examples of such DMEs include aldehyde dehydrogenase (ALDH) (Hjelle *et al.* 1983), uridine diphosphate glucuronosyl transferase (UDPGT) (Ikemoto *et al.* 1992, McGurk *et al.* 1998), N-acetyl transferases (NAT) (Hjelle *et al.* 1983) and cytochrome P4501A1 (CYP1A1) (Schaaf *et al.* 2001).

The next section presents a description of the most extensively studied phase I and II DMEs in the kidney, their localization in different segments of the kidney and how their activities compared with the activities of hepatic enzymes. Table 1 shows a detailed summary of the renal distribution of DMEs.

Renal phase I DMEs

Cytochrome P450 (CYP). The most extensively studied DMEs in the kidney are the CYPs. CYPs have been known to be expressed in the kidney since the early 1960s, but no active work was conducted on them until the 1980s (Lohr *et al.* 1998). CYP catalyses the oxidation of a large group of drugs and other xenobiotics. The renal CYP activity represents approximately 15–70% of the activity found in the liver (Ikemoto *et al.* 1992, Nagase *et al.* 1996, Lohr *et al.* 1998), depending on which region of the kidney is studied. The distribution of several CYP enzymes in the rat (Anders *et al.* 1980, Davis *et al.* 1981, Hjelle *et al.* 1983, Schaaf *et al.* 2001) and human (Ikemoto *et al.* 1992, Hakkola *et al.* 1994, Lock *et al.* 1998) kidney nephron segments has been determined. CYP enzymes are located in all segments of the nephron but are mainly concentrated in the microsomal fraction of the cortex region and the PT, with the highest concentration found in the convoluted (S₂) and straight (S₃) segments, and the lowest in the inner medulla (Cojocel *et al.* 1983, Foster *et al.* 1986).

CYP1 family. Renal CYP1A1 is detected only in the PT of the kidney and in higher concentrations than those of CYP1A2, the predominant CYP in the liver (Ikemoto *et al.* 1992, Schaaf *et al.* 2001). Ethoxyresorufin *O*-deethylase (EROD, CYP1A1 substrate) activity in human PT cells has been shown to be threefold higher than pentoxyresorufin *O*-deethylase (PROD, CYP2B) activity (Koop *et al.* 1991), indicating that CYP1A1 is a major CYP in the kidney. Furthermore, EROD activity is 44% higher in PT cells compared with rat liver homogenate and 400% higher than the activity of whole kidney microsomes, respectively (Schaaf *et al.* 2001). Surprisingly, the glomerular mesangial cells in the rat kidney have been shown to express atypical inducible forms of CYP1A1 and 1B1. Both CYP1A1 and 1B1 mRNA amounts are highly induced by benzo[a]pyrene more than by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), without any corresponding increase in EROD activity (Scarborough *et al.* 1999).

CYP2 family. A low level of CYP2B1/2 protein has been observed in rat PT and DT cells. Furthermore, treatment of rats with clofibrate induces the expression of CYP2B1/2 in whole kidney microsomes, but not in microsomes of the cortical, PT and DT cells (Cummings *et al.* 1999). Similarly, a recent study did not detect CYP2B protein and activity, measured by testosterone 16 β -hydroxylation, in rat PT cells (Schaaf *et al.* 2001).

CYP2C11 has been detected in the PT cells obtained from rat kidney, but not in DT cells (Cummings *et al.* 1999). The level of CYP2C11 expressed in the kidney is only 10% of that detected in the liver. Testosterone 2 α - and 16 α -hydroxylation, which are associated with CYP2C11 activity, has been demonstrated in rat PT cells. Another study, however, did not detect CYP2C11 mRNA in male rat kidneys (Schaaf *et al.* 2001).

Another subfamily detected in the kidney is CYP2D. This subfamily accounts for only 1.7% of the total CYP in the kidney compared with 9.8% in the liver, indicating its minor contribution in renal metabolism (Nagase *et al.* 1996). When rat renal microsomes were incubated with debrisoquine, a CYP2D substrate, the percentage of the renal 4-hydroxy metabolite was only 3% of that produced when incubated with liver microsomes. In addition, the mRNA expression of CYP2D1 in rat kidneys is much lower than in the liver (Nagase *et al.* 1996).

Studies have shown that the CYP2E1 protein content is 30% higher in the DT segments of rat kidneys when compared with the PT segments (Cummings *et al.* 1999). A threefold greater mRNA level in the DT cells has also been reported (Cummings *et al.* 1999, Schaaf *et al.* 2001). However, the activity of CYP2E1, using 6-chlorozoxazone as a substrate, was 2.7-fold higher in the PT than in the DT cells (Cummings *et al.* 1999). This is in agreement with other studies that showed that CYP2E1 is markedly localized in the PT of the mouse kidney (Ikemoto *et al.* 1992).

Members of the novel subfamily CYP2J are detected predominantly in extrahepatic tissues including the heart, intestines and the kidneys, although some, such as CYP2J5, are found in the liver (Krause *et al.* 2003). The human CYP2J2 is present in the kidney, but at a much lower level than the heart (Krause *et al.* 2003). It is mainly distributed in the PT, collecting duct and in the DT cells of rats, humans and mice. CYP2J2 was not detected in the glomerulus and the thin Loop of Henle (Lock *et al.* 1998).

Table 1. Regional and tubular localization of the drug-metabolizing enzymes in the kidney and nephron segments of different species.

Drug-metabolizing enzyme	Species	Regional			Tubular			Reference
		Cortex	Medulla	PT	Loop of Henle	DT	Collecting duct	
Phase I								
CYP	human, rat, rabbit, mouse, hamster	+++ ^O	++	+++ ^{S2/3}				Anders (1980), Davis <i>et al.</i> (1981), Gibson (1986), Lohr <i>et al.</i> (1998)
CYP1A	human, rat, rabbit	+		+				Koop <i>et al.</i> (1991), Ikemoto <i>et al.</i> (1992), Schaaf <i>et al.</i> (2001)
CYP2B1/2	rat	+		+		+		Cummings <i>et al.</i> (1999), Schaaf <i>et al.</i> (2001)
CYP2C	human, rat, rabbit	+		++		+		Cummings <i>et al.</i> (1999), Ikemoto <i>et al.</i> (1992), Schaaf <i>et al.</i> (2001)
CYP2D1	rat			++				Schaaf <i>et al.</i> (2001)
CYP2E1	rat, mouse	++		++		++		Cummings <i>et al.</i> (1999), Ikemoto <i>et al.</i> (1992)
CYP2J	human, rat, mouse		+	++ ^{S3}		+	++	Lock and Reed (1998), Krause <i>et al.</i> (2003)
CYP3A	human, rat	+++	+++	+++		+	+++	Ikemoto <i>et al.</i> (1992), Murray <i>et al.</i> (1999), Schaaf <i>et al.</i> (2001)
CYP4A	rat	++	+++	+++	+++	+	++	Ikemoto <i>et al.</i> (1992), Ito <i>et al.</i> (1998), Cummings <i>et al.</i> (1999)
NADPH-cytochrome reductase		+++ ⁱ	+	+				Lohr <i>et al.</i> (1998), Gibson (1986)
Flavin monooxygenase	human, rat, mouse	++	++	++		++	+	Krause <i>et al.</i> (2003), Lohr <i>et al.</i> (1998)
Monoamine oxidases	human, rat	++	+	++				Gibson (1986), Fernandes and Soares (1992)
MAO-A	human, rat	++	++	++				Fernandes and Soares (1992), Lohr <i>et al.</i> (1998)
MAO-B	human, rat, rabbit	++	+	+				Fernandes and Soares (1992), Lohr <i>et al.</i> (1998)

Alcohol dehydrogenase									Gibson (1986)
Aldehyde dehydrogenase									Hjelle <i>et al.</i> (1983)
Aldehyde reductase									Lohr <i>et al.</i> (1998)
Ketone reductase									Lohr <i>et al.</i> (1998)
Epoxide hydrolase								++	Ikemoto <i>et al.</i> (1992), McKay <i>et al.</i> (1995)
Carboxylesterase								+	Ikemoto <i>et al.</i> (1992)
Peptidases									Anders (1980), Gibson (1986)
Prostaglandin H synthase									Zenser and Davis (1984)
Phase II									
UDP-glucuronyl transferases								+	Gibson (1986), Schaaf <i>et al.</i> (2001), Ikemoto <i>et al.</i> (1992)
Sulfotransferases									Anders (1980), Ikemoto <i>et al.</i> (1992)
Glutathione S-transferases								+	Gibson (1986), Anders (1980), Schaaf <i>et al.</i> (2001), Ikemoto <i>et al.</i> (1992)
GST- α								+	Ikemoto <i>et al.</i> (1992), Overby <i>et al.</i> (1994), Rodilla <i>et al.</i> (1998)
GST- μ								++	Ikemoto <i>et al.</i> (1992), Rodilla <i>et al.</i> (1998)
GST- π								++	Rodilla <i>et al.</i> (1998), Ikemoto <i>et al.</i> (1992)
GST- ρ								+	Ikemoto <i>et al.</i> (1991, 1992)
N-acetyltransferases								+	Anders (1980), Gibson (1986)
Methyl transferases									Lohr <i>et al.</i> (1998)
COMT									Lohr <i>et al.</i> (1998)
γ -Glutamyl transpeptidase								+	Lock and Reed (1998), Marathe <i>et al.</i> (1979)
Cysteine conjugate β -lyase									Kim <i>et al.</i> (1997), Stevens (1985)

^O, Outer; ⁱ, inner; ^{S2}, convoluted PT; ^{S3}, straight PT; ND, not detected; +, low level of expression; ++, intermediate level of expression; +++, high level of expression.

CYP3 family. The expression of CYP3A enzymes in the kidney has been extensively studied, particularly CYP3A1 and CYP3A2 in rats and CYP3A4 and CYP3A5 in human (Scuets *et al.* 1992, Cummings *et al.* 1999, Schaaf *et al.* 2001). CYP3A1/2 protein level in rat kidney microsomes, however, is approximately 10% of that observed in liver microsomes (Cummings *et al.* 1999). There have been contradictory reports concerning the distribution of CYP3A in the kidney. Some studies have reported that CYP3A1/2 protein and 3A1 mRNA were not detected in rat PT or DT microsomes (Cummings *et al.* 1999). In addition, 6 β -hydroxylase activity, a marker for CYP3A activity, was not detected in the rat PT cell (Schaaf *et al.* 2001). It is likely, therefore, that members of the CYP3A subfamily are expressed in other areas of the rat nephron, such as the glomerulus (Lohr *et al.* 1998). In the human kidney, however, CYP3A4 has been shown to be expressed in the PT cells (Murray *et al.* 1988, Ikemoto *et al.* 1992).

CYP4 family. CYP4A2 represents the highest proportion of total CYP in the rat kidney (Nagase *et al.* 1996). However, CYP4A1, 4A2 and 4A3 are all highly expressed in all segments of the rat nephron including the glomerulus, the PT and the thick ascending limb of the Loop of Henle (Ito *et al.* 1998) with the highest expression being in the PT cells (Schaaf *et al.* 2001). The renal expression of CYP4A1, 4A3 and 4A8 mRNA was similar in female and male rats, whereas CYP4A2 mRNA expression was four times higher in male than female rats, indicating that the expression of CYP4A subfamilies in the rat kidney is sex dependent (Ito *et al.* 1998, Schaaf *et al.* 2001).

Flavin monooxygenases (FMOs). FMOs are microsomal enzymes that oxidize nucleophilic nitrogen-, sulphur- and phosphorus-containing xenobiotics. FMOs are found in the liver, kidneys and lungs (Lemoine *et al.* 1990, Baer *et al.* 1997, Krause *et al.* 2003). There are five different families of FMOs: FMO1–5. FMO1 is highly expressed in the human, mouse and rat kidneys, whereas FMO3 is high in the kidneys of rats and mice, but low in humans (Dolphin *et al.* 1991, Baer *et al.* 1997). Immunoblots performed on human kidney microsomes, using antibodies to FMO1, FMO3 and FMO5, have shown that the expression of FMO1 is 11- and 2.5-fold higher than FMO3 and FMO5, respectively (Krause *et al.* 2003). The distribution of FMO2 has been studied in rat kidneys and has been shown to be localized in the PT, DT and the collecting duct (Krause *et al.* 2003). There also seems to be ethnic differences in the expression of FMO1 in the kidneys. The expression of FMO1 in kidney samples from African-Americans is 18% higher than those obtained from Caucasians (Krause *et al.* 2003).

Renal FMOs have a minimal contribution to drug metabolism in comparison with their hepatic counterparts. However, the renal N-oxidation of tropsetron, a 5HT₃ antagonist, is considered to be the major renal metabolic pathway (Vickers *et al.* 1996). In addition, imipramine, a tricyclic antidepressant, was metabolized in the kidney only by FMO to its N-oxide metabolite (Lemoine *et al.* 1990).

Monoamine oxidases (MAOs). MAOs are mitochondrial oxidoreductases that catalyse the metabolism of aliphatic, aromatic, primary, secondary and tertiary amines. The total activity of MAO in the kidneys is approximately 30% of that in the liver in humans (Vogel *et al.* 1983). MAO-A and MAO-B are both found in the kidneys (Vogel *et al.* 1983, Fernandes and Soares 1992), with MAO-A being the

predominant form in rats and MAO-B being the predominant form in rabbits (Gargalidis-Moudanos *et al.* 1997, Lohr *et al.* 1998). Both enzymes are highly expressed in the PT cells in rats (Lohr *et al.* 1998). The Bowman's capsule and collecting duct, however, express only MAO-A (Rodriguez *et al.* 2001). In the human kidneys, the activity of MAO-A is similar in the cortex and the medulla, although MAO-B activity is higher in the cortex than the medulla. In contrast, the activity of MAO-A is higher than MAO-B in both the cortex and the medulla in rat kidneys (Fernandes and Soares 1992).

Alcohol dehydrogenases (ADHs). Alcohol oxidation occurs mainly in the liver; however, renal metabolism still plays an important role in its elimination (Hjelle *et al.* 1983). The human ADH family consists of seven isoenzymes: ADH1–7 (Lohr *et al.* 1998). The activity of ADH from baboon kidney extracts, using ethanol as a substrate, represented 30% of that observed in liver extracts (Holmes *et al.* 1986). In rat kidneys, ADH mRNA was expressed only in the inner medulla and cortex (Gibson 1986). The threefold increase in ADH activity and mRNA levels in rat kidneys, but not the liver, after treatment with oestradiol elucidates the role of hormones in controlling renal ADH (Qulali *et al.* 1993).

Aldehyde dehydrogenases (ALDHs). ALDHs are mitochondrial and cytosolic enzymes that oxidize aldehydes to their corresponding carboxylic acids (Hjelle *et al.* 1983, Lohr *et al.* 1998). Several ALDH isoenzymes in the kidneys have been detected and described in different species. ALDH1 is located in the cytosol, ALDH2 and ALDH4 are located in the mitochondria, and ALDH5 is located in both the mitochondria and the cytosol (Holmes *et al.* 1991). The overall ALDH activity in rat kidneys is about 20–80% of that in the liver (Dipple and Crabb 1993). ALDH activity in the subcellular fraction isolated from PT cells of rabbit kidneys was much higher than the activity observed in samples obtained from rabbit livers (Hjelle *et al.* 1983). The presence of ALDH in higher concentration than CYP in the PT highlights the protective role of ALDHs in metabolizing aldehydes, which are considered reactive substances generated from the metabolism, by CYP, of a variety of compounds (Hjelle *et al.* 1983, Lohr *et al.* 1998).

Prostaglandin H synthase (PHS). PHS is an enzyme responsible for the synthesis of prostaglandins in the kidneys. It contains both cyclooxygenase and peroxidase activities, therefore several xenobiotics, such as acetaminophen, non-steroidal anti-inflammatory agents, are substrates for the renal PHS (Davis *et al.* 1981, Lock and Reed 1998). It has been reported that PHS has a higher level of activity in the inner than the outer medulla in rabbit kidney, whereas it is barely detected in the cortex (Zenser and Davis 1984). At the mRNA level, Cowley *et al.* (1995) reported an increase in the expression of PHS-1 mRNA in dog renal medulla.

Renal phase II DMEs

Uridine diphosphate glucuronosyl transferases (UDPGTs). Extrahepatic glucuronidation is generally lower than that of the liver (McGurk *et al.* 1998). However, it has been observed that 25% of the endogenously formed glucuronide conjugate of *p*-nitrophenol is synthesized and excreted directly by the rat kidneys (Anders 1980). In addition, although uridine diphosphate glucuronic acid

(UDPGA) concentration in the kidneys is a 15th of that in the liver of humans, the UDPGT activity of the human kidneys is one-half of that of the liver (Cappiello *et al.* 1991). The kidney enzymes, therefore, seem to have a higher affinity for the substrate than the liver enzymes (Guidet and Shah 1989).

UDPGTs activity is greatest in the PT cells, with some activity observed in the DT cells (Gibson 1986, Ikemoto *et al.* 1992, Lohr *et al.* 1998). UDPGTs in foetal human kidneys are also distributed in the collecting duct (Ikemoto *et al.* 1992). The activity of UDPGTs in the kidneys is substrate- and species-dependent (Ander 1980, McGurk *et al.* 1998). Rat kidney UDPGTs cannot glucuronidate morphine, although UDPGTs in the human kidneys glucuronidate morphine at a similar rate as human liver UDPGTs (Ikemoto *et al.* 1992). Propofol, an anaesthetic, is also glucuronidated to a greater degree (fourfold more) by the human kidneys than by the human liver (McGurk *et al.* 1998). Interestingly, it has been observed that 3-methyl-cholanthrene (3MC) and phenobarbital (PB) induce hepatic, but not renal, UDPGTs in rats (Anders 1980). In contrast, TCDD induces the renal UDPGTs by twofold compared with 10-fold in the rat liver (McGurk *et al.* 1998).

Glutathione S-transferases (GSTs). GSTs are cytosolic enzymes that catalyse the conjugation of glutathione with an electrophilic substrate (Rodilla *et al.* 1998). Human kidneys contain the second highest activity of GSTs, after the liver (about 78% of the human liver) (Pacifi *et al.* 1988). Of the 20 GST isoenzymes located in the different human tissues, only a few are located in the kidney (Lohr *et al.* 1998). The rat kidney, on the other hand, all the major isoforms of GST are highly expressed (GST- α , - μ , - π and - ρ) (Ikemoto *et al.* 1992, Lohr *et al.* 1998). Among the human GSTs, the α isoform represents 50–75% of the total GST protein (Singh *et al.* 1987). GST- α is expressed mainly in the convoluted PT, but it is also found in the thin Loop of Henle of man (Singh *et al.* 1987, Rodilla *et al.* 1998) and rat (Ikemoto *et al.* 1992, Lohr *et al.* 1998) kidney. GST- μ and - π are localized in Bowman's capsule (Rodilla *et al.* 1998), the DT and the collecting duct in rat kidneys (Ikemoto *et al.* 1992, Rodilla *et al.* 1998). GST- ρ , on the other hand, was detected in the thin Loop of Henle, the collecting duct (Overby *et al.* 1994) and to a lesser extent in the DT (Ikemoto *et al.* 1992) in the rabbit kidney. A similar distribution of the GST classes in the rat kidney has been observed in the human kidney, with the absence of the GST- μ class (Ikemoto *et al.* 1992).

It has been reported that there is a significant difference between the hepatic and renal GSTs in their substrate specificity. One study reported that the K_m of the renal GSTs toward 1-chloro-2,4-dinitrobenzene (CDNB), a GST substrate, was significantly greater than the K_m of GST in the liver of rats. The V_{max} of renal GST activity, however, ranged from 34 to 56% of the V_{max} of the hepatic GST enzymes (Tarloff *et al.* 1991). Furthermore, hepatic GSTs are mainly regulated by xenobiotics, whereas renal GSTs are not (Pickett and Lu 1989). It has been shown that the hepatic, but not renal, μ - and π -class are significantly induced by 3MC and PB (Derbel *et al.* 1993).

N-acetyltransferases (NATs). NATs catalyse the acetylating reaction of aryl amines including isoniazide, procainamide and sulfamethazine. They have been shown to be localized predominantly in the DT of the rat kidney in both the cortex and medulla. NAT is found in the rat kidney homogenate at a higher concentration

than in other tissues, but at a lesser amount than in the liver (Lohr *et al.* 1998). Renal-to-hepatic NAT activity ratio, using *p*-aminobenzoic acid as a substrate, is >0.94 in mice and 1.0 in rabbits and guinea pigs (Gibson 1986). Interestingly, in the human kidney, the ratio is only 0.29 (Guidet and Shah 1989), indicating interspecies differences in the activity of NATs.

Sulfotransferases (SULTs). SULTs are cytosolic enzymes that catalyse the sulfation of many endogenous compounds, including bile acids, by transferring a sulfonyl group from 5'-phosphoadenosine 3'-phosphosulfate (PAPS) to an acceptor molecule. The renal concentration of PAPS is approximately $4.8 \mu\text{mol mg}^{-1}$ tissue, an amount 21% of that of the liver (Capiello *et al.* 1989). SULTs are greatly localized in the rat and human PT cells, higher by about twofold than that of the cortex, with some localization also in the collecting duct in the medulla (Ikemoto *et al.* 1992). SULTs activity, using 2-naphthol as a substrate, is minor compared with the activity of the liver: $48.2 \text{ pmol min}^{-1} \text{ mg}^{-1}$ microsomal protein compared with $592 \text{ pmol min}^{-1} \text{ mg}^{-1}$ microsomal protein in the human liver (Guidet and Shah 1989). It has also been reported that the sulphate conjugation of acetaminophen in isolated perfused human kidney cells is 5% of that of the liver (Lohr *et al.* 1998).

γ -Glutamyl transpeptidase (γ GT). γ GT is a membrane-bound enzyme located in many cell types including the kidney tubules, particularly the PT. The enzyme catalyses hydrolysis and transpeptidation reactions in the formation of mercapturic acids (Elfarra and Anders 1984). The enzyme is mainly located in the brush border membranes of the PT in the rat kidneys (Lock and Reed 1998) and, to lesser extent, in the glomeruli (Sochor *et al.* 1980). Microdissection of rat nephrons showed that straight PTs (S3) exhibit about twice as much γ GT activity as proximal convoluted tubules (S1 and S2) (Marathe *et al.* 1979). The kidney in several mammalian cells, including humans, has the highest activity of γ GT, as it has been reported that γ GT specific activity in rat kidney is twofold higher than that in the liver (Lock and Reed 1998).

Cysteine conjugate β -lyase (CC β L). CC β L is a pyridoxal phosphate-dependent enzyme that catalyses the β -elimination or transamination of cyteine conjugates. In human and rat kidneys, cortex exhibits the highest CC β L activity, particularly in the cytosolic fraction (Stevens 1985, Kim *et al.* 1997). In addition, CC β L has been found in all segments of the PT (Kim *et al.* 1997).

In general, the kidney contains all DMEs that are located in the liver and is therefore considered to be the organ with the second largest xenobiotic metabolizing activity, second only to the liver. Most renal DMEs are concentrated in the PT of the kidney either in the cytoplasmic or microsomal fraction. PT cells are the first part of the kidney to be exposed to the glomerular filtrates and they contain the transport systems of many drugs. The PT cells are the most active cells in the kidney in metabolizing xenobiotics and endogenous substances.

Effects of renal diseases on the expression and regulation of DMEs

Many studies have revealed a correlation between a decrease in creatinine clearance and a decrease in drug elimination in patients with renal disease (Uchida *et al.* 1995, Leblond *et al.* 2001). With recent evidence showing that the kidney possesses a considerable concentration of most DMEs, many researchers are now looking at the effect of renal diseases on these enzymes. Pathophysiological changes associated with renal diseases have been shown to alter DMEs in the kidneys, liver and intestines (Pacifici *et al.* 1988, Akahoshi *et al.* 1995, Guevin *et al.* 2002).

In the following section, the focus will be mainly on the effect of the highly prevalent renal diseases, such as RF and RCC, on hepatic, renal and intestinal phase I and II DMEs. Other renal diseases will also be discussed. Changes in these enzymes at the mRNA, protein and activity levels will be examined. A summary of these changes is produced in table 2.

Acute renal failure (ARF)

Unilateral nephrectomy has been used as an experimental model for studying the effect of ARF on DMEs in rats (Greenspan and Baron 1980). It has been observed that there is a 15–24% reduction in the hepatic total CYP activity 4 days post-unilateral nephrectomy (Greenspan and Baron 1980). The remaining kidney seems to increase its functional capacity by the process of renal hypertrophy. This may explain the observation that there is no significant change in the hepatic CYP content 15 days post-procedure.

Contradictory to the above results, ARF in rats, induced by intravenous administration of uranyl nitrate, produced a fourfold increase in CYP2E1 mRNA and a twofold increase in its protein level. The catalytic activity of CYP2E1, using 6-hydroxychlorzoxazone as a substrate, was also increased (Chung *et al.* 2002). This change was not seen, however, when *p*-nitrophenol was used as a substrate. The total CYP activity was also increased in rats with ARF, although CYP1A2, 2B1/2 and 3A23 activities were not significantly affected (Chung *et al.* 2002).

Similar to the phase I DMEs, UDPGT activity is also altered in ARF (Elston *et al.* 1993). There have been reports of both UDPGT inhibition and induction of UDPGT activity in azotemic rats (Patterson and Cohn 1984, Gibson 1986, Elston *et al.* 1993). A significant reduction in the rate of glucuronidation of phenolphthalein has been observed 1 day after total nephrectomy and 14 days after subtotal nephrectomy (Ali *et al.* 1979). The degree by which the glucuronidation rate is changed varies, depending on the severity and/or the duration of the acute renal problem (Patterson and Cohn 1984, Leblond *et al.* 2000). Recently, Westhuyzen *et al.* (2003) reported a significant increase in the urinary excretion of γ GT in ARF patients. Similarly, aminoglycoside-induced ARF in dog is also associated with a threefold increase in the mean urine γ GT-to-creatinine ratio (Rivers *et al.* 1996). γ GT activity has been shown to be increased in both cortical and medullar regions of ischaemic rat kidney (Cutrin *et al.* 2000).

Chronic renal failure (CRF)

DMEs are also altered in CRF. The degree by which these enzymes are altered is proportional to the severity of the renal impairment, as measured by increased blood urea nitrogen and creatinine (Chapman and Goldstein 1995, Uchida *et al.* 1995, Guevin *et al.* 2002).

Table 2. Effect of renal diseases on drug-metabolizing enzymes in various tissues.

DMEs	Renal impairment	Species	Tissue	Effects	Enzymes	Reference
Phase I Total CYP	acute renal failure	rat	H	↓		Greenspan and Baron (1980)
			H	↑ ↑↑		Chung <i>et al.</i> (2002)
	chronic renal failure	rat	H	↓		Patterson and Cohn (1984)
			H	↓		Uchida <i>et al.</i> (1995)
		rabbit	I	↓↓		Leblond <i>et al.</i> (2002)
	hydronephrosis	rat	R	↓↓↓		Zenser <i>et al.</i> (1984), Gibson (1986)
	nephrotoxicity	rat	H	↓		Mayer <i>et al.</i> (1989)
			R	↑↑↑		Mayer <i>et al.</i> (1989)
	uraemia	rat	H	↓		Guevin <i>et al.</i> (2002)
	acute renal failure	rat	H	↔	1A2	Chung <i>et al.</i> (2002)
CYP1 family	chronic renal failure	rat	I	↓↓	1A1	Leblond <i>et al.</i> (2002)
			H	↑		Uchida <i>et al.</i> (1995)
		human	H	↔		Leblond <i>et al.</i> (2000)
	renal cell carcinoma	human	RH	↑	1A1	McKay <i>et al.</i> (1995)
		rat	R	↓	1A1	Cheung <i>et al.</i> (1999)
		human	R	↑	1A1	Okamoto <i>et al.</i> (2001)
			R	↓	1A2	Cheung <i>et al.</i> (1999)
			R	↑	1B1	Cheung <i>et al.</i> (1999), Patterson and Murray (2002)
	acute renal failure	rat	H	↔	2B1/2	Chung <i>et al.</i> (2002)
			H	↓	2C11	Chung <i>et al.</i> (2002)
CYP2 family			H	↓	2D6,9	Lash and Tokarz (1989)
			H	↑↑↑	2E1	Chung <i>et al.</i> (2002)
	chronic renal failure	rat	H	↑↑↑	2A1	Pacifici <i>et al.</i> (1988)
			I	↔	B1/2	Leblond <i>et al.</i> (2002)
			H	↑↑	2C11	Uchida <i>et al.</i> (1995), Leblond <i>et al.</i> (2000), Leblond <i>et al.</i> (2001)
			I	↔	2C11	Leblond <i>et al.</i> (2002)
			H	↓↓	2C11	Pacifici <i>et al.</i> (1988)
			H	↓	2C6	Uchida <i>et al.</i> (1995)
			I	↔	2C6	Leblond <i>et al.</i> (2002)
			H	↑f, ↓ m	2C6	Pacifici <i>et al.</i> (1988)

Continued

Table 2. Continued

DMEs	Renal impairment	Species	Tissue	Effects	Enzymes	Reference
CYP3 family	uraemia	human	P	↓	2D6	Kevorkian <i>et al.</i> (1996)
		rat	H	↑	2E1	Pacifici <i>et al.</i> (1988)
		rat	H	↓↓	2C11	Guevin <i>et al.</i> (2002)
	acute renal failure chronic renal failure	rat	H	↔	2D1	Guevin <i>et al.</i> (2002)
		rat	H	↔	3A1/2	Chung <i>et al.</i> (2002)
		rat	H	↑↑f, ↓↓ m	3A2	Pacifici <i>et al.</i> (1988)
		rat	H	↓↓↓	3A1/2	Uchida <i>et al.</i> (1995), Leblond <i>et al.</i> (2000), Leblond <i>et al.</i> (2001), Leblond <i>et al.</i> (2002)
	renal cell carcinoma	rat	I	↓↓↓	3A1/2	Elston <i>et al.</i> (1993)
		human	H	↑	3A1/2	Murray <i>et al.</i> (1999)
		human	R	↑	3A4/5/6	Murray <i>et al.</i> (1999)
		rat	H	↓↓↓	3A1/2	Guevin <i>et al.</i> (2002)
		human	R	↓ (↑ mRNA juxtatumour) (4A2)		Goodman <i>et al.</i> (1997)
CYP4 family NADPH CYP reductase	adenocarcinoma	rat	R	↑↑↑↑		Chung <i>et al.</i> (2002)
	acute renal failure	rat	H	↑↑↑↑		Uchida <i>et al.</i> (1995)
	chronic renal failure		H	↔		Mayer <i>et al.</i> (1989)
	nephrotoxicity		R	↔		Zenser <i>et al.</i> (1984), Gibson (1986)
	Hydronephrosis	rabbit	R	↔		Patterson and Cohn (1984) Gibson (1986)
Monoamine oxidases	chronic renal failure	rat	H	↔		
	uraemia		H	↓		

Alcohol dehydrogenase	chronic renal failure uraemia	rat	H	↑ ↑ ↑	Patterson and Cohn (1984)
			H	↑ ↑ ↑ ↑	Patterson and Cohn (1984), Gibson (1986)
Aldehyde dehydrogenase	renal cell carcinoma	human	H	↑ ↑ ↑	Gibson (1986)
Phase II UDP-glucuronyl transferase	Uraemia	rat	R	↓	Balabanov <i>et al.</i> (2001)
			H	↓	Gibson (1986)
Glutathione S-transferase	chronic renal failure	rat	H	↓	Ali <i>et al.</i> (1979)
		human	P	↓	Verbeeck (1982)
	renal cell carcinoma	human	R	↓ ↓	Delbanco <i>et al.</i> (2001), Eickelmann <i>et al.</i> (1994)
			R	↓	
			R	↑	Rodilla <i>et al.</i> (1998)
			R	↓ ↓ ↓	Eickelmann <i>et al.</i> (1994)
N-acetyl transferases	chronic renal failure	human	H	↓	Eickelmann <i>et al.</i> (1994)
NAD(P)H:quinone oxidoreductase	renal cell carcinoma	human	R	↓ ↓	Eickelmann <i>et al.</i> (1994)
γ-glutamyltranspeptidase	acute renal failure	human	R	↑ ↑	Westhuyzen <i>et al.</i> (2003)
		dog	R	↑ ↑ ↑	Rivers <i>et al.</i> (1996)
	nephrotoxicity	mice	R	↑ ↑	de Ceaurriz and Ban (1990)
	renal cell carcinoma	human	R	↑ ↑	Sandock <i>et al.</i> (1997)
Cysteine conjugate β-lyase	nephrotoxicity	mice	R	↑ ↑	de Ceaurriz and Ban (1990)

↑, Increased; ↓, decreased; ↔, not significantly affected; H, hepatic; I, intestine; R, renal; RH, both kidney and hepatic; P, pharmacokinetic study using plasma and urine sample; ↓, mild change (<30%); ↓↓, moderate change (30–69%); ↓↓↓, marked changes (70–99%); ↓↓↓↓, extensive change (> 100%); f, female; m, male.

For severe CRF, patients are at high risk of drug accumulation and thus toxicity (Guevin *et al.* 2002), partly due to changes in DMEs. CRF has been shown to reduce the hepatic and/or renal CYP 20–80%, depending on the degree of renal impairment, the experimental method used for inducing CRF, and the type of cell and species examined (Patterson and Cohn 1984, Uchida *et al.* 1995, Baer *et al.* 1997, Leblond *et al.* 2000). CRF is usually accompanied by a reduction in the kidney mass and thus the number of functioning nephrons. To minimize this reduction, the kidney undergoes a compensatory mechanism as manifested by cellular hypertrophy of the epithelial cells in the cortex and collecting duct. These physiological and pathological changes in the kidney to some extent could contribute to the changes in the DMEs in CRF (McLaren *et al.* 1995). Several studies have examined the changes that occur in DMEs in patients with CRF. Only a few studies have examined the effect of CRF on CYP1A expression. Uchida *et al.* (1995) reported a slight increase in hepatic CYP1A2 in rats with CRF induced by partial nephrectomy. One study, however, failed to show a significant change (McLaren *et al.* 1995). Intestinal CYP1A1, on the other hand, has been reported to be reduced in rats with CRF (Leblond *et al.* 2002). The reduction in intestinal CYP1A1 has been shown to be a result of reduced gene expression, and not increased degradation (Guidet and Shah 1989, Guevin *et al.* 2002, Leblond *et al.* 2002).

CYP2C6 level was not significantly altered in rats with CRF, however *in vitro* incubation of normal rat hepatocytes with 10% serum from rats with CRF reduced the protein level of CYP2C6 and 2C11 by 38 and 43%, respectively, and the mRNA level of CYP2C11 by 27% (Guevin *et al.* 2002). On the other hand, a 65% reduction in CYP2C11 in male rats with CRF was observed, whereas CYP2C12, a typical female-specific form, has been shown to increase by 15% in female rats with CRF (Ikemoto *et al.* 1992). Differences in the regulation of these sex-specific enzymes may account for the differences in expression seen in patients with CRF.

Urine data analysis has shown a decrease in the clearance rate of sparteine, a CYP2D6 substrate, in patients with CRF (Kevorkian *et al.* 1996). It is not plausible to assume that this decrease is due to a reduction in CYP2D6 activity as many other confounding factors, such as reduced renal clearance and changes in protein binding, may account for some of these changes. CYP2D1 mRNA is not altered in rats with CRF compared with control animals (Leblond *et al.* 2001). More studies are needed to investigate the expression of this subfamily in CRF.

In addition, hepatic CYP3A1 and 3A2 proteins are significantly reduced by 70% in rats with CRF (Uchida *et al.* 1995, Leblond *et al.* 2001, Guevin *et al.* 2002). Surprisingly, the hepatic CYP3A2 has been shown to be significantly suppressed in male rats with CRF, but increased in female rats (Pacifi *et al.* 1988). The intestinal microsomal protein level of CYP3A2 is also reduced by 70% (Leblond *et al.* 2002) although the mRNA encoding CYP3A2 is reduced by only 36% (Guevin *et al.* 2002). The decrease in CYP3A2 may only be partially due to a decrease in the gene transcription and/or protein expression (Leblond *et al.* 2001, 2002). CYP3A1 and 3A2 correspond to the human 3A4 enzyme. A decrease in CYP3A4 in CRF patients may lead to significant drug accumulation and toxicity, since many drugs used by CRF patients are metabolized by the CYP3A4 enzyme.

The activity of phase II DMEs also seems to be altered in CRF. A clinical study on 50 children with mild RF reported a 2.5-fold increase in the urinary excretion of d-glucuronic acid, a marker for glucuronidation capacity (Nemeth and

Szelezcki 1981). In addition, the urinary excretion was increased by approximately fourfold when DME inducers, such as steroids or tranquillisers, were administered (Nemeth and Szelezcki 1981). In rats, CRF induced by bilateral nephrectomy resulted in an increase in glucuronyl conjugation (Elston *et al.* 1993). Azotemic rats demonstrate low UDPGT activity (Patterson and Cohn 1984, Lohr *et al.* 1998). Ali *et al.* (1979) observed a significant reduction in the hepatic glucuronidation of phenolphthalein in CRF rats, but no significant change on the 4-nitrophenol glucuronidation was observed. The alteration in UDPGT activity may be due to several complex mechanisms. Systemic deconjugation of the drug glucuronide may occur in CRF (Verbeeck 1982).

Renal cell carcinoma (RCC)

RCC represents 85–90% of all kidney cancers in the adult human (Rintala *et al.* 1998, Murray *et al.* 1999). The aetiology of RCC is not well understood although several environmental risk factors, including smoking and exposure to heavy metals, have been proposed (Murray *et al.* 1999). RCC occurs mainly in the PT epithelium, where most of the phase I and II DMEs are located (Eickelmann *et al.* 1994, Lohr *et al.* 1998, Murray *et al.* 1999). Although most of the experimental studies performed on patients with RCC have been conducted with the aim of developing and modifying therapy for RCC, they have also provided us with valuable information on the expression of these enzymes in tumour tissues.

Kidney tissue samples from 50 patients were examined for their CYP1A1 content (Rintala *et al.* 1998). The tumours were classified as grades I–IV based on the degree of cell differentiation and the number mitoses within the tumour, where grade IV tumours refers to a more aggressive tumour than grade I. The average tumour content of CYP1A1 is greater than the average content in normal tissue of healthy patients. Grade III tumours seem to have the highest enzyme concentration when compared with other tumour grades. Although there was no difference in the CYP1A1 activity of the tumours between patients with organ-confined disease and patients with metastatic tumours, the CYP1A1 activity in normal renal tissue in patients with metastatic disease was higher than in normal renal tissue in patients whose disease has not yet metastasized (Rintala *et al.* 1998). Normal kidney tissues adjacent to the tumours, obtained from kidneys with a grade III tumour, also have higher CYP1A1 enzyme activity when compared with other normal tissue adjacent to tumours with different grades (Rintala *et al.* 1998).

A study on 27 patients with RCC was conducted to identify the expression of CYP1A1 and 1B1 mRNA in both kidney tumour samples and their corresponding paired normal samples. Only 7.5% of the kidney tumour tissue over-expressed CYP1A1 compared with their corresponding normal tissues. There was no apparent difference in the expression of CYP1B1 between tumour and normal kidney tissue (Cheung *et al.* 1999). It has been reported that CYP1A1 has a variable expression pattern in different types of tumours, for example, CYP1A1 is expressed to a lesser degree in breast cancer cells compared with kidney cancer cells (Cheung *et al.* 1999). It has also been observed that the aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) are similarly expressed in tumour and normal kidney samples. This expression parallels the expression of CYP1B1, but not CYP1A1, suggesting that the AHR/ARNT

pathway is not the only pathway regulating the expression of the CYP1 family (Cheung *et al.* 1999).

CYP3A expression in RCC has also been studied. Renal tumours show a significantly higher expression of CYP3A mRNA (up to 70% higher) than in other type of tumours such as breast, colon and lung tumours (Murray *et al.* 1999). The over-expression of CYP3A in kidney tumours may contribute to the intrinsic multidrug resistance that is observed in this type of cancer (Chapman and Goldstein 1995, Patterson 2002).

In addition to phase I DMEs, phase II DMEs are also altered in kidney tumour tissues. NAD(P)H:quinone oxidoreductase (QOR) mRNA level is decreased by 66% in human kidney tumour tissue compared with histologically normal kidney tissue (Eickelmann *et al.* 1994). QOR activity is also suppressed in tumour tissue compared with normal kidney tissue (Eickelmann *et al.* 1994).

GST is another phase II DME that is altered in RCC. Most studies (Sundseth and Waxman 1992, Eickelmann *et al.* 1994, Rodilla *et al.* 1998, Delbanco *et al.* 2001) have reported a decrease in GST activity, using CDNB as a substrate, and mRNA levels in RCC. GST- α , - μ , - π and - θ have also been reported to be decreased by 69, 56, 45 and 69%, respectively (Delbanco *et al.* 2001). Interestingly, there have not been any reports of a significant alteration of GST- ρ in RCC (Sundseth and Waxman 1992). Although Rodilla *et al.* (1998) reported an increase in the GST- α protein in tumour tissue obtained from females compared with control; they observed a decrease in GST- α protein level in the male patients compared with control.

γ GT has also been reported to be increased in patients with RCC. In a clinical study of 53 patients with RCC, 70% of patients with metastatic RCC showed a significant increase in γ GT level compared with only 4% of those who have non-metastatic RCC, suggesting that γ GT could be a sensitive indicator of the disease progress (Sandock *et al.* 1997).

Other renal diseases

Few studies have addressed the effect of drug-induced nephrotoxicity on drug metabolism. Continuous treatment with 25–50 mg kg⁻¹ cyclosporine causes an increase in plasma blood urea nitrogen (BUN) concentration in the rat within 6 days. Such changes have been shown to decrease the hepatic CYP content to about 70% (Mayer *et al.* 1989). The renal CYP level is increased to about 160% of the control value. This increase in renal CYP may be due to the decrease in the haem oxygenase (HO) activity. The increase in the renal CYP is not accompanied by an increase in the drug metabolism capacity, however, since benzo[a]pyrene hydroxylase and NADPH-CYP reductase activities are not changed by nephrotoxicity (Mayer *et al.* 1989). Acute nephrotoxicity induced by gold sodium thiomalate is accompanied by a decrease in the renal CYP levels and an increase in the renal HO activity, with a decrease in benzo[a]pyrene hydroxylase activity (Eiseman *et al.* 1987).

Previous studies have correlated the nephrotoxicity with γ GT and CC β L activities. Inhibitors of γ GT and/or CC β L have been shown to protect the kidney from cisplatin-induced nephrotoxicity in both mice (Townsend and Hanigan 2002) and in human prostate carcinoma cell lines (Hanigan *et al.* 1999). In addition, pretreatment with γ GT or CC β L inactivators reduced the number of

damaged tubules by about 60% in hexachloro-1,3-butadiene-mediated nephrotoxicity in mice (de Ceaurriz and Ban 1990). All these observations indicate the role of these enzymes in renal toxicity.

The effect of unilateral hydronephrosis on several renal DMEs has been reported (Zenser *et al.* 1984, Gibson 1986, Lohr *et al.* 1998). CYP activity was not detected and biphenyl-4-hydroxylase activity was reduced in the hydronephrotic rabbit kidney compared with normal kidney tissue. NADPH-CYP reductase was not significantly altered (Zenser *et al.* 1984, Gibson 1986) upon treatment of the hydronephrotic rabbit kidney with 3MC; only 20% induction in the CYP and biphenyl-4-hydroxylase activity was observed (Zenser *et al.* 1984). However, a significant increase in methyltransferase activity, especially histamine methyltransferase, has been observed after hydronephrosis (Barth *et al.* 1975).

RF is considered as end-stage organ complication of many other diseases. Therefore, diseases in organs distance from kidney could, directly or indirectly, modulate renal and hepatic DMEs and hence xenobiotic metabolism in kidney. This hypothesis has been supported by several studies that showed that the expression of renal and hepatic DMEs is altered in diabetes, pregnancy, hepatorenal syndrome and hypertension.

Hypertension is multisystem disease that ends up with end-stage RF. The kidney is a rich reservoir of arachidonic acid (AA), which is metabolized by CYPs in the kidney to produce epoxyeicosatrienoic acids (EETs) and 19- and 20-hydroxy-eicosatrienoic acids (19- and 20-HETE), which generates vasodilating effect and inhibit Na^+/K^+ -ATPase and rennin activities, respectively. Thus, there is increasing evidence that development of hypertension is associated with abnormalities in AA metabolism (Omata *et al.* 1992, Carroll *et al.* 2003). Administration of high dietary salt for 14 days results in increased renal CYP2C11, 2C23 protein and decreased CYP4A protein level. However, administration of angiotensin II plus the salt results in down-regulation of these enzymes (Zhao *et al.* 2003). Previous studies showed a significant increase in hepatic testosterone 6 β -hydroxylation and renal lauric acid ω - and (ω -1)-hydroxylation activity in hypertensive rats (Imaoka and Funae 1991).

Several studies have reported an alteration in the hepatic and renal DMEs when rats develop diabetes. However, little information is available for other species, including humans. It has been reported that hepatic CYP2B10, 3A11, 4A10 and 4A14 mRNA levels are increased in streptozotocin-induced diabetic mouse, whereas hepatic CYP1A2 and 2E1 and renal CYP2E1 and 4A were not changed (Sakuma *et al.* 2001). In streptozotocin-induced diabetic rats, the catalytic activities of hepatic aniline hydroxylation, EROD, testosterone 2 β -, 6 β -, and 16 β -hydroxylation, and renal CYP2E1 and 4A2 were elevated (Shimojo *et al.* 1993). These changes were reversed by insulin treatment (Shimojo *et al.* 1993, Sakuma *et al.* 2001). The precise mechanism by which diabetes regulates CYP remains unclear. However, it could be attributed to changes in sex hormones and ketosis (Schenkman 1990).

Mechanisms involved in the expression and regulation of CYP in renal failure

It has been observed that during the course of RF and other renal impairments, many intrarenal changes occur including changes in cell polarity, signalling pathways and gene expression (Lash and Tokarz 1989, Ma *et al.* 1999).

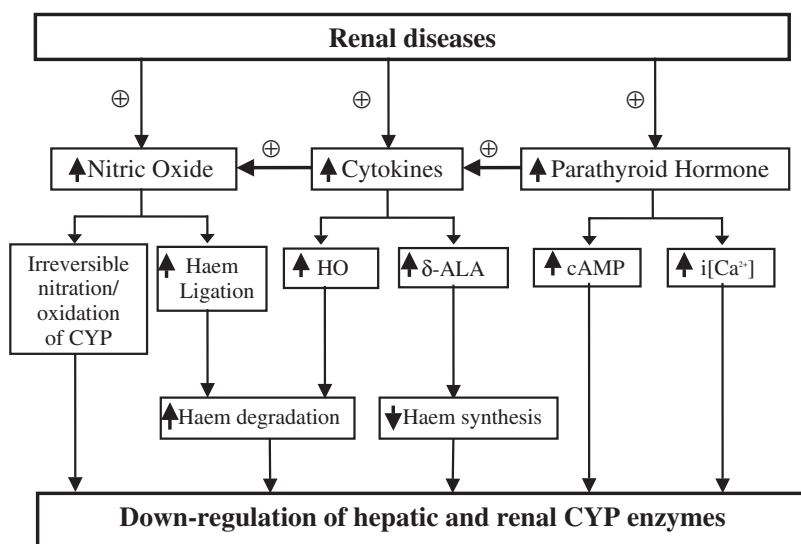


Figure 1. Different mechanisms involved in the down-regulation of CYP in renal diseases.

Unfortunately, the precise molecular mechanism(s) involved in the alteration of DMEs in RF remain(s) unknown. Many theories have been proposed (Gibson 1986, Uchida *et al.* 1995, Clyne *et al.* 1996, Lam *et al.* 1997, Leblond *et al.* 2000, Guevin *et al.* 2002) and figure 1 summarizes some of the proposed mechanisms.

Protein synthesis

RF usually results in lower protein body content due to a reduction in protein synthesis and chronic malnutrition (Leblond *et al.* 2001). Chronic protein restriction has been shown to reduce CYP synthesis. Protein restriction from 23 to 5% of casein for 4 weeks significantly decreases rat hepatic CYP1A2, 2C11, 2E1 and 3A1/2 levels (Cho *et al.* 1999). Northern blot analysis showed a significant decrease in CYP1A2 and 2C11 mRNA levels, with a slight decrease in CYP2E1 and 3A1/2 mRNA (Cho *et al.* 1999). Furthermore, EROD activity in protein-restricted rats is reduced by 40% compared with the control rats, although PROD activity is not altered significantly (Yoshida *et al.* 2002). Cysteine supplementation to protein-restricted rats restored the major CYP protein toward normal levels and completely prevented the reduction in EROD activity (Yoshida *et al.* 2002). Contrary to the above results, epoxide hydrolase and GSTA3/5 mRNA levels are increased in protein restricted rats (Cho *et al.* 1999, Yoshida *et al.* 2002).

Serum mediators

The theory that circulating mediators in the serum of RF patients causes down-regulation of CYP came after the observation that perfusion of livers from healthy rats with blood from uraemic rats significantly decreased the intrinsic clearance of propranolol (Elston *et al.* 1993, Uchida *et al.* 1995, Lam *et al.* 1997, Leblond *et al.* 2000, Guevin *et al.* 2002). Guevin *et al.* (2002) reported that the CYP3A2 protein level in hepatocytes of healthy rats is suppressed when the liver is perfused with serum of rats with RF. In addition, these latter authors reported that circulating proteins in the serum of rats with RF, by a factor having a molecular

weights in the range of 10–30 kDa, suppressed CYP3A2 activity. Interleukins 1 (IL-1) and 6 (IL-6), granulocyte inhibitory protein (Guevin *et al.* 2002), monocyte chemotactic and activating factor (Akahoshi *et al.* 1995), testosterone and growth hormone (Pacifci *et al.* 1988) have all been reported to be increased in RF. One or more of these circulating factors may therefore be responsible for the changes in CYP during RF.

Role of cytokines

Cytokines could be the serum factors mediating the suppression of the CYP during RF for many reasons. First, RF increases the plasma levels of cytokines including IL-1, tumour necrosis factor (TNF)- α and IL-6 (Cooper *et al.* 1994, Yamashita *et al.* 1994), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferons α and γ (IFN- α/γ) (El-Kadi and Souich 1998). Second, cytokines can also depress CYP activity and mRNA *in vitro* and *in vivo* (El-Kadi *et al.* 1997, Kurdi *et al.* 1999, Bleau *et al.* 2001). The incubation of cytokines with hepatocytes decreases the activity of selected CYP enzymes and the mRNA encoding for these apoenzymes with IL-1 β , IL-6 and TNF being the most potent suppressors. Interestingly, IFN- γ reduced CYP1A2 and CYP2E1 mRNA, but had no effect on CYP3A and epoxide hydrolase mRNA levels. Conversely, IL-4 was found to increase CYP2E1 mRNA level by fivefold (Muntane-Relat *et al.* 1995), while selectively inducing the GST- α isoenzyme (Bertilsson *et al.* 2001). The potency of these cytokines in altering enzyme activity depends upon the CYP form targeted. Unfortunately, the exact mechanisms through which cytokines alter CYP expression are not fully understood. Suppression of δ -aminolevulinic acid (δ -ALA) synthase and dehydrase and the activation of HO-1 have been suggested as plausible mechanisms (Lash and Tokarz 1989, Uchida *et al.* 1995).

Role of haem oxygenase (HO)

HO catalyses the degradation of haem proteins such as CYP to CO, iron, and biliverdin (Rodgers and Stevenson 1990). Recently, Yoshida *et al.* (2002) demonstrated an up-regulation of HO-1 gene expression in ARF rat. Similarly, it was reported that HO-1 mRNA was extensively and rapidly induced in rats exposed to the nephrotoxicant mercuric chloride (Horikawa *et al.* 1998). In rats with CRF, a significant increase in HO activity has been reported (Uchida *et al.* 1995). Furthermore, HO activity in human adenocarcinoma tissue is significantly higher than the activity in normal kidney tissue adjacent to the tumour (Goodman *et al.* 1997). The increase in HO activity in the renal carcinoma and RF may be attributed solely to HO-1 gene over-expression. It will be important to determine in the future the correlation between HO and the CYP in ARF and CRF.

Role of nitric oxide (NO) and reactive oxygen species (ROS)

NO is an inflammatory mediator derived from L-arginine. It is synthesized by NO synthases (NOS) in many segments of the nephron, especially the PT cells (Rahman and Wright 1992, Iber *et al.* 2001). Two of the NOS are constitutive — neuronal (nNOS) and endothelial (ecNOS) — and one is inducible (iNOS) (Noris and Remuzzi 1999). The role of NO in hepatic metabolism has been reviewed by

Alexander (1998). Studies using experimental models and human subjects have shown an enhanced production and release of NO in RF (Aiello *et al.* 1997, 1999, Aoyagi *et al.* 2001, Fayed *et al.* 2002). NO decreases the activity of CYP1A1, 1A2, 2B1, 2B2, 2C11 and 3A2 and completely inactivates CYP2E1 (Aoyagi *et al.* 1996, Barakat *et al.* 1997, 2001, Donato *et al.* 1997, El-Kadi *et al.* 2000). It has been reported that iNOS is activated in RF, thus increasing the production of NO. NO decreases CYP activity, either by binding reversibly with the haem moiety of CYP, forming an iron-nitrosyl complex or through the thiol pathway, resulting in an irreversible modulation of gene expression (Aoyagi *et al.* 1996, Minamiyama *et al.* 1997).

ROS may also play a role in the down-regulation of CYPs. Nagase *et al.* (1996) reported that the rate of hepatic microsomal production of thiobarbituric acid, a marker of oxidative damage, in rats with RF is significantly higher than in control. The hydrogen peroxide (H_2O_2) level was elevated in rats with glycerol-induced RF (Guidet *et al.* 1989). The possibility that H_2O_2 might directly decrease the activity of selected CYP enzymes was already proposed some years ago, although the precise mechanism remains unclear. H_2O_2 formed in the haemoprotein active centre can interact with the enzyme-associated Fe^{2+} , leading to haem destruction and enzyme inactivation (Karuzina *et al.* 1994). Although this may be one mechanism by which H_2O_2 decreases CYP activity, it may also act as a second messenger in a complex pathway that leads to the inactivation and/or down-regulation of selected CYP enzymes (Lowe *et al.* 1998).

Role of cAMP, intracellular Ca^{2+} and parathyroid hormone (PTH)

RF is associated with an increase in the cytosolic Ca^{2+} level of the hepatocyte. Elevation of intracellular Ca^{2+} has been shown to modulate CYP expression, but the mechanisms of modulation remain unknown (Klin *et al.* 1995). Studies have shown that an increase in intracellular Ca^{2+} is associated with a reduction in protein synthesis of CYP2B2 (Guevin *et al.* 2002), CYP11B2 (Clyne *et al.* 1996), CYP2C11 and CYP3A2 (Uchida *et al.* 1995, Baer *et al.* 1997). The elevation of intracellular Ca^{2+} in RF is most likely due to the increased levels of PTH observed in patients with RF (Mkrtchian and Andersson 1990, Clyne *et al.* 1996, Klin *et al.* 1996, Guevin *et al.* 2002). The stimulatory effect of PTH on the Ca^{2+} level of the DT cells is significantly higher than on PT cells (Ma *et al.* 1999). It has been observed that isolated PT cells, primarily, are responsive to stimulation by PTH, which increased the cAMP production fourfold (Koop *et al.* 1991).

cAMP levels, which are also stimulated by PTH, regulate the biotransformation system of controlling the phosphorylation and degradation rate of CYP (Taniguchi *et al.* 1985, Pyerin and Taniguchi 1989, Klin *et al.* 1995). An increase in cAMP causes activation of kinases that effectively phosphorylate CYP, resulting in inactivation of these enzymes (Klin *et al.* 1995). The mechanism by which phosphorylation inactivates CYP is unclear. Phosphorylation may increase the uncoupling of the NADPH-dependent hydroxylation system, which includes: CYP, CYP reductase and cytochrome b_5 (Pyerin and Taniguchi 1989).

In conclusion, the mechanisms by which renal impairment down- or/up-regulates DMEs are complex. The complexity arises from the fact that individual CYPs appear to respond differently, most likely because of the indirect effects of many other unknown mediators, in addition to the presence of other factors such as

the degree of renal impairment, the kidney tissue, and the synergism and antagonism between these mediators. Therefore, one cannot conclude a fixed rule to be applied to all mediators for all CYPs. Further studies are needed to elucidate the exact role of these mediators.

Significance of changes of the drug metabolic pathways in renal diseases and some important examples

Renal diseases alter the metabolism of xenobiotics by either increasing or decreasing the hepatic and/or extrahepatic DMEs, particularly the renal enzymes. In addition, other pharmacokinetic parameters may be altered due to changes in protein binding, hepatic blood flow and drug transport systems (Elston *et al.* 1993).

It has been established that some endogenous substances such as insulin, growth hormone, angiotensin, vasopressin, prolactin and leutinizing hormone are hydrolysed in the kidney, mainly in the PT and cortex (Gibson 1986). The alteration in metabolism of these endogenous substances in renal impairment could play a role in the pathogenesis of some secondary diseases (Rahman and Wright 1992, Lock and Reed 1998).

Considerable evidence has accumulated about the role of the renal CYP metabolites of arachidonic acid in the pathogenesis of hypertension. It has been observed that the renal localization of the CYP4A subfamily, particularly in the PT, is well correlated with the renal distribution of arachidonic acid ω -hydroxylase (Lock and Reed 1998). Furthermore, it has been suggested that the distribution of CYP2J5 and its metabolite in the PT plays a significant role in angiotensin II action, as this segment has the highest concentration of angiotensin II receptors (Lock and Reed 1998). ADH distribution in the PT might protect against exposure to the reactive, cytotoxic intermediates (Hjelle *et al.* 1983). These examples reveal that a disturbance in the DMEs in RF could alter the metabolism of these endogenous substances, leading to secondary diseases.

It has been well documented that renal impairment dramatically increases the risk of drug accumulation and toxicity for most drugs (Lam *et al.* 1997). Most clinical studies, however, have not studied changes in DMEs. The effect of renal diseases on most commonly used drugs is summarized in table 3.

Conclusions and remarks

Scientific investigations within the last decade have provided vital information on the role of extrahepatic DMEs in xenobiotic metabolism. It is now known that of all the extrahepatic organs, the kidneys play the greatest role in xenobiotic metabolism. Furthermore, pathological alteration of the kidneys is now known to alter DMEs not only in the kidneys, but also in the liver and intestines. Disease states such as acute and chronic RF and RCC mainly down-regulate the expression of phase I and II DMEs, especially CYP. The down-regulation of the DMEs is due to a selective inhibition of the expression of some CYP enzymes. Although there are many theories about the mechanisms by which such alterations occur, the exact mechanisms are yet to be determined. Such information may lead to the development of strategies that may decrease the incidence of drug side-effects and toxicity in patients with renal disease. Further clinical studies are needed to elucidate the full mechanism responsible for the changes of hepatic and extrahepatic DMEs in renal impairment.

Table 3. Summary of the effects of renal diseases on the metabolism of some drugs and the DMEs involved.

Drug name	DME involved	Species	Tissue	Changes in metabolism	Reference
Aminopyrine	CYP3A	rat	liver	↓	Guevin <i>et al.</i> (2002), Ushida <i>et al.</i> (1995)
Amiodarone	CYP3A4	human	liver	↓	Fillastre <i>et al.</i> (1993)
Antipyrine	CYP1A, 2B	human	plasma	↑	Elston <i>et al.</i> (1993), Maddocks <i>et al.</i> (1975)
AQ4N (phase I trial)	CYP3A			↑	Patterson (2002)
Bosentan	CYP2C9, 3A4	human	plasma	↑	Dingemanse <i>et al.</i> (2002)
Bufuralol	CYP2D6	human	plasma	↓	Balant <i>et al.</i> (1980)
Chloramphenicol (chronic)	UDPGT			↓	Verbeeck (1982)
Clofibrat	UDPGT	human	plasma	↓	Verbeeck (1981, 1982)
Codeine	CYP2D6	human	plasma/ urine	↔	Guay <i>et al.</i> (1988)
Diazepam	hydroxylation			↓	Gibson (1986)
Erythromycin	CYP3A3	human, rat	liver	↓	Elston <i>et al.</i> (1993), Guevin <i>et al.</i> (2002)
Fosinopril	CYP2C9, 3A2	human	plasma	↑	Hui <i>et al.</i> (1991), Elston <i>et al.</i> (1993)
Isoniazid	NAT	human	plasma	↓	Kim <i>et al.</i> (1994)
Isradipine	CYP3A4			↔	Elston <i>et al.</i> (1993)
Lidocaine	CYP3A4	human	liver	↓	Fillastre <i>et al.</i> (1993)
Lignocain	CYP3A4	human	kidney	↔	Sotaniemi <i>et al.</i> (1995)
Lorazepam (chronic)	UDPGT			↓	Verbeeck (1982)
Metoclopramide	CYP1A, 2B	human	plasma	↓	Lehmann <i>et al.</i> (1985)
Metoprolol	CYP2D6	human	plasma	↓↔	Lloyd <i>et al.</i> (1990)
Midazolam	CYP3A4	human	liver	↓	Elston <i>et al.</i> (1993)
Morphine	UDPGT	rabbit	kidney	↓	Gibson (1986)
Mycophenolic acid	UDPGT	human	plasma	↓	Wigger <i>et al.</i> (2002)
Nicadipine	CYP3A4	human	plasma	↓	Ahmed <i>et al.</i> (1991)
Nifedipine	CYP3A4	human	plasma	↑	Odor-Cederlof <i>et al.</i> (1990), van Bortel <i>et al.</i> (1989)
Nimodipine	CYP3A4	human	plasma	↓	Kirch <i>et al.</i> (1984)
Nitrendipine	CYP3A4			↓	Elston <i>et al.</i> (1993)
Nortriptyline	CYP2D6	human	plasma	↔	Dawling <i>et al.</i> (1982)
Oxazepam	UDPGT	human	plasma	↓	Verbeeck (1981) (1982)
Phenytoin	CYP2C9, 3A2			↑	Greenspan and Baron (1980), Uchida <i>et al.</i> (1995)
Procainamide	NAT	human	plasma	↓	Gibson <i>et al.</i> (1977)
Propafenone	UDPGT	human	plasma	↔	Fromm <i>et al.</i> (1995)
Propranolol	CYP2D6	rat	liver	↓	Terao and Shen (1984)
Sulphadimidine	NAT	human	plasma	↑	Elston <i>et al.</i> (1993)
Theophylline	CYP1A2/2E1	rat	plasma	↑	Yu <i>et al.</i> 2002
Tolbutamide	CYP2C9	human	liver	↓	Elston <i>et al.</i> (1993)
Verapamil	CYP3A4	human	plasma	↓	Storstein <i>et al.</i> (1984)
Zidovudine	UDPGT	human	plasma	↓	Kimmel <i>et al.</i> (1995)

↓, Decreased; ↑, increased; ↔, no changes.

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Comparison of the metabolism of ethylene glycol and glycolic acid *in vitro* by precision-cut tissue slices from female rat, rabbit and human liver

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1. The metabolism of [1,2-¹⁴C]-ethylene glycol and [1,2-¹⁴C]-glycolic acid was studied *in vitro* using precision-cut tissue slices prepared from the livers of female Sprague–Dawley rats, New Zealand white rabbits and humans. The time-course for production of metabolites formed from ethylene glycol at concentrations from 3 to 40 mM was determined to compare quantitatively the differences between species in the rates and amounts of formation of glycolic acid, the presumed developmental toxicant of ethylene glycol. The rates of metabolism of glycolic acid to glyoxylic acid at concentrations from 0.05 to 16 mM by liver tissue from the different species were also determined. The apparent $V_{\max}/K_m$ for the metabolic conversions of ethylene glycol to glycolic acid and for glycolic acid to glyoxylic acid in liver tissue from the different species were obtained.

2. There were qualitative differences in the metabolic profiles and quantitative differences in the formation of glycolic acid between the mammalian liver systems. There was an average of 10-fold less glycolic acid produced by liver slices from rabbits compared with rats. With the human liver, the formation of glycolic acid was not detectable using tissue from three of four human donors. A low level of glycolic acid was detected in one liver slice incubation from one of the four subjects, but only at one extended time point; glyoxylate was detected with liver slices from all four humans.

3. Liver slices prepared from female Sprague–Dawley rats, female New Zealand White rabbits and three female human subjects all metabolized glycolic acid to glyoxylic acid. Human liver tissue was the most effective at further metabolizing glycolic acid to glyoxylic acid. The ratios of $V_{\max}/K_m$, representing the relative clearance of glycolic acid from liver tissue, were approximately 14:9:1 for human, rat and rabbit liver, respectively.

4. Precision-cut liver slices maintained in dynamic organ culture are good predictors of metabolism by liver tissue *in vivo*. The results of the present study therefore indicate that levels of glycolic acid, if formed *in vivo*, following exposures to similar concentrations of ethylene glycol, would be lower in humans than in rabbits and rats.

Introduction

There have been a number of metabolic and toxicological studies to investigate the mechanism of toxicity of ethylene glycol (Mulinos *et al.* 1943, Gessner *et al.* 1961, Coen and Weiss 1966, Richardson 1973, Marshall 1982, Jacobsen *et al.* 1982, 1984, Jacobsen and McMartin 1986). From these investigations, the metabolic degradation of ethylene glycol by mammalian systems has been largely elucidated showing that conversion to glycolate, glyoxylate, oxalate and carbon dioxide and

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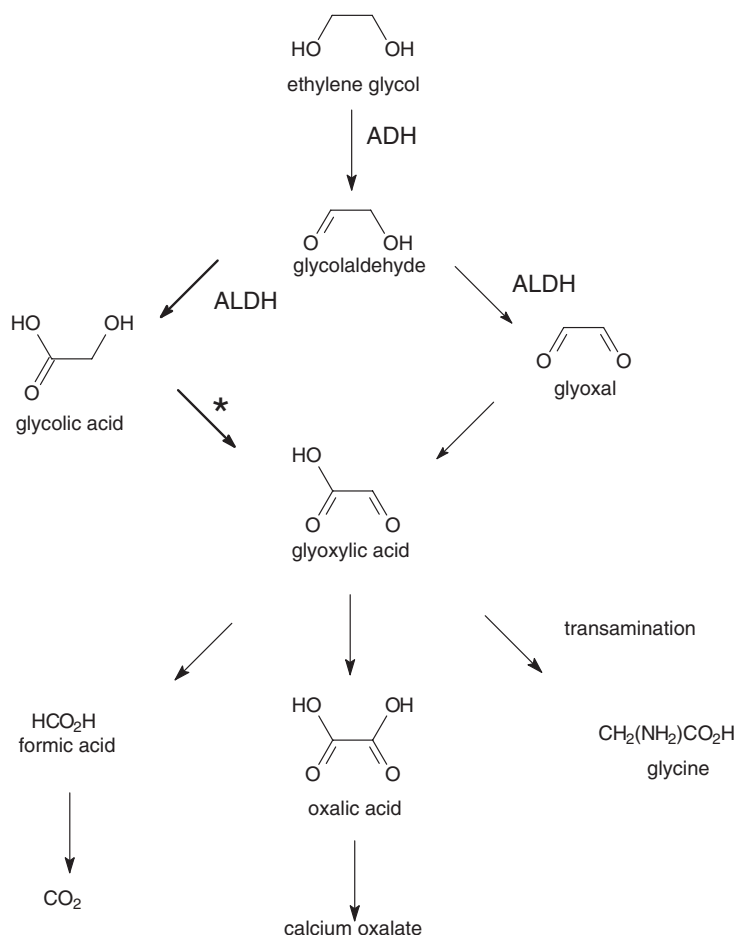


Figure 1. Metabolism of ethylene glycol. The major pathway is indicated by bold arrows. *Rate determining step. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

incorporation into glycine occurs via a number of intermediates. The overall pathway of metabolism is shown in figure 1. In more recent studies on the developmental toxicology of ethylene glycol, it was found that there are significant species differences in the expression of developmental toxicity following exposure of experimental animals to this compound. Sprague-Dawley rats responded at oral gavage dose levels ranging from 1000 to 5000 mg kg⁻¹ day⁻¹ (Price *et al.* 1985, Neeper-Bradley *et al.* 1995), whereas no developmental toxicity was observed in New Zealand white rabbits at up to lethal oral doses of 2000 mg kg⁻¹ day⁻¹ (Tyl *et al.* 1993). Several studies have also compared the developmental toxicity (Neeper-Bradley *et al.* 1995) and pharmacokinetics (Frantz *et al.* 1996a-c) of ethylene glycol in rats and mice.

From the general understanding of the metabolism of ethylene glycol, it is accepted that metabolism is necessary for developmental toxicity and it is considered that glycolic acid, one of the acidic metabolites of ethylene glycol (figure 1), is responsible for the developmental effects (Carney 1994, Carney *et al.* 1996a, b, 1999, Pottenger *et al.* 2001). Whilst the overall profile of metabolism of

ethylene glycol has been investigated in different species (Gessner *et al.* 1961, Marshall 1982), it is possible that there are significant quantitative differences in the relative amounts of potentially developmentally toxic metabolites formed between different species. Some *in vitro* studies have already been carried out to investigate the metabolism of ethylene glycol (Coen and Weiss 1966), but a quantitative comparison of the metabolites formed, particularly glycolic acid, across the range of species rat, rabbit and man has not been reported previously.

Liver slices have been used for many years for the study of xenobiotic metabolism (Storey 1950). More recently precision-cut tissue slices have become useful tools in toxicology and metabolism studies (Smith *et al.* 1985, 1986, 1987; for reviews, see Olinga *et al.* 1997, Lerche-Langrand and Toutain 2000). The use of liver slices has thus become a reliable *in vitro* method for the assessment of species differences in hepatic biotransformations. The main advantage of the use of liver slices is to provide a system that maintains coupled phase I (e.g. oxidation) and II (e.g. glucuronidation, sulphation) xenobiotic metabolism activity for extended periods (Barr *et al.* 1991a, b). Typically, liver slices remain viable for at least 12–24 h (Smith *et al.* 1987) and depending on the culture conditions can be maintained for longer periods. The development of dynamic organ culture systems has overcome the major problems associated with incubation of slices, namely the lack of adequate nutrient and gaseous exchange as a result of diffusional barriers (Smith *et al.* 1986). An effective system rotates a tissue slice in the incubation medium in the sealed vial in a cyclical manner (Barr *et al.* 1991a, b, Salyers *et al.* 1994). Liver slices maintained under these dynamic conditions have been shown to be stable and viable for extended periods (Sipes *et al.* 1987). For example, this type of dynamic *in vitro* system using rat or human liver slices has produced the same metabolites of theophylline as were found in urine (Salyers *et al.* 1994). The system is therefore useful in comparing the metabolism of xenobiotics in different species including man.

Following either oral or skin exposure to ethylene glycol, the major metabolic pathways involving alcohol dehydrogenases occur in the liver. On this basis, it was considered that a reliable comparative analysis of the metabolism of ethylene glycol in rat, rabbit and man could be achieved using *in vitro* techniques based on liver preparations. The approach of the present study was to compare, qualitatively and quantitatively, the metabolism of ethylene glycol by rats, rabbits and humans using *in vitro* techniques based on precision-cut liver slices as described above. The overall aim was to establish if there are differences in the metabolism of ethylene glycol and the detoxication of its metabolites, in particular glycolic acid, between the experimental species and man in order to help determine the relevance of toxicological findings in experimental animals for humans.

Materials and methods

Standards and radiochemicals

[1,2-¹⁴C]-ethylene glycol (50 mCi mmol⁻¹, ≥ 99%, 1.9 mCi ml⁻¹ in aqueous solution, 38 mM) was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). [1,2-¹⁴C]-glycolic acid (calcium salt) (30–50 mCi mmol⁻¹, ≥ 99% was purchased from ICN Pharmaceuticals, Inc., via ICN Biomedicals BV (Zoetermeer, the Netherlands). Ethylene glycol, glycolaldehyde (dimer), glycolic acid, glyoxylic acid, glyoxal, oxalic acid, glycine and formic acid were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Sulphuric acid (98%) was from Merck BV (Amsterdam, the Netherlands). All solutions were prepared using high-purity water.

Animal tissues

Rats. Female Sprague–Dawley rats (280–300 g) were obtained from Harlan (Horst, the Netherlands). Animals were housed at the Free University, Amsterdam, and acclimatized for at least 5 days, maintained on a 12-h light/dark cycle. Animals were permitted water and standard laboratory diet *ad libitum*. On the day of the incubations, the animals were sacrificed by decapitation and the livers removed and washed with ice-cold Krebs–Henseleit buffer (pH 7.5) [NaCl (118 mM), KCl (4.7 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.64 mM), KH_2PO_4 (1.18 mM), NaHCO_3 (24.88 mM), glucose (11.10 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2.52 mM) in distilled water]. Liver cores (cylinders approximately 8 mm in diameter) were prepared immediately for slicing as described below.

Rabbits. Female New Zealand white rabbits (approximately 2500 g) were from ENKI (Someren, the Netherlands). Animals were sacrificed by a blow to the head. Following removal of livers, these were transported on ice in transit buffer [a modification of Sack's buffer (Monden and Fortner 1982) containing KH_2PO_4 (5.07 mM), NaHCO_3 (14.30 mM), K_2HPO_4 (54.50 mM), KHCO_3 (5.99 mM), mannitol (206 mM) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (8.00 mM), the pH was adjusted to 7.75 with NaOH] to the Free University before slicing. Cores were prepared for slicing immediately after arrival at the laboratory (within 1.5 h after sacrificing the animals) as described below.

Preparation of rat and rabbit liver slices. Cylinders of liver tissue from dissected lobes of livers were produced using a sharpened stainless steel tube (internal diameter 8 mm) attached to a drill press. Slices were produced using a Krumdieck tissue slicer. The sectioning process was carried out in cold Krebs–Henseleit buffer (pH 7.5, 3°C). The immobilized liver core was drawn across a motor driven oscillating blade and the sectioned slices (200–300 μm thickness as determined by the slicer) were swept into a collecting chamber with buffer and then loaded into a glass incubation vial.

In vitro incubation procedure for rat and rabbit liver slices. The incubation medium was Krebs–Henseleit buffer (1.5 ml) supplemented with 25 mM HEPES (pH 7.5) under Carbogen (5% CO_2 in medical air, BOC Edwards Pharmaceutical Systems, Dongen, The Netherlands). The vials containing tissue were rotated at 3 rpm in a water bath at 37°C for a 90-min pre-incubation. The tissues were then transferred to new vials (one slice/vial, duplicates) containing substrate and fresh medium (1.5 ml). Samples (30 μl) of the medium were removed at various times, frozen quickly at –20°C, then stored for analysis at –80°C. After each sampling, the medium was gassed with Carbogen for 10–20 s.

Human tissue

Female human livers were obtained from the pathology laboratory of the Free University Hospital. Post-mortem delay was < 8 h. All studies performed with human liver tissue were done in collaboration with the pathologists of the Free University Hospital, after acceptance of the studies by the hospital's Ethical Committee, and in accordance with the Declaration of Helsinki V (South Africa 1996).

Age and cause of death of the human subjects. Subject 1: 84 years 5 months, lung emboly and heart rhythm defect; subject 2: 49 years 9 months, viral myocarditis; subject 3: 42 years 2 months, myocardial infarction; subject 4: 48 years 1 months, cerebral infarction.

Human liver collection. The liver sample from subject 1 was transported on ice in transit buffer. Liver samples from subjects 2–4 were transported on ice, then placed in Krebs–Henseleit buffer. Cores were then prepared immediately for slicing.

Preparation of human liver slices. Cylinders of human liver tissue were prepared as described above for the rat and rabbit liver and then slices (200–300 μm thickness) were produced using a Krumdieck tissue slicer.

In vitro incubation procedure with human liver slices. The pre-incubation and incubation procedure for human livers slices was the same as described for rat and rabbit liver.

Control incubations

[1,2- ^{14}C]-ethylene glycol (9.6 mM, specific activity 5 mCi mmol^{-1}) in incubation medium, Krebs–Henseleit buffer (1.5 ml) under Carbogen was incubated at 37°C for 24.0 h. Samples (30 μl) of the medium were removed at 0.5, 1.0, 2.0, 4.0, 6.0, 9.0, 12.0 and 24.0 h and stored at –80°C until analysed. After every sampling, the medium was gassed with Carbogen for 10–20 s. Similar controls were performed for [1,2- ^{14}C]-glycolic acid (0.05 mM, specific activity 15 mCi mmol^{-1} ; 0.48 mM, specific activity 5 mCi mmol^{-1}) in incubation medium and buffer.

Protein assay

The protein contents of homogenates of slices, or representative slices prepared at the same time and in the same manner as those used in the *in vitro* incubations, were determined (Bradford 1976) to enable direct quantitative comparisons of metabolite levels.

High-performance liquid chromatography (HPLC) system and radiocounting

Components in incubations were separated using an Aminex HPX-87H ion exclusion column (4.5 × 250 mm; Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) eluted isocratically with 6 mM H₂SO₄ at a flow rate of 0.3 ml min⁻¹. Measurements were made on media from duplicate slices. The column temperature was maintained at 65 ± 1°C using a temperature-controlled column oven. The non-radiolabelled standards, [1,2-¹⁴C]-ethylene glycol and [1,2-¹⁴C]-glycolic acid, were detected using an Altex Model 156 Refractive Index Detector (Anachen Ltd., Luton, UK). Radiolabelled metabolites, [1,2-¹⁴C]-ethylene glycol and [1,2-¹⁴C]-glycolic acid were detected using a Raytest Ramona-D/90 online radioactivity detector (Raytek Scientific Ltd., Sheffield, UK) fitted with a single-tube solid scintillant cell (200 µl volume). Data were acquired and analysed using Ramona 90 V11.4 software. In addition to online radioactivity counting, for selected samples from the study with [1,2-¹⁴C]-ethylene glycol, fractions (150 µl) were collected and radioactivity in the fractions was determined by liquid scintillation counting (Packard 2400CA TRI-CARB Liquid Scintillation Analyser, Canberra Packard, Groningen, The Netherlands).

System calibration

The retention times of standard solutions (approximately 1 mg ml⁻¹) of ethylene glycol, glycolaldehyde, glycolic acid, glyoxylic acid, glyoxal, oxalic acid, glycine and formic acid in 6 mM H₂SO₄ were determined by HPLC coupled with refractive index detection using the conditions described above. Co-chromatography of mixtures of standards was performed to confirm that all the compounds could be resolved and there was no co-elution of components. Calibration of the HPLC system was carried out at the start of each working day using a mixture of standards containing ethylene glycol and the metabolites of interest. Sample analyses were performed only after confirming the retention time and resolution of the standards (figures 2 and 3). The refractive index and radiochemical detectors were connected in series, which resulted in a small difference (about 1 min) in the retention time measured by each detector. This difference was used to calculate the actual retention time for all the radiolabelled metabolites. [1,2-¹⁴C]-Ethylene glycol was detectable by both the refractive index and radiochemical detectors and the retention time for this compound was therefore used as a reference to calculate the retention time of radiolabelled metabolites. To distinguish between glycolaldehyde and glycolic acid peaks in the HPLC chromatogram, the retention times of the standards relative to ethylene glycol, or [1,2-¹⁴C] metabolites relative to [1,2-¹⁴C]-ethylene glycol, were determined. Glycolaldehyde had a relative retention time of 0.77; glycolic acid had a relative retention time of 0.74 (ethylene glycol = 1.00).

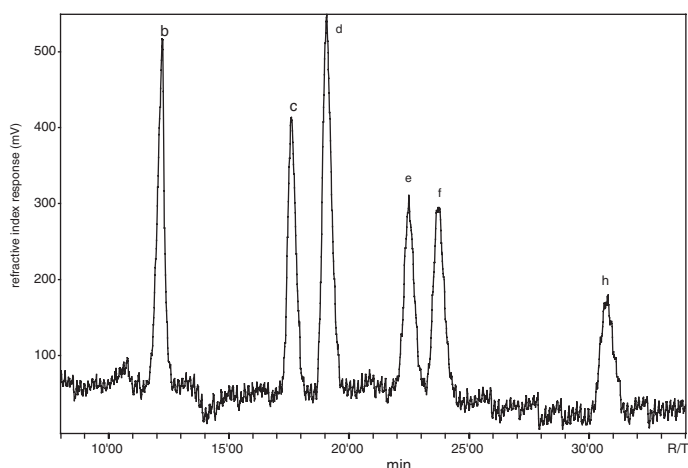


Figure 2. Chromatographic profile of reference standards of ethylene glycol metabolites. (b) Oxalic acid, (c) glyoxylic acid, (d) glyoxal, (e) glycolic acid, (f) glycol aldehyde, (h) ethylene glycol used for identifying metabolites from [1,2-¹⁴C]-ethylene glycol.

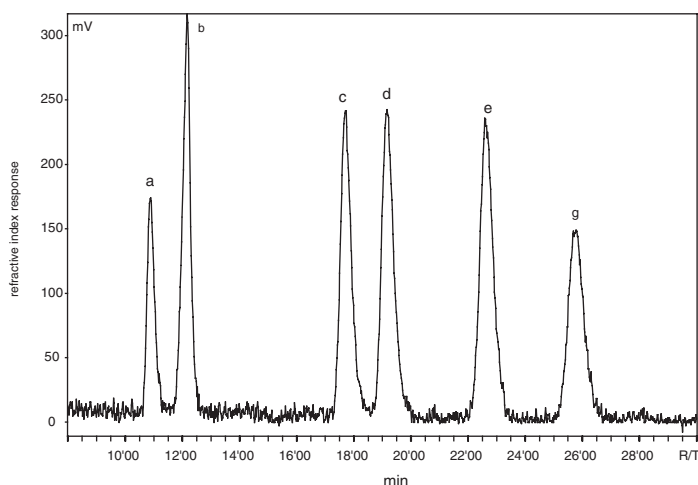


Figure 3. Chromatographic profile of reference standards of ethylene glycol metabolites. (a) Glycine, (b) oxalic acid, (c) glyoxylic acid, (d) glyoxal, (e) glycolic acid, (g) formic acid used for identifying metabolites from $[1,2-^{14}\text{C}]$ -glycolic acid.

Analyses of metabolites

Analysis of *in vitro* liver slice incubation medium. Samples of the incubation medium (30 μl) from liver slices incubated with either $[1,2-^{14}\text{C}]$ -ethylene glycol or $[1,2-^{14}\text{C}]$ -glycolic acid taken at selected time points were frozen at -20°C , then stored at -80°C until analysed. Shortly before analysis, each sample was thawed, centrifuged and then a subsample (7.5 or 10 μl) was analysed by radio-HPLC using the conditions described above. For incubations with $[1,2-^{14}\text{C}]$ -ethylene glycol, the total protein content for each liver slice (rat) or average total protein content for a set of liver slices (rabbit) were determined and the values used to express the $[1,2-^{14}\text{C}]$ -glycolic acid levels formed as mmol mg^{-1} protein, thus allowing direct comparison of results between the different species. For the metabolism studies with $[1,2-^{14}\text{C}]$ -glycolic acid, average protein weights for representative additional liver slices were determined and the values used to express the $[1,2-^{14}\text{C}]$ -glyoxylic acid formed as mmol mg^{-1} protein. In the glycolic acid study, the amounts of radioactivity remaining in liver slices at the end of the incubation were determined.

Analysis of metabolites from *in vitro* incubations of $[1,2-^{14}\text{C}]$ -ethylene glycol or $[1,2-^{14}\text{C}]$ -glycolic acid with rat liver slices. Samples of medium from *in vitro* incubations of rat liver slices from three animals with $[1,2-^{14}\text{C}]$ -ethylene glycol (in duplicate) (3.0 and 9.6 mM, specific activity 5 mCi mmol^{-1} ; 30 and 40 mM, specific activity 2.5 mCi mmol^{-1}) or $[1,2-^{14}\text{C}]$ -glycolic acid (0.05 mM, specific activity 15 mCi mmol^{-1} ; 0.16 mM, specific activity 5 mCi mmol^{-1} ; 0.48 mM, specific activity 5 mCi mmol^{-1} ; 3.2, 8.0 and 16 mM, specific activity 2.5 mCi mmol^{-1}) were analysed according to the HPLC-radiochromatographic procedures described above to determine the content metabolite profiles. The time points for sampling were 0, 0.5, 1.0, 2.0, 4.0, 6.0 and 9.0 h.

Analysis of metabolites from *in vitro* incubation of $[1,2-^{14}\text{C}]$ -ethylene glycol or $[1,2-^{14}\text{C}]$ -glycolic acid with rabbit liver slices. Samples of medium from *in vitro* incubations of $[1,2-^{14}\text{C}]$ -ethylene glycol (3.0 and 9.6 mM, specific activity 5 mCi mmol^{-1} ; 30 and 40 mM, specific activity 2.5 mCi mmol^{-1}) or $[1,2-^{14}\text{C}]$ -glycolic acid (0.16 mM, specific activity 5 mCi mmol^{-1} ; 3.2, 8.0 and 16 mM, specific activity 2.5 mCi mmol^{-1}), with rabbit liver slices from three animals were analysed for metabolites using the HPLC-radiochromatographic procedures described above.

Analysis of metabolites from *in vitro* incubation of $[1,2-^{14}\text{C}]$ -ethylene glycol or $[1,2-^{14}\text{C}]$ -glycolic acid with human liver slices. Samples of medium from *in vitro* incubations of $[1,2-^{14}\text{C}]$ -ethylene glycol (3.0 and 9.6 mM, specific activity 5 mCi mmol^{-1} ; 30 and 40 mM, specific activity 2.5 mCi mmol^{-1}) with human liver slices from tissue from human subjects 1–4 were analysed in the same way as described for incubations with rat or rabbit liver slices. Incubations of ethylene glycol with liver slices from the first

human subject were carried out in duplicate. For samples from human subjects 2–4, single incubations were performed. In incubations of liver slices from subjects 3 and 4, samples were collected up to 12 h. Samples of medium from *in vitro* incubations of human liver slices from tissue obtained from human subjects 2–4 with [1,2-¹⁴C]-glycolic acid (0.16 mM, specific activity 5 mCi mmol⁻¹; 3.2, 8.0 and 16 mM, specific activity 2.5 mCi mmol⁻¹) were analysed as described for incubations with rat or rabbit liver slices.

Determination of apparent kinetic parameters for the formation of glycolic acid from ethylene glycol by rat and rabbit liver slices. Time-course data sets for the formation of glycolic acid at different concentrations of [1,2-¹⁴C]-ethylene glycol (3, 9.6, 30 and 40 mM for rat or rabbit) incubated with isolated liver slices were collected for six different sets of liver slices at the time points 0, 0.5, 1.0, 2.0, 4.0, 6.0, 9.0 (rat and rabbit), 12.0 and 24 h (rabbit). Graphical plots of concentration of glycolic acid, expressed as mmol glycolic acid measured in the incubation medium per mg liver protein, versus time were constructed. A best-fit curve through each plot was generated. From the curve fitting, the initial rate for the formation of glycolic acid was determined as the first derivative of the function at time zero. Using the derived initial rate data a Michaelis–Menten plot:

$$V_0 = V_{\max}[S]/K_m + [S]$$

was then obtained. With the results from rabbit liver slices, the Michaelis–Menten plots showed approximate linearity and therefore $V_{\max}/K_m$ was obtained as the tangent to the Michaelis–Menten plot.

Determination of apparent kinetic parameters for the formation of glyoxylic acid from glycolic acid by rat, rabbit and human liver slices. Time-course data sets for the formation of glyoxylic acid at different concentrations of [1,2-¹⁴C]-glycolic acid (0.05, 0.16, 0.48, 3.2, 8.0 and 16 mM for rat or 0.16, 3.2, 8.0 and 16 mM for rabbit and human) incubated with isolated liver slices were collected for three different sets of liver slices at the time points 0, 0.25, 0.50, 0.75, 1.0, 2.0, 4.0 and 6.0 h. Graphical plots of concentration of glyoxylic acid, expressed as mmol glyoxylic acid measured in the incubation medium per mg liver protein, versus time were constructed. A best-fit curve for each plot was generated. From the curve fitting, the initial rate for the formation of glyoxylic acid was determined as the first derivative of the function at time zero. Using the derived initial rate data a Michaelis–Menten plot was then obtained.

Results and discussion

The metabolism and disposition of ethylene glycol in rats *in vivo* has already been described in several studies. In a study in rats by Lenk *et al.* (1989) about 65% of the administered oral dose of ethylene glycol was excreted in the urine as the unchanged parent compound and 29% as glycolic acid. Marshall (1982) reported that the metabolic disposition of ethylene glycol in exposed rats was dose dependent with a greater production of CO₂ at low doses and a greater excretion of urinary glycolic acid at higher doses of ethylene glycol. Comparative studies on the dose-dependent urinary elimination of ethylene glycol by rats and mice have also been carried out (Frantz *et al.* 1996a). Considerable *in vivo* reference data are therefore available for comparison with data generated in *in vitro* test systems. In the present study, the metabolism of ethylene glycol has been examined in liver slices prepared from livers of rats, rabbits and humans. The concentrations of ethylene glycol used in the liver slice incubations of the present study were selected to permit comparison with *in vivo* data for blood concentrations of metabolites formed from exposures to ethylene glycol (Carney *et al.* 1996b, Pottenger *et al.* 2001). In the current study overall, for liver tissue from the three species, the amount of radiolabelled metabolites present in the incubation medium relative to unmetabolized ethylene glycol was in the range 2–5%. For the studies with glycolic acid, the amount of radiolabelled metabolites relative to unmetabolized glycolic acid ranged from 1.5 to 13%.

Metabolism of [1,2-¹⁴C]-ethylene glycol by rat liver slices in vitro

Rat liver slices from three animals were incubated in duplicate with ethylene glycol at four different concentrations and the incubations were analysed by radio-HPLC at selected time points. A representative HPLC chromatogram of metabolites present in the incubation medium after 4 h is shown in figure 4A. Radioactive peaks with the same retention times as the reference standards for formic, glycolic, glyoxylic and oxalic acids were detected in the incubation medium. The peak (e) representing glycolic acid, the presumed developmental toxicant and metabolite of interest, was quantified at each time point. Glycolic acid was quantitatively the major metabolite detected, with smaller amounts of radioactivity corresponding to oxalic, glyoxylic and formic acids, in decreasing order of abundance.

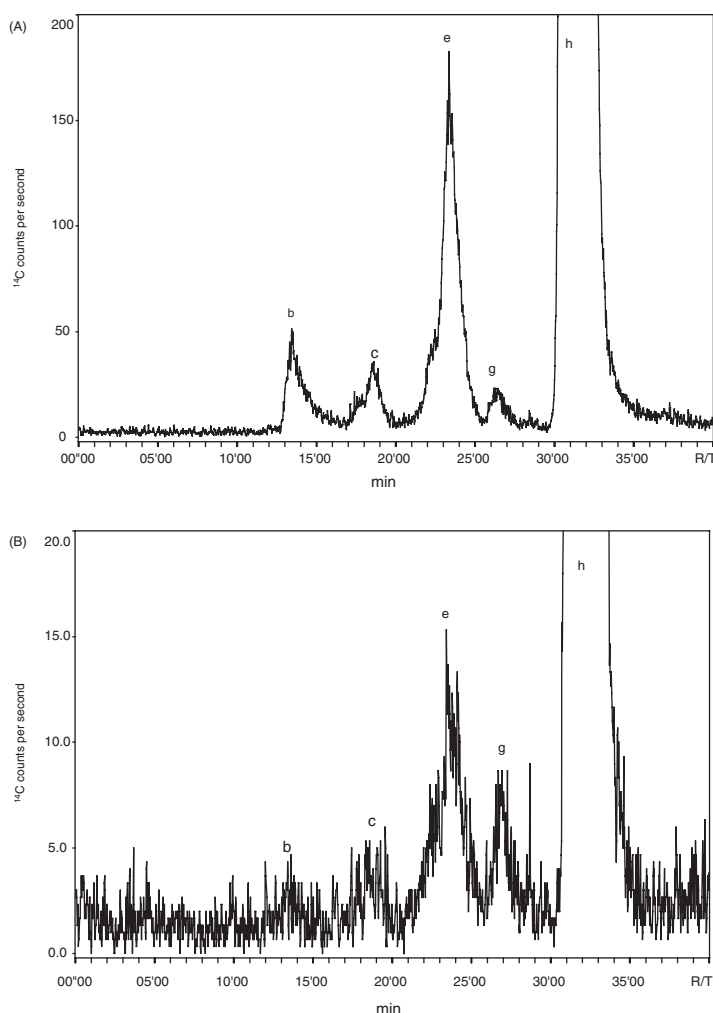


Figure 4. *In vitro* metabolism of 9.6 mM [1,2-¹⁴C]-ethylene glycol in (A) rat liver slices and (B) rabbit liver slices. HPLC profiles of the incubation medium sampled after 4 h. (b) Oxalic acid, (c) glyoxylic acid, (e) glycolic acid, (g) formic acid, (h) ethylene glycol.

Metabolism of [1,2-¹⁴C]-ethylene glycol by rabbit liver slices in vitro

Rabbit liver slices prepared from livers of three animals were incubated in duplicate with ethylene glycol at four different concentrations and incubations were analysed by radio-HPLC at selected time points. A representative HPLC chromatogram of the metabolites detected in the incubation medium after 4 h is shown in figure 4B. Radioactive peaks with the same retention times as formic, glycolic, glyoxylic and oxalic acids were observed in the incubation medium. Glycolic acid (e) was detected in incubations at the three highest concentrations of ethylene glycol and was quantitatively the major metabolite with smaller amounts of formic, glyoxylic and oxalic acids. In incubations performed at the lowest concentration (3.0 mM) of ethylene glycol, glycolic acid was detected above the background in only one sample.

Metabolism of [1,2-¹⁴C]-ethylene glycol by human liver slices in vitro

Four sets of human liver slices from four different donors were incubated *in vitro* with [1,2-¹⁴C]-ethylene glycol:

- Human 1A/B. The incubations with the liver slices from subject 1 were carried out in duplicate and were designated Human 1A and 1B. Up to 12 h, no glycolic acid was detected at any of the concentrations of ethylene glycol. In only one of the liver slice incubations carried out with ethylene glycol was there some evidence of glycolic acid formation. In the incubation medium sampled at 24 h from a liver slice from Human 1A incubated with 9.6 mM ethylene glycol, radioactivity corresponding to glycolic acid was detected at 0.25 $\mu\text{mol mg}^{-1}$ protein. For both Human 1A and 1B low levels of glyoxylic acid were detected in the 9.6, 30 and 40 mM ethylene glycol liver slice incubations for the 4–24-h samples. Oxalic acid was also detected in the 24-h samples from the same incubations.
- Human 2. Glycolic acid was not detected at any of the concentrations of [1,2-¹⁴C]-ethylene glycol used in the *in vitro* incubations. Liquid scintillation counting of fractions of the HPLC elutant confirmed the absence of radioactivity eluting at the same retention time as glycolic acid. Glyoxylic acid was detected in the 9.6, 30 and 40 mM ethylene glycol incubations after 1 h and reached a maximum at 6, 9 and 9 h, respectively. Oxalic acid was present in all the 24-h samples.
- Human 3. Glycolic acid was not detected at any of the concentrations of [1,2-¹⁴C]-ethylene glycol used in the *in vitro* incubations. Samples were collected and analysed up to 12 h. Glyoxylic acid was detected in the incubations with 9.6 (12 h), 30 (9 and 12 h) and 40 mM (6, 9 and 12 h) ethylene glycol.
- Human 4. Glycolic acid was not detected at any of the concentrations of [1,2-¹⁴C]-ethylene glycol used in the *in vitro* incubations. Samples were collected and analysed up to 12 h. Glyoxylic acid was detected in incubations of ethylene glycol at all four concentrations. In incubations with 3.0, 9.6 and 30 mM ethylene glycol, glyoxylic acid was detected in the 1–6-h samples and in the 40 mM incubation it was present in the 1–9-h samples. Oxalic acid was detected in the 30 mM incubation at 12 h.

Comparison of metabolism of ethylene glycol in rat, rabbit and human liver slices

The metabolism of ethylene glycol to glycolic acid occurred in liver slices *in vitro* from both rat and rabbit. In order to permit direct quantitative comparisons

Table 1. Ratio of glycolic acid determined in the incubation medium at set time points following *in vitro* incubation of rat and rabbit liver slices with 3.0, 9.6, 30 and 40 mM [1,2-¹⁴C]-ethylene glycol.

Ethylene glycol concentration (mM)	Time (h)	Ratio of glycolic acid rat: rabbit
3.0	0	— ¹
3.0	0.5	—
3.0	1	—
3.0	2	—
3.0	4	—
3.0	6	—
3.0	9	—
9.6	0	—
9.6	0.5	5
9.6	1	7
9.6	2	15
9.6	4	20
9.6	6	25
9.6	9	22
30	0	—
30	0.5	4
30	1	4
30	2	5
30	4	10
30	6	12
30	9	13
40	0	—
40	0.5	3
40	1	4
40	2	5
40	4	8
40	6	12
40	9	15

Glycolic acid concentrations were the average of analyses of duplicate incubations from livers of three different animals, normalized to the level of protein in liver slice for each species.

¹Ratio of glycolic acid not determinable.

between species, the amounts of glycolic acid were normalized with respect to the level of protein present in the liver slices from the different species. Both quantitative and qualitative differences in metabolism were observed between rat and rabbit. At all concentrations of ethylene glycol, the levels of glycolic acid produced in the incubations with rabbit liver slices were lower than those produced in the corresponding rat liver slice incubations (table 1). The ratio of glycolic acid produced by rat liver compared with rabbit liver ranged from three to 25 and was on average about one order of magnitude greater for rat liver. The finding that greater amounts of glycolic acid are formed *in vitro* by rat liver compared with rabbit liver is consistent with the observation of developmental toxicity of ethylene glycol in the rat *in vivo* compared with an absence of toxicity in the rabbit (Tyl *et al.* 1993). From the kinetic determinations and Michaelis-Menten plot (figure 5), the apparent $V_{\max}$ and K_m for the formation of glycolic acid from ethylene glycol by rat liver tissue were $1.64 \times 10^{-4} \text{ mmol mg}^{-1} \text{ h}^{-1}$ and 23.83 mM, respectively. $V_{\max}/K_m$ was $6.9 \times 10^{-6} \text{ l h}^{-1} \text{ mg}^{-1}$. The corresponding $V_{\max}/K_m$ determined from results obtained with rabbit liver tissue was $7.08 \times 10^{-7} \text{ l h}^{-1} \text{ mg}^{-1}$. The ratio of $V_{\max}/K_m$ for rat liver versus rabbit liver was 9.8:1 indicating a significantly greater turnover of ethylene glycol to glycolic acid in rat liver compared with rabbit liver tissue. The glycolic acid formed from ethylene

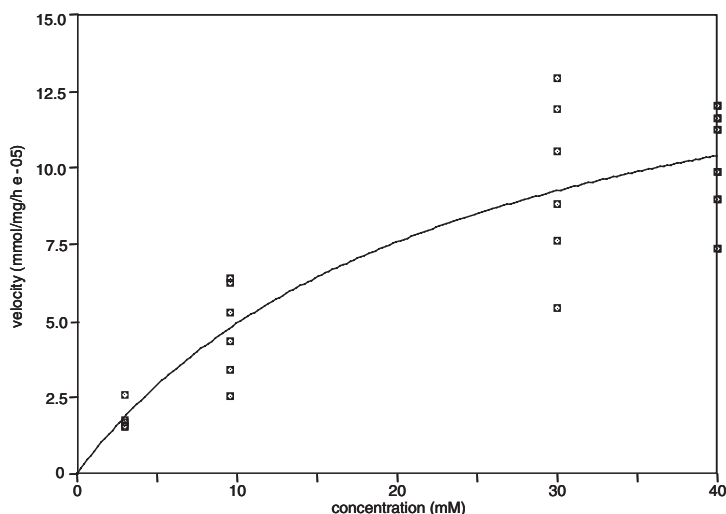


Figure 5. Michaelis-Menten plot for the formation of glycolic acid from ethylene glycol by rat liver slices. Individual data points for each concentration are shown.

glycol is effectively further metabolized to glyoxylic acid and so these values do not represent absolute values for the kinetic parameters but nevertheless are useful in a comparative manner for species comparisons.

Glycolic acid could not be determined in kinetic incubations of ethylene glycol with human liver slices and therefore the turnover for this reaction by human liver is considerably less than for both rabbit or rat liver. With the exception of the analysis of one liver sample from Human 1 and only at one extended time point (24 h), glycolic acid formation was not detected in the incubation medium from the human liver slices. The detection limit for glycolic acid in the liver slice incubation medium was 1.5 μM . In developmental toxicity studies using a rat whole embryo culture system, it was found that concentrations of glycolic acid as high as $\geq 12.5 \text{ mM}$ were necessary to inhibit embryo growth and development (Carney *et al.* 1996a). For the one human sample where glycolic acid was detected, there was no evidence for a concentration-related formation of glycolic acid as was observed with rat and rabbit liver. In addition, the glycolic acid was only detected after 24 h and could not be detected in the duplicate incubation at the same time point. This lack of time dependence, dose-response and reproducibility suggests that the peak detected was an artefact.

The metabolic viability of the human liver slices was established in the studies to investigate the biotransformation of glycolic acid by liver slices. In studies with liver slices from three of the four subjects (Humans 2–4), all of these metabolized glycolic acid to glyoxylic acid and other metabolites indicating the metabolic viability of the tissue. This is good evidence for the metabolic activity of liver slices in which the formation of glycolic acid from ethylene glycol was not detected. Fresh liver slices from Human 1 were not available for use in the metabolism study on glycolic acid, but there was no evidence to indicate an impairment of metabolic activity in the liver slices from this subject. Hepatic alcohol dehydrogenase activity has been reported to be not affected by age or gender (Wynne *et al.* 1991). It was therefore considered unnecessary to take the age of the donor subjects into consideration.

The failure to detect glycolic acid in the incubations with human liver slices could be attributed to its effective further metabolism to glyoxylic acid. The presence of glyoxylic acid, the primary metabolite of glycolic acid, in samples of the liver slice incubation medium is an indication that glycolic acid may have formed during the incubations, but it does not distinguish between this pathway of formation and the route via glyoxal (figure 1). In principle, glyoxylic acid could also be formed from glyoxal (figure 1). However, glyoxal was not detected in the human liver slice incubations. Low levels of glyoxal can also be formed by autoxidation of [1,2- ^{14}C]-ethylene glycol. In a control incubation of [1,2- ^{14}C]-ethylene glycol carried out in the absence of liver tissue for an extended period, low levels of glyoxal were detected in the 6–24-h incubation samples.

The differences in metabolism of ethylene glycol between rats and rabbit noted in the present study are consistent with previous observations from studies *in vivo* with these species. Metabolism studies *in vivo* carried out by Lenk *et al.* (1989) confirmed earlier findings (McChessney *et al.* 1971) that in rats unchanged ethylene glycol was the major excretory product from oral doses of ethylene glycol, and that glycolic acid was the major excretory metabolite. In comparative studies, Gessner *et al.* (1961) found that in rats and rabbits, the two main excretory products were CO_2 in the expired air and unchanged ethylene glycol in the urine, with traces of oxalic acid and urea in the urine. The amount of unchanged ethylene glycol in the urine was dose dependent with greater amounts being excreted unchanged at higher doses. The proportion of CO_2 in expired air decreased with increasing doses of ethylene glycol. However, in rats the urinary excretion of oxalic acid was greater at low doses of ethylene glycol, whereas in rabbits excretion of oxalic acid increased with the dose of ethylene glycol. In further studies by Marshall (1982) and Frantz *et al.* (1996a), there was a greater production of CO_2 at low doses of ethylene glycol and a greater urinary excretion of glycolic acid at higher doses of ethylene glycol.

Metabolism of [1,2- ^{14}C]-glycolic acid by rat liver slices in vitro

Liver slices prepared from the liver of each of three animals were incubated *in vitro* with [1,2- ^{14}C]-glycolic acid at six different concentrations and the incubations were analysed at selected time points by HPLC coupled with online radioactivity detection. The metabolites glyoxylic acid and oxalic acid were the only metabolites clearly detectable in the incubation medium together with unmetabolized glycolic acid. A representative HPLC chromatographic profile of metabolites in the incubation medium after 2 h is shown in figure 6A. The major metabolite glyoxylic acid was detected in the incubation medium at all time points for all concentrations of glycolic acid used. A trace level peak corresponding to glyoxylic acid was detectable in some samples at the time-zero points, and values obtained at subsequent time points of incubation were therefore corrected for this background before the data were used. The amount of residual radioactivity measured in rat liver slices after the incubations ranged from 0.3 to 4%. Determination of the time-course profiles for the formation of glyoxylic acid and derivation of the Michaelis–Menten plot (figure 7) allowed determination of the apparent K_m (0.30 mM) and $V_{\max}$ ($0.084 \mu\text{mol mg}^{-1} \text{h}^{-1}$) for the conversion of glycolic acid to glyoxylic acid.

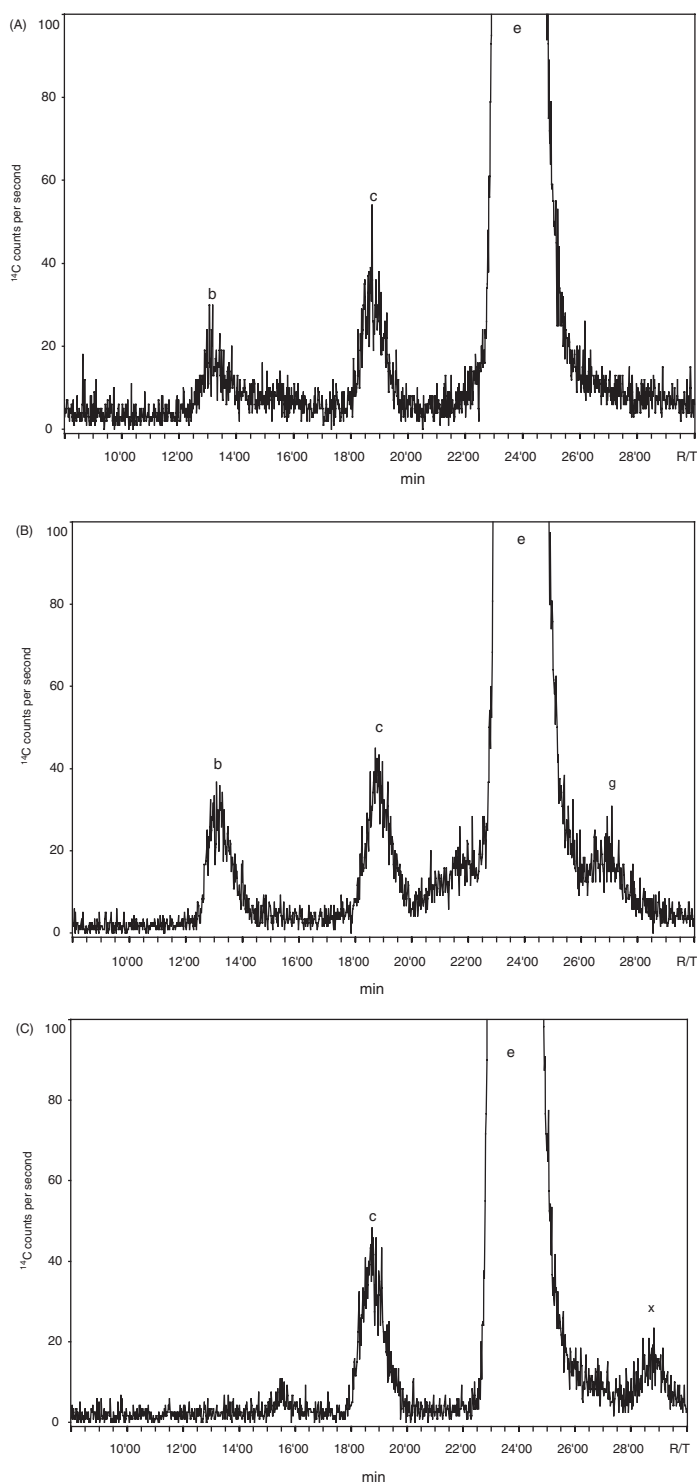


Figure 6. *In vitro* metabolism of 3.2 mM [1,2-¹⁴C]-glycolic acid in (A) rat liver slices, (B) rabbit liver slices and (C) human liver slices. HPLC profiles of incubation medium sampled after 2 h. (b) Oxalic acid, (c) glyoxylic acid, (e) glycolic acid, (g) formic acid, (x) unidentified.

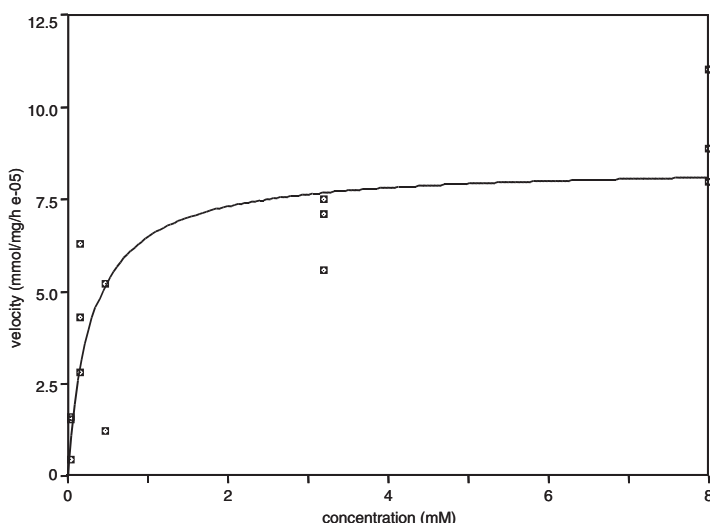


Figure 7. Michaelis-Menten plot for the formation of glyoxylic acid from glycolic acid by rat liver slices. Individual data points for each concentration are shown.

Metabolism of [1,2- ^{14}C]-glycolic acid by rabbit liver slices in vitro

Rabbit liver slices prepared from each of the livers of three animals were incubated *in vitro* with [1,2- ^{14}C]-glycolic acid at four different concentrations and the incubation medium was analysed by HPLC coupled with online radioactivity detection at selected time points. The metabolites glyoxylic acid, oxalic acid and formic acid were detected in the incubation medium together with the unmetabolized parent, glycolic acid. A representative HPLC chromatogram of the profile of the metabolites detected in the incubation medium after 2 h is shown in figure 6B. The amount of glyoxylic acid present in the incubation medium was quantified and time-course profiles for the formation of glyoxylic acid from glycolic acid obtained. The data at individual time points were corrected for a trace level background measurement detected at the time-zero point before these data were used. The amount of residual radioactivity in rabbit liver slices after the incubations ranged from 0.6 to 7%. K_m and $V_{\max}$ for the metabolism of glycolic acid to glyoxylic were obtained essentially as described for the rat liver incubations. From the Michaelis-Menten plots, apparent $V_{\max}$ and K_m of $0.03 \mu\text{mol mg}^{-1} \text{h}^{-1}$ and 0.98 mM, respectively, were obtained.

Metabolism of [1,2- ^{14}C]-glycolic acid by human liver slices in vitro

Human liver slices prepared from each of the livers from three different female donors (subjects 2–4) were incubated *in vitro* with [1,2- ^{14}C]-glycolic acid at four different concentrations and incubations were analysed at selected time points by HPLC coupled with online radioactivity monitoring. Radioactivity corresponding to glyoxylic acid was detected as the principal metabolite in the incubation medium together with the unmetabolized parent compound, glycolic acid and an unidentified metabolite x. A representative HPLC chromatogram of the metabolites detected in the incubation medium after 2 h is shown in figure 6C. Glyoxylic acid present in the incubation medium was quantified and time-course profiles for the formation of glyoxylic acid from glycolic acid obtained. The data at individual

time points were corrected for a background measurement detected at the time-zero point before these data were used. The amount of residual radioactivity in human liver slices after the incubations ranged from 0.5 to 1%. At the highest concentration of glycolic acid (16 mM), the level of glycolic acid remained essentially unchanged, indicating that this concentration was toxic to the liver tissue, and therefore the data for this group were excluded from the calculation of kinetic parameters. From the derived Michaelis–Menten plot, apparent $V_{\max}$ and K_m of $0.31 \mu\text{mol mg}^{-1} \text{h}^{-1}$ and 0.72 mM, respectively, were obtained.

Comparison of metabolism of glycolic acid in rat, rabbit and human liver slices

In the metabolic studies using $[1,2-^{14}\text{C}]$ -glycolic acid with liver slices from different species, the metabolic conversion of glycolic acid to glyoxylic acid was shown to occur *in vitro* in tissue slices prepared from rat, rabbit and human liver. A comparison of the profile of metabolites formed by liver tissue from the different species showed that after 2 h the profiles were broadly similar for both rat and rabbit liver (figure 6A, B), although some formic acid was observed with rabbit liver slices. There were also some qualitative differences when these profiles were compared with the profile obtained from incubations with human liver slices (figure 6C). The main difference was the presence of an unidentified metabolite in the human liver incubations compared with those from rat and rabbit liver incubations. Greater amounts of glyoxylic acid were formed in human liver slices compared with the rat and rabbit liver tissue. Overall, the results indicated that there were some qualitative differences in the metabolic profiles but that there were greater quantitative differences in the rate of production of glyoxylic acid between the different mammalian systems. With rabbit liver tissue the conversion of glycolic acid to glyoxylic acid occurred at a lower rate than for rat liver tissue. The conversion of glycolic acid to glyoxylic acid was observed with human liver tissue from all three donors. The comparable magnitude of the apparent K_m obtained for the different species indicated that the metabolizing enzyme system in each species was similar. The $V_{\max}/K_m$ for the conversion of glycolic acid to glyoxylic acid for rat versus rabbit versus human liver tissue were 0.28 versus 0.03 versus $0.43 \text{ l h}^{-1} \text{ g}^{-1}$. A correction was not made for the small amount of residual radioactivity measured in slices after incubations were complete. The relative values obtained for rat and human liver tissue, however, were broadly similar to results obtained in a recent *in vitro* study using ^{13}C -labelled glycolic acid and cytosol from female rat and human liver (Bartels and Clark 2001). The ratio of $V_{\max}/K_m$, which represent the relative clearance of glycolic acid from liver tissue of the different species, was approximately 14:9:1 for human, rat and rabbit liver tissue, respectively. These ratios indicated that liver tissue from human was therefore the most effective of the three species for the transformation of glycolic acid to glyoxylic acid. Hepatic clearance of glycolic acid *in vivo* would therefore be expected to be greater for human compared with that occurring for rat or rabbit.

Conclusions

The present study generated relative quantitative data on the *in vitro* metabolism of ethylene glycol to glycolic acid, the presumed developmental toxicant, and its further metabolism to glyoxylic acid by liver tissue from female rats, rabbits and humans. The results demonstrated that there are clear differences in the

metabolism of ethylene glycol by liver tissue from rats, rabbit and human, particularly in respect of the quantitative production of glycolic acid and its further biotransformation to glyoxylic acid. The precision-cut liver slice test system is considered to behave as a good mimic for xenobiotic metabolism in liver *in vivo*. Therefore, the differences between species in the rates and amounts of metabolism of ethylene glycol and its metabolite, glycolic acid, observed in the present study are also expected, by extrapolation, to occur *in vivo*. Thus, levels of glycolic acid, if formed *in vivo* following exposures to similar concentrations of ethylene glycol, would be lower in humans than in rabbits and rats, and therefore these results caution against the direct extrapolation of the developmental toxicity observed in rodents to predict the response in humans.

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Optimizing bacterial expression of catalytically active human cytochromes P450: comparison of CYP2C8 and CYP2C9

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1. Methods for the co-expression in *Escherichia coli* of human cytochrome P450 (CYP) 2C8 and CYP2C9 with NADPH-cytochrome P450 reductase (OxR) to produce a catalytically active system were compared.

2. Approaches assessed were expression of a CYP:OxR fusion construct, bicistronic plasmids, simultaneous transformation with CYP and OxR plasmids, and separate expression of CYP and OxR with reconstitution of activity by mixing the bacterial membranes. Two N-terminal modifications ($\Delta 3-20$ and 17α -leader) of the individual P450s were additionally investigated.

3. Each approach gave efficient expression of CYP2C8 and CYP2C9, but the bicistronic constructs under the expression conditions used gave low OxR expression and low catalytic activity. CYP expression was higher with the $\Delta 3-20$ construct for CYP2C9 and with the 17α -presequence construct for CYP2C8.

4. Using torsemide as substrate, all methods gave catalytically active systems with K_m values similar to human liver microsomes. Mixing bacterial membranes containing separately expressed CYP and OxR reconstituted a catalytically active system with the $\Delta 3-20$ construct for CYP2C9 but not for CYP2C8, and with neither of the 17α -presequence constructs. OxR co-expressed with CYP in the same membrane interacted with CYP to reconstitute activity more effectively than addition of exogenous OxR membranes.

5. Expression construct and OxR co-expression strategy should be individualized for CYP isoforms.

Introduction

Cytochrome P450 (CYP) is a haem monooxygenase that uses NADPH-cytochrome P450 reductase (OxR) for the transfer of electrons from NADPH to the haem moiety (Guengerich 2001). CYP exhibits remarkable substrate diversity, catalysing the metabolism of structurally diverse substrates that include drugs, environmental chemicals (e.g. dietary constituents, environmental pollutants) and endogenous compounds (e.g. steroids, eicosonoids, fatty acids) (Rendic 2002). This versatility arises from CYP heterogeneity. Thus, CYP exists as a superfamily of independently regulated forms (isoforms) that tend to exhibit distinct, but sometimes overlapping, substrate and inhibitor selectivities (Rendic 2002). Given

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the differences in regulation, genetic polymorphism and drug–drug interactions between isoforms, considerable attention has focused on the development of systems for expression of human P450s to allow characterization of substrate and inhibitor selectivities and of the effects of amino acid substitutions on enzyme activity (Fujita and Kamataki 2002).

Recombinant CYP proteins have been expressed in several heterologous systems, including mammalian cell lines, yeast, insect cells and bacteria. Of the heterologous host systems, bacteria are considered the simplest to use. They also have the advantages of readily accommodating foreign genetic material, rapid expression and producing very high yields of protein (Fujita and Kamataki 2002). Bacteria, however, do not contain a suitable endogenous redox partner to catalyse efficiently the transfer of electrons to CYP. Hence, a mammalian NADPH-cytochrome P450 reductase (OxR) must be co-expressed with the P450 or subsequently added in order to generate an active system (Fujita and Kamataki 2002). Several methods have been employed for generating active systems in bacteria. These include the expression of P450-OxR fusion proteins (Fisher *et al.* 1992, Shet *et al.* 1993, Chun *et al.* 1996), bicistronic expression systems (Parikh *et al.* 1997), co-expression using separate but compatible co-transformed plasmids (Pritchard *et al.* 1998), or co-expression via a single plasmid with separate promoters for each cDNA (Iwata *et al.* 1998).

In studies on the functional effects of changes in the primary structure of CYP enzymes, it is important to determine both the K_m and V_{max} of the altered enzyme, as together these determine the *in vitro* intrinsic clearance. This can be particularly difficult in the case of V_{max} , as the OxR:CYP ratio to achieve maximal activity can vary markedly between expression systems. It is necessary, therefore, to use a system that expresses a stable OxR:CYP ratio or which allows reconstitution of a system with an optimal OxR:CYP ratio.

CYP2C9 is of major importance in drug metabolism (Miners and Birkett 1998), and there is increasing awareness of the contribution of CYP2C8 to the metabolic clearance of drugs and endogenous compounds (Zeldin *et al.* 1995, Fisslthaler *et al.* 1999, Ong *et al.* 2000). In many experiments where CYP2C8 and CYP2C9 were co-expressed with OxR in bacteria and used in activity assays, kinetic parameters have not been firmly established (Sandhu *et al.* 1993, Richardson *et al.* 1995, Parikh *et al.* 1997, Iwata *et al.* 1998, McGinnity *et al.* 1999, Cuttle *et al.* 2000). In those studies that have reported K_m and/or V_{max} values, the effects of adding OxR to the system have generally not been investigated (Iwata *et al.* 1998, McGinnity *et al.* 1999, Dai *et al.* 2001). Recently it has been shown that the addition of OxR to CYP2C8 and CYP2C9 co-expressed with OxR can increase relative activity by up to twofold (Yamazaki *et al.* 2002).

The present study compared systematically several methods for the co-expression in *Escherichia coli* of human CYP2C8 and CYP2C9 with OxR to produce a stable catalytically active system. With each P450 enzyme, two cDNA constructs differently engineered to enhance bacterial expression were compared in several co-expression systems. A reconstitution method that consisted of mixing bacterial membranes containing separately expressed CYP and OxR was also investigated. Two plasmid expression resulted in high catalytic activity for both CYP2C8 and CYP2C9, but the mixed membrane approach was effective only for CYP2C9. Importantly, the results show that the cDNA construct and co-expression system used should be ‘customized’ for the individual CYP enzyme.

Materials and methods

Materials

Restriction enzymes were purchased from New England Biolabs, Inc. (Hitchin, Hertfordshire, UK). Torsemide (1-isopropyl-3-[[4-methylphenylamino]pyridine]-3 sulfonyl]urea) and its principal metabolite hydroxytorsemide (1-isopropyl-3-[(3-hydroxymethylamino)-3-pyridyl]-sulfonylurea) were obtained from Boehringer Mannheim International (Mannheim, Germany). All other chemicals and reagents were purchased from Sigma (Sydney, Australia) unless otherwise stated. *E. coli* DH5 α cells were purchased from Life Technologies (Melbourne, Australia).

N-terminus modifications to the CYP2C8 and CYP2C9 cDNAs

N-terminus modifications previously shown to promote high levels of bacterial expression of human P450s were incorporated in the CYP2C8 and CYP2C9 cDNAs (Sandhu *et al.* 1993, Richardson *et al.* 1995). The desired modifications were introduced using polymerase chain reaction (PCR). Both the CYP2C8 and CYP2C9 cDNAs were modified in two ways to enhance bacterial expression and recovery. The first strategy involved replacing the second codon with GCT (substitution of Asp for Ala), deleting codons 3–20, and modifying codons 21–26 for bacterial codon bias (Sandhu *et al.* 1993). These cDNAs are subsequently referred to as CYP2C8 (Δ 3–20) and CYP2C9 (Δ 3–20). Dr E. Gillam (University of Queensland, Brisbane, Australia) kindly provided the CYP2C9 cDNA with these Δ 3–20 N-terminus modifications. The second strategy involved replacing the native leader sequence (codons 1–33) with a modified sequence derived from bovine CYP17A followed by a short CYP2C consensus sequence (Richardson *et al.* 1995). These cDNAs are referred to as CYP2C8 (17 α) and CYP2C9 (17 α). The modified sequence includes silent mutations in the first eight codons of CYP17A designed to reduce formation of stable secondary structures in the RNA transcript plus a portion designed to function as a universal primer for CYP2C cDNAs.

N-terminus modifications to the rOxR cDNA

The cDNA coding for the rat NADPH-cytochrome P450 reductase (rOxR) used in all constructs, except the CYP:rOxR fusions, consisted of the OmpA signal sequence fused upstream of the full length native rOxR sequence (Shen *et al.* 1989). This construct was kindly provided by Professor R. Estabrook (University of Texas South Western Medical Center, Dallas, TX, USA).

Construction of CYP2C8, CYP2C9 and rOxR expression and co-expression plasmids

All expression constructs were generated using the pCW ori (+) plasmid (provided by Professor R. Estabrook) unless indicated otherwise. Constructs used for the expression of CYP alone were generated by inserting the PCR amplified cDNA (incorporating the corresponding N-terminal modifications and deletion of the 3' non-coding sequence) into the appropriate restriction sites in pCW ori(+). Bicistronic constructs were generated by inserting the PCR amplified OmpA-rOxR cDNA into a restriction site just downstream of the CYP cDNA. The stop codon of the CYP and start codon of OmpA-rOxR cDNAs were separated by 15 nucleotides. Only one bicistronic construct was generated for each CYP. The N-terminal modification containing the (Δ 3–20 deletion or 17 α presequence) that gave the highest expression for each CYP when expressed alone was used in the bicistronic constructs.

The CYP2C9-rOxR fusion cDNA was created by fusion PCR (Yon and Fried 1989) in a similar manner to that reported by Fisher *et al.* (1992). It comprised the CYP2C9 cDNA bearing the Δ 3–20 deletion followed by a truncated rOxR cDNA (residues 1–56 deleted) that incorporated the catalytic domain. To provide flexibility for protein–protein interactions between the CYP and OxR, the respective cDNAs were separated by a linker that coded for two amino acids (Ser and Thr).

The rOxR construct used in the two-plasmid co-expression system was constructed in the plasmid pACYC184. The pACYC184 plasmid has a different origin of replication and selective marker conferring compatibility with co-transformed pCW based expression plasmids. The construct was generated according to Pritchard *et al.* (1998), except that the OmpA-rOxR fusion cDNA was used in the present study.

Protein expression and preparation of membrane fractions of *E. coli*

Plasmids were transformed into *E. coli* DH5 α cells. Overnight cultures (5 ml) grown in Luria–Bertani broth with ampicillin (50 mg l⁻¹) and/or chloramphenicol (50 mg l⁻¹) at 37°C were used to inoculate a 50 ml culture of Terrific broth containing 50 mg l⁻¹ ampicillin and/or 50 mg l⁻¹ chloramphenicol. Cultures were grown at 37°C with shaking (200 rpm) for 4 h, or until an optical density of approximately 0.7 at 600 nm was attained, at which time isopropyl- β -D-thiogalactopyranoside (1 mM) and δ -aminolevulinic acid (0.5 mM) were added. Cultures were subsequently grown at 30°C with shaking at 200 rpm for an additional 24 h. Cells were then harvested and membranes prepared according to an established protocol (Gillam *et al.* 1993). Membranes were stored at –20°C in a 1:1 mixture of TES (Tris/EDTA/sucrose buffer) and water. Membranes in which both CYP and OxR were expressed were diluted in water

at 0°C to give an appropriate CYP concentration before being added to the reaction mix. For reconstitution experiments, membranes containing CYP and OxR were mixed on ice to the required CYP:OxR ratio and then added to the reaction mix.

Torsemide hydroxylation assay

Torsemide (TA) tolylmethylhydroxylation was used to assess the catalytic activity of expressed CYP2C8 and 2C9 proteins. Although TA is hydroxylated primarily by CYP2C9 in human liver microsomes, recombinant CYP2C8 also exhibits activity towards this compound and hence TA serves as a convenient probe substrate for both enzymes in expression systems (Miners *et al.* 1995, 2000). TA hydroxylase activity was measured according to the procedure of Miners *et al.* (1995). Briefly, the reaction mixtures contained expressed CYP2C8 or CYP2C9 (10–50 pmol) and OxR (0.1–10 molar ratio with CYP), TA (1–750 µM), NADPH-generating system (1 mM NADP⁺, 10 mM glucose 6-phosphate and 2 IU glucose 6-phosphate dehydrogenase) and phosphate buffer (0.1 M, pH 7.4) in a total volume of 1 ml. Incubations were performed at 37°C, and terminated after 20 min by the addition of perchloric acid (0.01 ml, 11.6 M) and cooling on ice. Following addition of 4-methylumbelliferone (4 MU; 4 nmol, the assay internal standard), reaction mixtures were saturated with ammonium sulfate (1.5 g) and hydroxy-TA was extracted with dichloromethane-*isopropanol* (85:15, 2 × 4 ml). The combined extracts were evaporated under a stream of dry N₂ and reconstituted in 0.2 ml HPLC mobile phase. An aliquot was injected onto the HPLC column (Waters Nova-Pak C18, 15 cm × 3.9 mm i.d., 4 µm particle size; Millipore, Milford, MA, USA), which was eluted with acetate buffer (pH 4.3, 50 mM)-acetonitrile (86:14) at a flow rate of 2 ml min⁻¹. Peaks were monitored using a UV-Vis detector set at 290 nm. Retention times for hydroxy-TA and 4 MU were 2.5 and 8.0 min, respectively. Standard curves for hydroxy-TA were constructed in the concentration range 0.1–5 µM, and unknown concentrations were determined by comparison of peak height ratios with those of the standard curve.

Rate of hydroxy-TA formation versus substrate concentration data were model-fitted using the program EnzFitter (BioSoft, Cambridge, UK), and in all experiments a single enzyme Michaelis-Menten model best described kinetic data.

Other methods

Protein concentrations were determined according to Lowry *et al.* (1951). The CYP content of membrane fractions was determined spectrophotometrically (Omura and Sato 1964). The rate of reduction of cytochrome c (Yasukochi and Masters 1976) was used as a measure of OxR activity in membrane fractions.

Results

All constructs expressed CYP2C8 or CYP2C9 holoenzyme as determined by the spectral P450 content, or active NADPH-cytochrome P450 reductase as determined by cytochrome c reductase activity (table 1). With CYP2C9, the Δ3–20 construct expressed approximately four times higher levels of haemoprotein than the construct bearing the 17α presequence. The reverse was true for CYP2C8, where the construct containing 17α presequence was more efficient than the Δ3–20 construct. The OmpA-rOxR cDNA expressed active reductase at a high level. Further expression constructs used the Δ3–20 leader sequence for CYP2C9 and the 17α leader sequence for CYP2C8 as these were expressed most

Table 1. Expression levels for CYP2C8, CYP2C9 and OxR when expressed alone.

Construct	CYP or OxR content (nmol mg ⁻¹ protein)	CYP or OxR content (nmol l ⁻¹ culture)
CYP2C8 (Δ3–20)	0.286 ± 0.024	133 ± 10
CYP2C9 (Δ3–20)	0.800 ± 0.109	419 ± 25
CYP2C8 (17α)	0.873 ± 0.075	399 ± 47
CYP2C9 (17α)	0.248 ± 0.042	114 ± 31
OmpA-rOxR	0.504 ± 0.162	207 ± 72

Values are the mean of three or more replicates ± 1 SD.

Table 2. Expression levels of CYP2C8, CYP2C9 and OxR from co-expression systems and CYP:OxR molar ratio for each system.

Construct	CYP (nmol l ⁻¹ culture)	OxR (nmol l ⁻¹ culture)	CYP to OxR (molar ratio)
CYP2C9 two plasmid	244 ± 26	268 ± 84	1:1.10
CYP2C9 bicistronic	352 ± 43	97 ± 19	1:0.27
CYP2C9 fusion	120 ± 27	247 ± 35	1:2.06
CYP2C8 two plasmid	351 ± 79	292 ± 69	1:0.83
CYP2C8 bicistronic	390 ± 57	69 ± 13	1:0.18

Values are the mean of three or more separate expression experiments ± 1 SD.

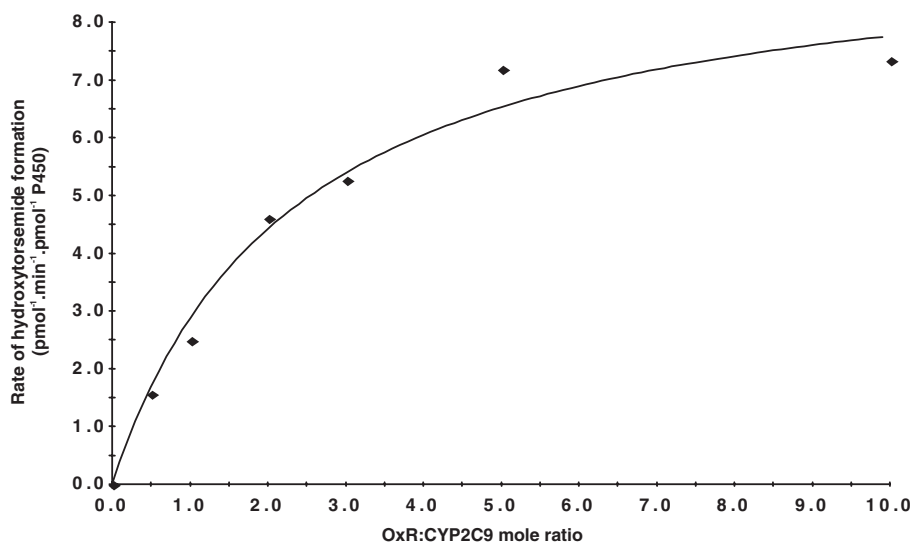


Figure 1. Rate of torsemide hydroxylation versus the molar ratio of OxR to CYP2C9 for the mixed membrane system with torsemide as the substrate. Data are the average of duplicate estimations.

efficiently. Table 2 shows the expression levels for P450 and reductase using various co-expression strategies. CYP expression was highest with the bicistronic constructs, but reductase expression was low so that the CYP:OxR ratio was of the order of 1:0.2 for both CYP2C9 and CYP2C8. Transformation simultaneously with CYP and OxR plasmids resulted in colonies that expressed high levels of both CYP haemoprotein and OxR with a ratio of approximately 1:1. The fusion construct showed relatively low levels of expression of CYP and an apparent CYP:OxR ratio of 1:2 where, by definition, the ratio should be unity. This may be due to expression of active reductase linked to misfolded CYP resulting in apoprotein that has failed to incorporate haem.

Another strategy investigated here for producing an active CYP system was the mixing of bacterial membranes separately containing expressed CYP or OxR. Figure 1 shows the activity of the CYP2C9 ($\Delta 3-20$) reconstituted system using torsemide as substrate. Activity increased as reductase membranes were added to the CYP2C9 membranes, reaching a maximum at a CYP:OxR ratio of approximately 1:5. This ratio was used in subsequent kinetic experiments.

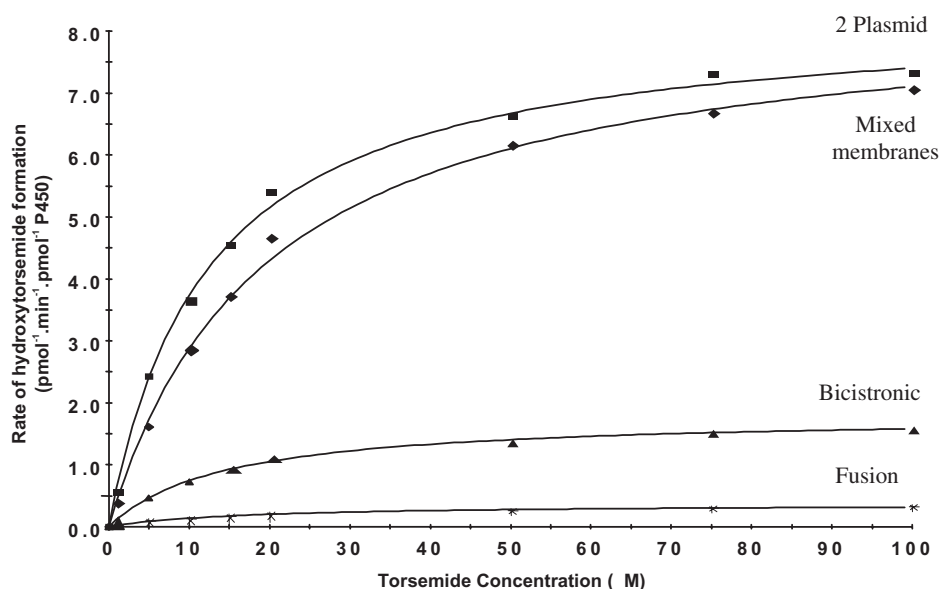


Figure 2. Kinetic plots for CYP2C9 expression systems with torsemide as the substrate. Data are the average of duplicate estimations.

Table 3. Kinetic parameters (K_m , V_{max} , V_{max}/K_m) for torsemide hydroxylation by CYP2C9/OxR co-expression systems.

Expression system	K_m (μM)	V_{max} ($\text{pmol min}^{-1} \text{pmol}^{-1} \text{P450}$)	V_{max}/K_m ($\mu\text{l min}^{-1} \text{pmol}^{-1} \text{P450}$)
Two plasmid	11.9 ± 0.9	7.6 ± 1.4	0.64 ± 0.08
Bicistronic	14.5 ± 0.9	1.8 ± 0.1	0.12 ± 0.004
Fusion	17.4 ± 1.6	0.36 ± 0.05	0.021 ± 0.003
Mixed membrane 1:5	20.2 ± 1.2	8.6 ± 0.2	0.43 ± 0.03

Values are the mean of three or more assays ± 1 SD.

The activity and functional characteristics of the CYP2C9 ($\Delta 3-20$) expression systems were similarly investigated with torsemide as substrate. Representative kinetic plots are shown in figure 2 and the derived kinetic parameters in table 3. Mean apparent K_m values for the different constructs fell within a narrow range (11.9–20.2 μM). V_{max} values, however, varied by more than 20-fold, and *in vitro* intrinsic clearance (V_{max}/K_m) varied more than 30-fold. The bicistronic construct had a low V_{max} reflecting the low content of reductase in this expression system. The fusion construct, despite having an apparent K_m similar to the other systems, had low activity with a V_{max} 20-fold lower than the two plasmid system which had a similar CYP:OxR ratio.

For CYP2C8, co-expression using the two plasmid system (CYP:OxR ratio of 1:0.8) gave an apparent K_m of $147 \pm 9 \mu\text{M}$ and V_{max} of $1.8 \pm 0.1 \text{ pmol min}^{-1} \text{pmol}^{-1} \text{P450}$. The bicistronic system (CYP:OxR ratio of 1:0.18) gave an apparent K_m of 126 μM and V_{max} of $0.31 \text{ pmol min}^{-1} \text{pmol}^{-1} \text{P450}$ (single determination). Using the mixed membrane approach with the CYP2C8(17 α) protein, the reconstituted activity was too low for determination of the kinetic constants.

Mixing bacterial membranes containing separately expressed CYP and OxR gave effective reconstitution of activity with the CYP2C9 ($\Delta 3-20$) construct, but very low activity with the CYP2C8 (17 α) construct. To investigate whether this was a function of the specific P450 or the leader sequence used, the various combinations of P450, leader sequence and expression system were systematically investigated (table 4). With both CYP2C9 and CYP2C8, the constructs containing the 17 α -leader showed very low activity when the CYP and OxR membranes were mixed, but the $\Delta 3-20$ constructs effectively reconstituted activity in both cases. At the same CYP:OxR ratio, OxR co-expressed in the same membrane (two plasmid or bicistronic co-expression) supported activity more efficiently than when the membranes were mixed. This was particularly apparent for the 17 α -containing CYP2C9 and CYP2C8 constructs, but was also seen with the $\Delta 3-20$ constructs of both isoforms (table 4). The activity of these co-expressed systems could not be enhanced by increasing the CYP:OxR ratio with membranes containing OxR. This was the case for both isoforms and both leader sequences (table 4). Addition of membranes containing OxR enhanced the activity of the fusion construct but the activity achieved at a CYP:OxR ratio of 1:5 was much lower than with the other systems.

Discussion

The studies reported herein were performed to select an optimal bacterial expression system for structure–function studies of CYP2C8 and CYP2C9. Full assessment of the functional effects of changes in the primary structure of the CYP2C enzymes requires determination of effects on both substrate affinity (apparent K_m) and substrate turnover (V_{max}). This latter parameter in turn is dependent on the presence of the CYP electron transfer partner OxR.

While expression of CYPs in *E. coli* has become routine, different expression systems give variable yields of CYP holoenzyme and very different CYP:OxR ratios. Cytochromes P450 require manipulation of the amino terminus for efficient expression in bacterial systems. Two approaches have generally been employed. The first involves replacing the second codon with GCT (coding for Ala), deleting codons 3–20, and modifying codons 21–26 to accommodate for bacterial codon bias (Sandhu *et al.* 1993). The second involves replacing the native leader sequence (corresponding to codons 1–33) with a modified sequence derived from bovine CYP17A followed by a short CYP2C consensus sequence (Richardson *et al.* 1995). Although CYP2C8 and CYP2C9 are 85% identical in amino acid sequence, the first strategy produced higher expression with CYP2C9, and the second higher expression with CYP2C8 (table 1). Sandhu *et al.* (1993) reported a identical preference for the $\Delta 3-20$ construct with CYP2C9, although the overall expression levels were much lower (5–11 nmol l⁻¹ for the 17 α construct and 9–19 nmol l⁻¹ for the $\Delta 3-20$ construct). In contrast to the present results, Iwata *et al.* (1998) reported very high CYP2C8 expression levels (1620 nmol l⁻¹) with the $\Delta 3-20$ construct. Overall expression levels for the two enzymes (table 1) were similar to those found previously, although levels of up to 1210 nmol l⁻¹ for CYP2C9 using the $\Delta 3-20$ construct (Iwata *et al.* 1998) and 1500 nmol l⁻¹ for CYP2C8 using the 17 α construct (Richardson *et al.* 1995) have been reported. The OxR was efficiently expressed as the OmpA construct (table 1).

Table 4. Rate of torseamide hydroxylation by CYP2C9/OxR expression systems with and without the addition of separately expressed OxR.

CYP system	Molar ratio CYP:OxR as expressed	No added OxR	Rate (pmol min ⁻¹ pmol ⁻¹ p450) ^a		
			CYP: OxR molar ratio after addition of OxR		
			1:1	1:3	1:5
CYP2C9 (Δ3-20) mixed membranes	not applicable	not applicable			
CYP2C9 (Δ3-20) + OxR two plasmid co-expression	1:1	not applicable	3.0	5.6	6.2
CYP2C9 (Δ3-20) bicistronic co-expression	1:0.3	7.6	7.6	7.5	7.7
CYP2C9 (Δ3-20) fusion	1:2.0		3.0	5.6	6.2
CYP2C9 (17α) mixed membranes co-expression	not applicable	not applicable	0.3	0.6	0.9
CYP2C9 (17α) + OxR two plasmid co-expression	1:3	29	0.05	0.14	0.21
CYP2C8 (Δ3-20) mixed membranes	not applicable	not applicable	not applicable	not applicable	31
CYP2C8 (Δ3-20) + OxR two plasmid co-expression	1:1	0.76	0.16	0.25	0.31
CYP2C8 (17α) mixed membranes	not applicable	not applicable	0.76	0.75	0.74
CYP2C8 (17α) + OxR two plasmid co-expression	1:0.8	1.5	0.02	0.02	0.04
			not applicable	1.9	not applicable

^aAt 100 μM torseamide for CYP2C9 and at 750 μM torseamide for CYP2C8. Values are single determinations with duplicate samples.

Strategies for producing a functional CYP in a heterologous system have included purification and reconstitution of CYP and OxR, expression of a CYP:OxR fusion construct (Murakami *et al.* 1987, Fisher *et al.* 1992, Shet *et al.* 1993, Chun *et al.* 1996), bicistronic plasmids (Dong and Porter 1996, Parikh *et al.* 1997), co-expression via a single plasmid with separate promoters for the two cDNAs (Blake *et al.* 1996, Iwata *et al.* 1998) and reconstitution by mixing bacterial membranes containing separately expressed P450 and reductase (Yamazaki *et al.* 1999). In the current study, the specific cDNA constructs shown to promote high levels of expression for each individual P450 were used to produce CYP and OxR co-expression systems. These were systematically compared for their expression efficiency (table 2), for their ability to generate a reproducible catalytically active system, and for their kinetic parameters (K_m and V_{max}) with the substrate torsemide. Two strategies, bicistronic and two plasmid constructs, were assessed for both CYP2C8 and CYP2C9, whereas a CYP:OxR fusion construct was assessed only for CYP2C9. In addition, separate expression of the CYP and OxR with reconstitution by simply mixing the bacterial membranes was investigated.

All systems showed efficient expression of CYP but the bicistronic system under the conditions used here produced relatively low expression of the OxR resulting in low CYP:OxR ratios for both P450s. The functional integrity of the P450s was retained in all systems including the CYP2C9 fusion construct, as the K_m values for torsemide were similar to those reported previously for CYP2C9 in human liver microsomes (Miners *et al.* 1995) and for CYP2C8 and CYP2C9 in a reconstituted system (Miners *et al.* 2000).

CYP:OxR fusion proteins were first reported by Murakami *et al.* (1987) and have been explored extensively (Fisher *et al.* 1996). Although efficiently expressed, the CYP2C9:OxR fusion product has relatively low activity indicating suboptimal intramolecular electron transfer. The activity was only marginally improved by addition of bacterial membranes containing separately expressed OxR. Other reports have similarly usually found no effect of added OxR on CYP:OxR fusion products (Shet *et al.* 1994, Chun *et al.* 1996, Parikh and Guengerich 1997), although Deeni *et al.* (2001) reported some stimulation of the activity of a CYP2D6:OxR fusion protein with added OxR. Interestingly, the CYP2C9:OxR ratio for the fusion construct based on cytochrome c reductase activity was approximately 1:2 rather than the expected 1:1. The reason for this is not clear, but could be due to a higher than expected cytochrome c reductase activity of the truncated OxR in the fusion product. Alternatively, there may have been expression of products containing CYP apoprotein with active reductase. Shet *et al.* (1994) have reported a lower than expected (from the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) content) cytochrome c reductase activity of a bovine CYP17A fusion product.

With the CYP2C9 ($\Delta 3-20$) construct, activity was effectively reconstituted simply by mixing bacterial membranes containing separately expressed CYP2C9 and OxR (figure 1 and table 4). The effect of added OxR reached a maximum at an OxR:CYP ratio of 5:1 (figure 1). Yamazaki *et al.* (1999, 2002) similarly reported that adding bacterial membranes containing OxR to membranes containing expressed CYP3A4 stimulated CYP3A4 activity with the effect reaching a maximum at an OxR:CYP ratio of about 4:1. Other authors have reported optimal OxR:CYP ratios in various systems including 2:1 (a reconstituted system) to 8:1

(bacterial membranes) for bacterial expressed CYP1B1 (Shimada *et al.* 2000), and 2:1 to 4:1 for reconstituted CYP1A2 (Venkatakrishnan *et al.* 2000).

Yamazaki *et al.* (2002) reported a highly variable effect of adding exogenous OxR membranes to bacterial expressed CYP membranes depending on the CYP used. Stimulation by added OxR membranes was marked for CYP3A4 and CYP2C9, moderate for CYP2C8 and absent for CYP2E1, CYP1A1, CYP1A2, CYP1B1 and CYP2D6. In the present study, adding exogenous OxR membranes effectively reconstituted activity with CYP2C9 ($\Delta 3-20$) but not with CYP2C8 (17 α leader sequence). A systematic study was performed to determine whether this was a function of the N-terminal modification or of the particular CYP (table 4). Interestingly, the answer seems to be both. Unlike the $\Delta 3-20$ construct of CYP2C9, neither the 17 α construct of CYP2C9 nor the $\Delta 3-20$ construct of CYP2C8 could effectively be reconstituted by addition of OxR membranes. Another notable feature was that OxR co-expressed with CYP in the same membrane (by the two plasmid system) was more effective than addition of exogenous OxR membranes at interacting with CYP to reconstitute activity. This was apparent with the both CYP2C9 and CYP2C8, and with both types of N-terminal modification (table 4). Shimada *et al.* (2000) also reported that OxR co-expressed in bacterial membranes with CYP2B1 was more effective than added purified OxR in producing an active system.

For ongoing structure–function studies with CYP2C9, the CYP2C9 ($\Delta 3-20$) mixed membrane approach was chosen because it has proven to be robust and simple. The CYP2C9 (17 α) two plasmid co-expression system has a relatively high OxR:CYP ratio producing high activity per pmol CYP, but a relatively low level of CYP expression. In contrast, with CYP2C8 the two plasmid co-expression with the 17 α construct provides the most reproducible OxR:CYP ratio (approximately 1:1) and stable catalytic activity for this enzyme. From the results of the present work and previous studies of CYP expression in *E. coli*, it is clearly not realistic to produce a single standard ‘optimal’ CYP expression system as the most appropriate strategy will vary for each enzyme. However, it is feasible to produce a system for a particular CYP that has stable catalytic characteristics and is suitable for structure–function studies.

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Oxidative one-carbon cleavage of the octyl side chain of olanexidine, a novel antimicrobial agent, in dog liver microsomes

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1. The oxidative one-carbon cleavage reaction in the octyl side chain of olanexidine [1-(3,4-dichlorobenzyl)-5-octylbiguanide], a new potent biguanide antiseptic, was characterized in dog liver microsomes.

2. Olanexidine was initially biotransformed to a monohydroxylated metabolite, 8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2-octanol (DM-215), and DM-215 was subsequently oxidized to the diol derivative, 8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-1,2-octandiol (DM-220). DM-220 was further biotransformed to 2-hydroxy aldehyde derivative, 2-hydroxy carboxylic acid derivative, and an oxidative C-1–C-2 bond cleavage metabolite, 7-[5-(3,4-dichlorobenzyl)-1-biguanidino] heptanoic acid [DM-223 (C7), a seven-carbon chain derivative], after incubation with dog liver microsomes.

3. DM-223 formation required NADPH as a cofactor and was inhibited by quinidine and quinine, relatively selective inhibitors of CYP2D subfamilies in dogs.

4. The results suggest that the one-carbon fragment of the octyl side chain of olanexidine could be removed by the oxidative C-C bond cleavage with the possible involvement of cytochrome P450 systems such as CYP2D subfamily. This oxidative C-C bond cleavage reaction by cytochrome P450s could play an important role in the removal of one-carbon fragment of other drugs or endogenous compounds containing aliphatic chains.

Introduction

Olanexidine monohydrochloride hemihydrate is a newly synthesized antimicrobial agent (Tsubouchi *et al.* 1997) with potent microbicidal activity against fungi (yeast), Gram-negative bacteria (*Pseudomonas aeruginosa*), and Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) at low concentrations (Ohguro *et al.* 1997, Sakagami *et al.* 1999, 2000).

The absorption, distribution, metabolism and excretion of olanexidine have been reported for rats and dogs after subcutaneous administration (Kudo *et al.* 1998a–d). Olanexidine is eliminated principally by metabolism in rats and dogs (Kudo *et al.* 1998c, d) and four metabolites have been identified in dog urine as 6-[5-(3,4-dichlorobenzyl)-1-biguanidino] hexanoic acid [DM-210 (C6), a six-carbon chain derivative], 4-[5-(3,4-dichlorobenzyl)-1-biguanidino] butanoic

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acid [DM-212 (C4), a four-carbon chain derivative], 5-[5-(3,4-dichlorobenzyl)-1-biguanidino] pentanoic acid [DM-213 (C5), a five-carbon chain derivative], and 3,4-dichlorobenzoic acid (figure 1) (Kudo *et al.* 1998c). Interestingly, each metabolite, except for 3,4-dichlorobenzoic acid, is a methylene chain shorter with a carboxy end and is a predominant metabolite of olanexidine in both rats (Kudo *et al.* 1998d) and dogs (Kudo *et al.* 1998c).

In previous *in vitro* metabolism studies using rat and dog liver preparations, the monohydroxy metabolites {8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2-octanol (DM-215), 8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-3-octanol (DM-218), and 8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-4-octanol (DM-217)}, a ketone derivative {8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2-octanone (DM-219)}, and the dihydroxy metabolites {8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-1,2-octandiol (DM-220), *threo*-8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2,3-octandiol (DM-221), and *erythro*-8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2,3-octandiol (DM-222)} were produced (Umehara *et al.* 2000a). Diols (DM-221 and DM-222) were further biotransformed to a ketol derivative and a C-C bond cleavage metabolite [DM-210 (C6)], an *in vivo* end product, after incubation with dog liver microsomes. Thus, the degradation products of olanexidine are likely to be produced by C-C bond cleavage following sequential oxidative reactions (monohydroxylation, dihydroxylation and ketol formation at the octyl side chain; figure 1), but not β -oxidation after ω -hydroxylation and oxidation to the metabolite with octanoic acid (Umehara *et al.* 2000a). In addition, these oxidative reactions were significantly inhibited by cytochrome P450 inhibitors (i.e. carbon monoxide, *n*-octylamine, SKF 525-A, metyrapone, α -naphthoflavone and quinine), indicating cytochrome P450 (CYP2D subfamily) as an enzyme possibly involved in the metabolism of olanexidine (Umehara *et al.* 2000a, b).

Diol derivatives are important intermediates when cytochrome P450s catalyse C-C bond cleavage of aliphatic chains of drugs or endogenous compounds such as steroids (Akhtar *et al.* 1993, Ortiz de Montellano 1995). Oxidative cleavage reactions of the octyl side chain of olanexidine were characterized using DM-221 and DM-222 as in Umehara *et al.* (2000b). 1,2-Octandiol (DM-220) was also observed in the incubation mixture and oxidative C-1–C-2 bond cleavage reaction is anticipated as with the oxidative cleavage reactions of the octyl side chain of DM-221 and DM-222 (figure 1).

In the current study, the *in vitro* metabolism of olanexidine metabolite 1,2-octandiol (DM-220) was examined using dog liver microsomes to characterize the oxidative C-1–C-2 bond cleavage of the octyl side chain of olanexidine.

Materials and methods

Chemicals

DM-220 dihydrochloride hemihydrate (chemical purity 97.4%) and DM-223 dihydrochloride (99.0%) were obtained from Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan). β -NADPH, β -NADH, β -NAD, β -NADP, α -naphthoflavone, troleandomycin, quinidine and sulfaphenazole were from Sigma Chemical Co. (St Louis, MO, USA). *S*-mephenytoin was from ULTRAFINE Chemical Co. (Manchester, UK). Quinine was from Extrasynthese (Genay, France). Pentobarbital was from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Sodium N,N-diethyldithiocarbamate trihydrate and sodium phenobarbital were from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and solvents were of analytical grade.

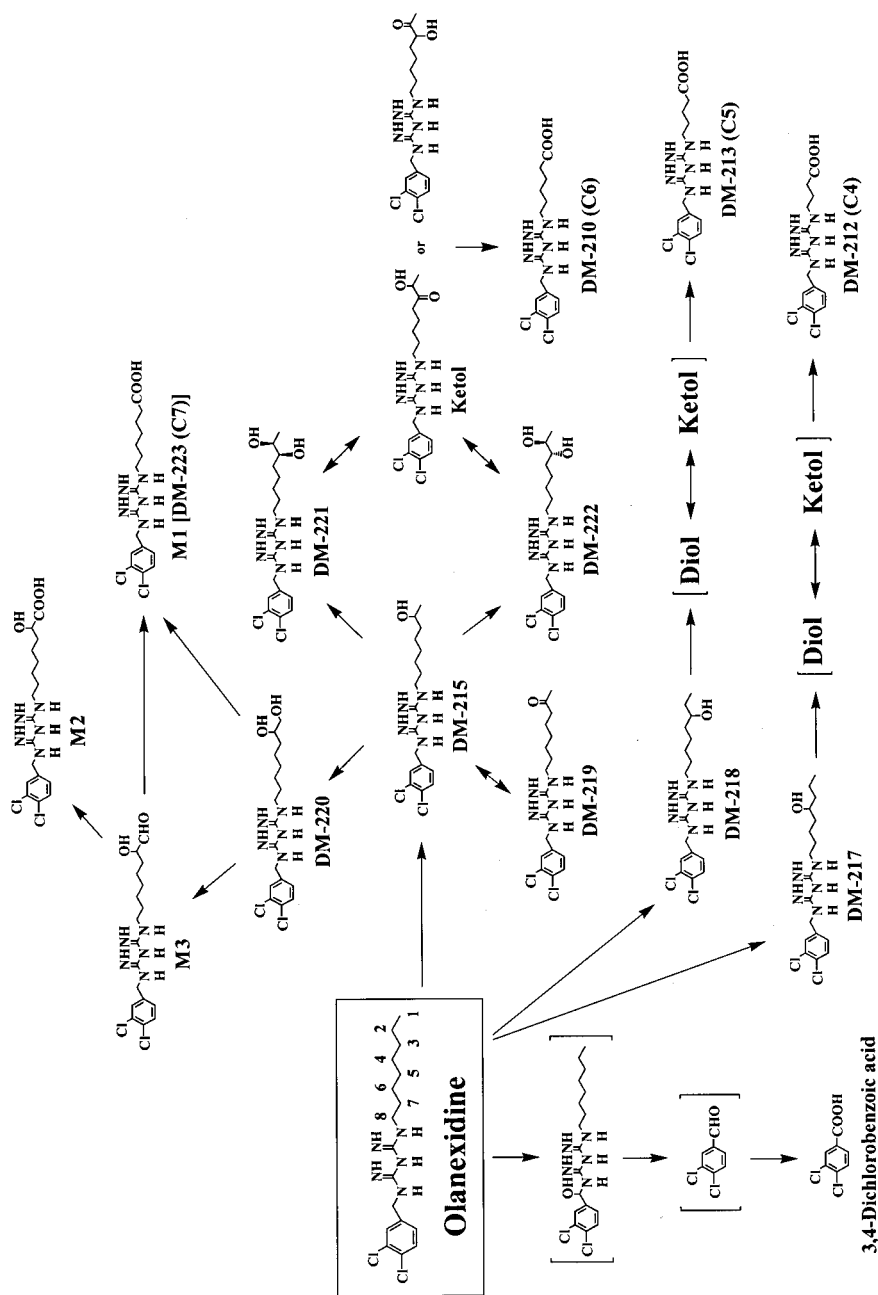


Figure 1. Proposed metabolic pathway of olanzexidine in rat and dog. Pathways of metabolism are hypothetical and compounds shown in brackets were not observed. The pathway from DM-220 to DM-223 (C7) was investigated.

Animal treatments and preparation of microsomes

Three male beagle dogs from Hazelton Research Products, Inc. (Cumberland, VA, USA) were acclimatized in a controlled area maintained at $23 \pm 2^\circ\text{C}$ for temperature and $60 \pm 10\%$ for relative humidity during 12-h light/dark cycles. The animals were fed laboratory chow CD-5 (Japan Clea Co., Ltd, Tokyo, Japan) and water was available *ad libitum* during acclimatization. Dogs of 19–23 months of age were used. They were sacrificed by exsanguination under anaesthesia with pentobarbital. The livers were resected and washed with ice-cold saline and homogenized in 3 vols/liver weight of 1.15% KCl solution with a Waring Blender. The homogenate was centrifuged at 9000g for 30 min at 4°C . To prepare liver microsomes, the 9000g supernatant was centrifuged at 105 000g for 60 min at 4°C . The microsomal pellet thus obtained was resuspended in 0.05 M phosphate buffer (pH 7.4) and the microsomes were stored at -80°C . The protein concentration was determined using Bio-Rad DC Protein Assay Kits (Hercules, CA, USA).

Assay of DM-223 formation activity

The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), 2.5 mM β -NADPH, DM-220 dihydrochloride hemihydrate, and dog microsomal protein (1 mg) in a final incubation volume of 0.5 ml. DM-220 dihydrochloride hemihydrate was dissolved in methanol. The reaction was initiated by the addition of cofactor, and was carried out in air at 37°C in a shaking water bath for 20 min. Under these conditions, the reaction rate was linear for at least 60 min at the enzyme concentration used.

The effects of the following relatively selective CYP inhibitors on the DM-223 formation were examined: α -naphthoflavone for CYP1A1/2 (Tassaneeyakul *et al.* 1993), sulfaphenazole for CYP2C8/9 (Back *et al.* 1988), *S*-mephenytoin for CYP2C19 (Wrighton *et al.* 1993), quinidine and quinine for CYP2D (Otton *et al.* 1988, Kobayashi *et al.* 1989, Roussel *et al.* 1998), diethyldithiocarbamate for CYP2E1 (Guengerich *et al.* 1991, Chauret *et al.* 1997), and troleandomycin for CYP3A (Watkins *et al.* 1985, Sonderfan *et al.* 1987, Cook *et al.* 2002). The concentration of DM-220 dihydrochloride hemihydrate was $5 \mu\text{M}$. The inhibitors were pre-incubated with dog liver microsomes and cofactor for 15 min, and the reaction was initiated by the addition of the substrates. The concentrations of inhibitors ranged from 1 to $100 \mu\text{M}$.

For all experiments, the reaction was terminated by adding 0.5 ml methanol. An aliquot (50 μl) of the supernatant obtained after centrifugation to pellet the denatured protein was analysed by high-performance liquid chromatography (HPLC) as described below.

High-performance liquid chromatography

The HPLC system consisted of two model 510 high-pressure HPLC pumps, a model 717 automatic sample processor, a model 486 ultraviolet light (UV) detector, a model 680 automatic gradient controller (all Waters Associates, Milford, MA), and a model C-R7A Chromatopac (Shimadzu, Kyoto, Japan).

In the assay for DM-223 production, a TSKgel ODS-80_{TM} column (4.6 mm i.d. $\times$ 150 mm; Tosoh, Tokyo, Japan) was used at a flow rate of 1 ml min^{-1} with UV detection at 240 nm. As the mobile phase, 10% acetonitrile in water containing 0.1% acetic acid was used as solution A and 80% acetonitrile in water containing 0.1% acetic acid as solution B. The metabolites were analysed using a linear gradient developed from 15 to 35% solution B over 30 min. The retention times for DM-220 and DM-223 were 15.4 and 20.5 min, respectively. The calibration curve of DM-223 was established by an absolute standard method, based on the peak area of DM-223.

Characterization of M1–3

The reaction mixture with DM-220 and dog liver microsomes was extracted by methanol, and the extract was analysed by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) to characterize the chemical structures of the metabolites.

HPLC analysis was carried out with a Shimadzu HPLC system equipped with a model CLASS-LC10 system controller, a model LC-10AD pump, a model SIL-10A_{XL} automatic sample processor with sample cooler S, a model CTO-10AC column thermoregulation bath, a model FCV-14AH column switching valve, and a model DGU-14A in-line degasser. A YMC-Pack Pro C18 column (2.0 mm i.d. $\times$ 150 mm; YMC Co., Ltd, Kyoto, Japan) was used at a flow rate of 0.2 ml min^{-1} . The metabolites were analysed using a linear gradient developed from 0 to 50% solution B over 45 min.

MS/MS analysis was performed using a Finnigan MAT (San Jose, CA, USA) triple-stage quadrupole TSQ-7000 mass spectrometer equipped with an ESI source. The interface and mass spectrometer were operated under the following conditions: ion mode, positive; capillary temperature, 240°C ; sheath gas pressure of nitrogen, 70 psi; auxiliary nitrogen gas flow rate, 10 U; electron multiplier voltage, 1.4 kV; collision gas (argon), about 2.0 mTorr; and collision energy, -24 eV .

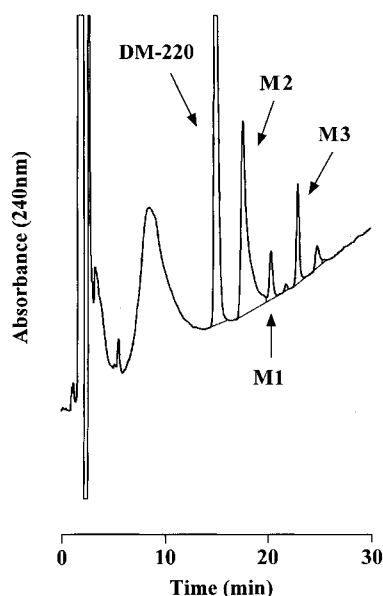


Figure 2. HPLC chromatogram of the methanol extract obtained from the incubation mixture of DM-220 with dog liver microsomes. DM-220 (100 μ M) was incubated at 37°C with dog liver microsomes in the presence of NADPH (2.5 mM) in 0.1 M phosphate buffer (pH 7.4) for 60 min.

Results

In vitro metabolism of DM-220 by dog liver microsomes

DM-220 was incubated with dog liver microsomes in the presence of NADPH, and the methanol extract was analysed by HPLC-UV and LC/ESI-MS/MS. In the reaction mixture, three metabolites were observed: M1–3 (figure 2).

M1 was identified as DM-223 since its $[M+H]^+$ ions in the precursor ion mass spectra, the product ion mass spectra (figure 3A) and HPLC retention time were in good agreement with those for the authentic standard. M2 displayed molecular ions of m/z 418 $[M+H]^+$ in the precursor ion mass spectrum, indicating that this metabolite had a molecular weight of 417, this being 14 greater than that of the unchanged DM-220. In addition, the characteristic fragment ions at m/z 372 and 218 were observed in the product mass spectrum (figure 3B). M2 would thus appear to be 2-hydroxy carboxylic acid derivative. M3 displayed molecular ions of m/z 402 $[M+H]^+$ in the precursor ion mass spectrum, indicating that this metabolite had a molecular weight of 401, this being two smaller than that of the unchanged DM-220. In addition, the characteristic fragment ions at m/z 218 and 202 were observed in the product mass spectrum (figure 3C). M3 would thus appear to be a ketol derivative of DM-220 or 2-hydroxy aldehyde derivative. Since a 2-hydroxy carboxylic acid derivative was observed in the reaction mixture, M3 was thought to be the 2-hydroxy aldehyde derivative.

DM-223 formation activity in dog liver microsomes

The cofactor requirement for microsomal DM-223 formation was examined (table 1). The reactions required NADPH for maximal activity. NADH was much less effective for the catalytic activity, and NAD^+ and $NADP^+$ were ineffective

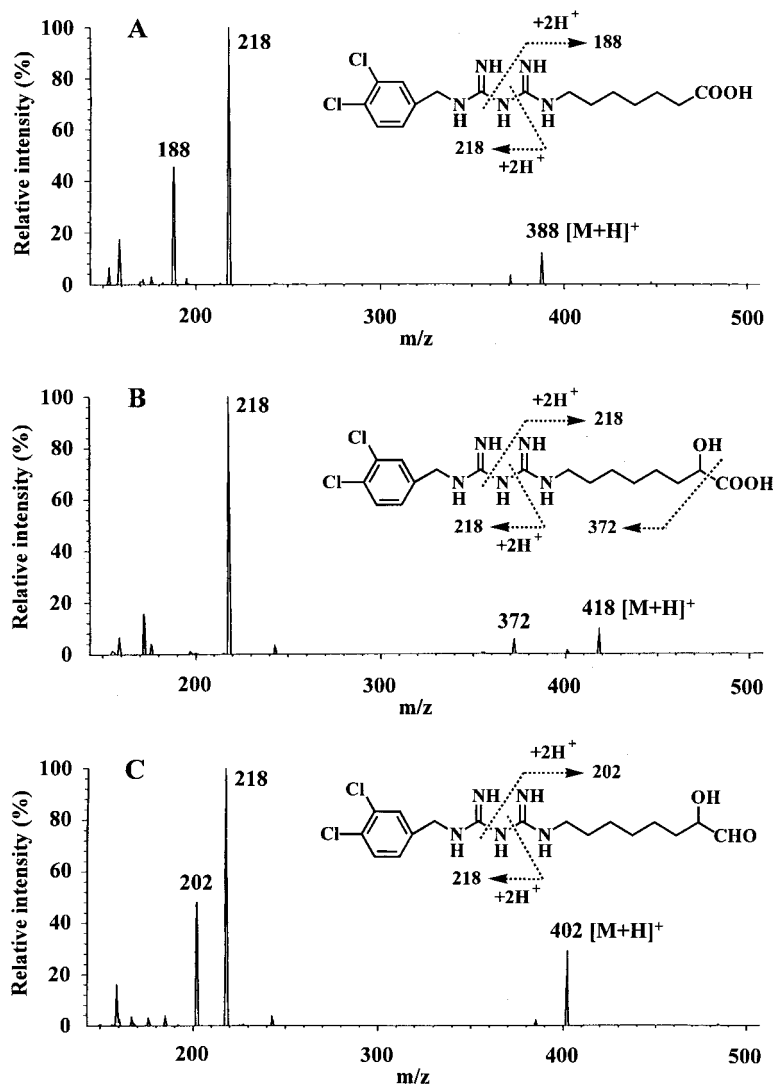


Figure 3. ESI product ion mass spectrum of [M + H]⁺ of M1 at *m/z* 388 (A), M2 at *m/z* 418 (B) and M3 at *m/z* 402 (C).

Table 1. Cofactor requirement for DM-223 formation with dog liver microsomes.

Cofactors	DM-223 formed (pmol min ⁻¹ mg ⁻¹)
None	n.d. (n.d.)
NAD ⁺	n.d. (n.d.)
NADP ⁺	n.d. (n.d.)
NADH	7.4 ± 0.5 (45)
NADPH	16.5 ± 0.5 (100)

Enzyme incubation and metabolite analysis were carried out in triplicate and the data expressed as means ± SD. Each cofactor was added to the incubation mixture at 2.5 mM. Numbers in parentheses are the relative activities.
n.d., Not detected.

Table 2. Effects of various inhibitors on DM-223 formation with dog liver microsomes.

Inhibitors	DM-223 formed (pmol min ⁻¹ mg ⁻¹)
Control	3.7 ± 0.2 (100)
α-Naphthoflavone (1 μM)	3.7 ± 0.3 (100)
Sulfaphenazole (100 μM)	3.4 ± 0.2 (92)
S-mephenytoin (100 μM)	3.9 ± 0.6 (105)
Quinidine (10 μM)	n.d. (n.d.)
Quinidine (100 μM)	n.d. (n.d.)
Quinine (10 μM)	n.d. (n.d.)
Quinine (100 μM)	n.d. (n.d.)
Diethyldithiocarbamate (100 μM)	4.2 ± 0.5 (111)
Troleandomycin (100 μM)	4.3 ± 0.5 (114)

DM-220 was incubated with microsomes for 20 min at 37°C in the presence of various inhibitors. Enzyme incubation and metabolite analysis were carried out in triplicate and the data expressed as means ± SD. Numbers in parentheses are the relative activities.
n.d., Not detected.

as cofactors. DM-223 formation was significantly inhibited by the addition of *n*-octylamine, a typical inhibitor of cytochrome P450 system (Minato *et al.* 1999), and SKF 525-A and metyrapone, known to be inhibitors of phenobarbital inducible cytochrome P450 enzymes (Lu *et al.* 1972, Buening and Franklin 1974) (data not shown).

A number of known cytochrome P450 inhibitors were used to determine the potential roles of cytochrome P450 enzymes on DM-223 formation (table 2). Quinidine and quinine, relatively selective inhibitors of CYP2D subfamilies in rats, dogs and man, completely inhibited DM-223 formation. α-Naphthoflavone (an inhibitor of CYP1A1/2 subfamilies in human), sulfaphenazole (an inhibitor of CYP2C8/9 subfamilies in human), *S*-mephenytoin (an inhibitor of CYP2C19 subfamily in human), diethyldithiocarbamate (an inhibitor of CYP2E1 subfamily in dogs and human), and troleandomycin (an inhibitor of CYP3A subfamilies in rats, dogs and human) had no inhibitory effect on DM-223 formation activity in male dog liver microsomes.

Discussion

The present study has demonstrated that a one-carbon fragment of the octyl side chain of olanexidine is removed by sequential oxidative reactions. Olanexidine was initially monhydroxylated to DM-215, which was subsequently oxidized to a diol derivative (DM-220). DM-220 was further biotransformed to the 2-hydroxy aldehyde derivative, the 2-hydroxy carboxylic acid derivative and DM-223 (heptanoic acid derivative) after incubation with dog liver microsomes. Low amounts of DM-223 have been detected in the excreta of rats and dogs following subcutaneous administration of olanexidine (Kudo *et al.* 1998c, d). DM-223 formation required NADPH as a cofactor and was inhibited by typical cytochrome P450 inhibitors such as *n*-octylamine, SKF 525-A and metyrapone (data not shown). In addition, the reaction was inhibited by quinidine and quinine, relatively selective inhibitors of CYP2D subfamilies in dogs, indicating this reaction to be catalysed by the cytochrome P450 system such as the CYP2D subfamily.

Some cytochrome P450 enzymes integrated into biosynthetic pathways have catalytic activity not only in conventional hydroxylation reactions, but also C-C bond cleavage reactions (Akhtar *et al.* 1993, Ortiz de Montellano 1995). A mitochondrial P450 enzyme (CYP11A1) cleaves the C-20–C-22 bond of cholesterol, and the activated oxygen complex ($[\text{Fe}=\text{O}]^{3+}$) of CYP11A1 is thought to react with a diol intermediate of cholesterol to sever the C-20–C-22 bond (Shikita and Hall 1974, Byon and Gut 1980, Ortiz de Montellano 1995). In addition, CYP17 cleaves the C-17–C-20 bond of progesterone, and a ferric peroxide complex ($\text{Fe}^{3+}\text{-O-OH}$) of CYP17 is thought to react with a carbonyl intermediate of progesterone to sever the C-17–C-20 bond (Corina *et al.* 1991, Miller *et al.* 1991). The same mechanisms involving the intermediate formation of a high-valent oxoiron complex ($[\text{Fe}=\text{O}]^{3+}$) for the bond cleavage catalysed by CYP11A1 and/or a ferric peroxide complex ($\text{Fe}^{3+}\text{-O-OH}$) for the bond cleavage catalysed by CYP17 may be applicable to oxidative C-1–C-2 bond cleavage of the octyl side chain of olanexidine, because the diol and carbonyl compounds were observed in the reaction mixture.

The inhibition data in this study using quinine and quinidine are consistent with the proposed oxidative metabolism of olanexidine by the CYP2D subfamily (Roussel *et al.* 1998). In CYP2D subfamily-mediated catalytic reactions, it has been suggested that the CYP2D subfamily requires ion-pair formation between a substrate and the enzyme for effective catalytic activity (Smith 1991, Smith and Jones 1992) and hypothetically, a positively charged basic nitrogen ($pK_a > 7.5$) located 5–7 Å from the site of oxidation for typical CYP2D subfamily substrate (Strobl and Wolff 1991). DM-220 has two basic nitrogen atoms in the biguanide structure, which are highly ionized at physiological pH, and therefore may be a typical substrate of the CYP2D subfamily.

α -Oxidation, as well-known in the metabolism of branched-chain fatty acids such as phytanic acid, results in C-C bond cleavage of successive one-carbon fragment starting from the carboxyl-terminal, leading to fatty acids with an odd number of carbon atoms in the methylene chain (Vamecq and Draye 1988, Wanders and Van Roermund 1993). Although there is little information about the α -oxidation mechanism and the enzyme(s) involved in this reaction system, recently, the α -oxidation mechanism in peroxisomes has been resolved (Verhoeven *et al.* 1998, Mannaerts *et al.* 2000, Jansen *et al.* 2001). The α -oxidation mechanism of phytanic acids includes a series of four reactions: (1) activation of phytanic acid to phytanoyl-CoA, (2) hydroxylation of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA, (3) cleavage of 2-hydroxyphytanoyl-CoA to pristanal and formyl-CoA and (4) oxidation of pristanal to pristanic acid. 2-Hydroxy carboxylic acid derivative was also observed in the incubation of DM-220 with dog liver microsomes. If the 2-hydroxy carboxylic acid derivative was activated to its CoA-ester, it may be catalysed by α -oxidation system leading to DM-223.

The present study showed that one-carbon fragment of aliphatic chains could be removed by the CYP-catalysed sequential oxidative reactions in dog liver microsomes other than the classical α -oxidation system in peroxisomes. As with the metabolic pathway of olanexidine, there are compounds whose metabolites contain side chains in which one-carbon fragment has been removed by metabolism (Harvey 1989, Takahashi *et al.* 1992). The pentyl side chain of cannabidiol and its derivatives are reduced to four carbon atoms by removal of one carbon atom (Harvey and Leuschner 1985, Harvey 1989, 1990, Samara *et al.* 1990).

Although the mechanism for removal of one carbon fragment still remains to be fully determined, the metabolic routes are considered to involve intermediates derived from monohydroxylation, dihydroxylation and 2-hydroxy carboxylic acid derivative (Harvey 1989, Samara *et al.* 1990). In analogy with the production mechanism of oxidative cleavage metabolite of olanexidine, oxidative C-C bond cleavage by cytochrome P450s may be essential for the removal of a one-carbon fragment in the metabolism of these compounds.

In summary, the present *in vitro* study demonstrates that a one-carbon fragment of the octyl side chain of olanexidine could be removed by the oxidative C-C bond cleavage with the possible involvement of cytochrome P450 systems (the CYP2D subfamily). Such oxidative C-C bond cleavage by cytochrome P450s could play an important role in the removal of one-carbon fragment of other drugs or endogenous compounds containing aliphatic chains.

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NMR and QSAR studies on the transacylation reactivity of model 1 β -O-acyl glucuronides. I: Design, synthesis and degradation rate measurement

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1. The products arising from intramolecular acyl migration reactions of drug ester glucuronides are reactive towards cellular proteins and can potentially cause toxic side-effects. The relationship between molecular structure and the degradation rates (k_d) of 1 β -O-acyl glucuronides were investigated systematically using a series of model compounds based on 4-substituted benzoic acids.

2. A rational method for selecting suitable compounds for inclusion was used and 10 glucuronide esters, predicted to produce a wide range of transacylation rates, were synthesized via a simple 'one-pot' method using an imidazole intermediate. The 10 substituents, where X = NO₂, CN, I, Br, F, H, nPr, Et, OMe, O-nPr, had degradation rate half-lives ($t_{1/2} = \log_e(2)/k_d$) ranging from 0.9 to 106.6 h. The reactions resulted in mixtures, which predominantly consisted of the desired 1 β -O-acyl glucuronides.

3. It was demonstrated that further purification was unnecessary for determination of k_d of the synthetic 1 β -O-acyl glucuronides. Degradation rates (k_d) were calculated by following the disappearance of the ¹H-NMR signal from the 1 β -anomeric proton of the glucuronic acid moiety as the reaction progressed in pH 7.4 buffer inside a nuclear magnetic resonance tube. Each measured degradation rate represents a pseudo-first-order rate constant, which is a combination of the transacylation rate (1 β to 2 β isomer) and the hydrolysis rate.

4. Degradation rates show a clear relationship with substituent properties, with half-life increasing as the substituent becomes more electron-donating, e.g. 4-nitro $t_{1/2}$ = 0.9 h and 4-propoxy $t_{1/2}$ = 106.6 h.

Introduction

A major route for the elimination of drugs with a carboxylic acid functional group is conjugation with β -D-glucuronic acid to form 1 β -O-acyl glucuronides with an ester linkage (Jakoby *et al.* 1982, Mulder 1990, Mulder 1992, Fenselau 1994, Gibson and Skett 1994, Gavaghan 1997). Such 1 β -O-acyl glucuronides show a range of reactivities and are known to be unstable in aqueous solution under acidic, neutral and alkaline pH conditions (Gavaghan 1997). In physiological conditions, at pH 7.4, the carbonyl carbon of the acyl ester is susceptible to nucleophilic attack and the 1 β -O-acyl glucuronide can either hydrolyse, liberating the aglycone, or alternatively the molecule can undergo an intramolecular rearrangement, via a tetrahedral intermediate (figure 1), involving acyl migration

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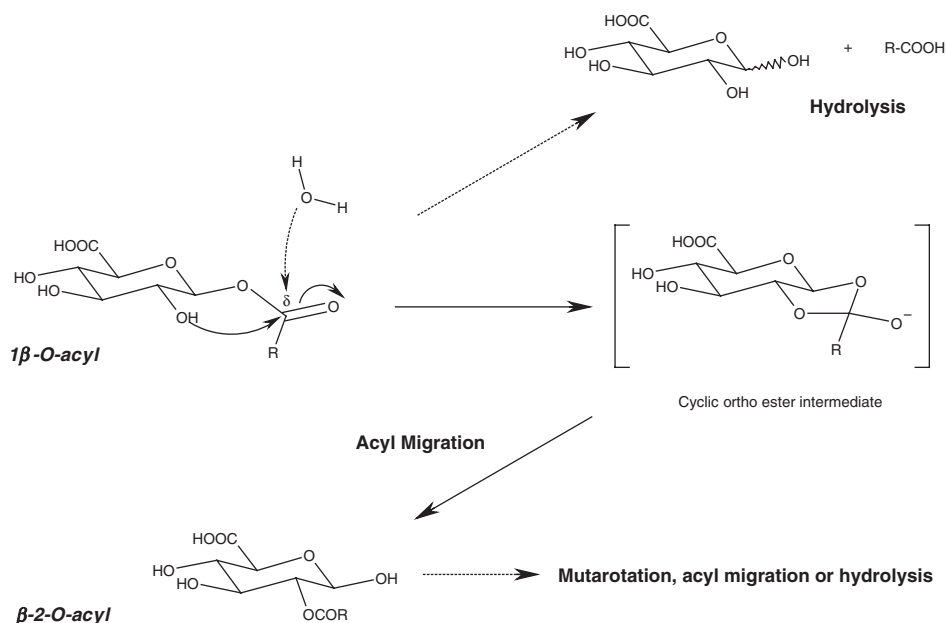


Figure 1. Mechanism of transacylation and hydrolysis for ester glucuronides.

to the 2-, then subsequently, 3- and 4-*O*-acyl positions. The initial transition from the 1- to 2-*O*-acyl position was thought to be an irreversible reaction but recent experiments have shown the formation of 1α-*O*-acyl glucuronides *in vitro*; these are presumably formed from the α-2-*O*-acyl glucuronides (themselves formed after transacylation from the 1β-*O*-acyl anomers followed by mutarotation of the β-2-*O*-acyl isomers) via a cyclic *ortho*-acid ester intermediate with 1,2-*cis*-(ax,eq) fused rings (Corcoran *et al.* 2001). In light of this new evidence, the analogous reaction of formation of 1β-*O*-acyl from the β-2-*O*-acyl isomers (via cyclic *ortho*-acid ester intermediates with 1,2-*trans*-(eq,eq) fused rings) cannot be ruled out and was in fact first proposed by Hansen-Moller *et al.* (1998) on the basis of HPLC/UV analysis of diflunisal 1β-*O*-acyl glucuronides and enzyme hydrolysis experiments.

Following migration of the acyl group from the 1-position, the carbohydrate ring can open and recyclize via the aldehyde isomer, enabling mutarotation to occur and yielding the α- and β-anomers of the 2-, 3- and 4-*O*-acyl positional isomers. The resulting transacylation equilibrium mixture contains six positional isomers and anomers, plus residual amounts of 1β-*O*-acyl/1α-*O*-acyl isomers formed by back-reaction. In addition, all of these isomers are, irreversibly, undergoing hydrolysis. In total, these interconversions and hydrolyses involve 26 individual rate constants. The overall situation is summarized in figure 2. It is thought that the resulting glucuronides (particularly the isomers in the 3- and 4-position) may act as haptens (Park and Kitteringham 1990) and stimulate immune responses/allergic reactions *in vivo* as they can react, irreversibly, to form covalently bound adducts with cellular macromolecules, via imine formation/Amadori rearrangement or nucleophilic displacement (Park *et al.* 1987, Smith *et al.* 1990). The ability to undergo these rapid acyl migrations appears to be related to the incidence of hypersensitivity in many cases and these reaction products have also recently been linked to hepatotoxicity (Wade *et al.* 1997).

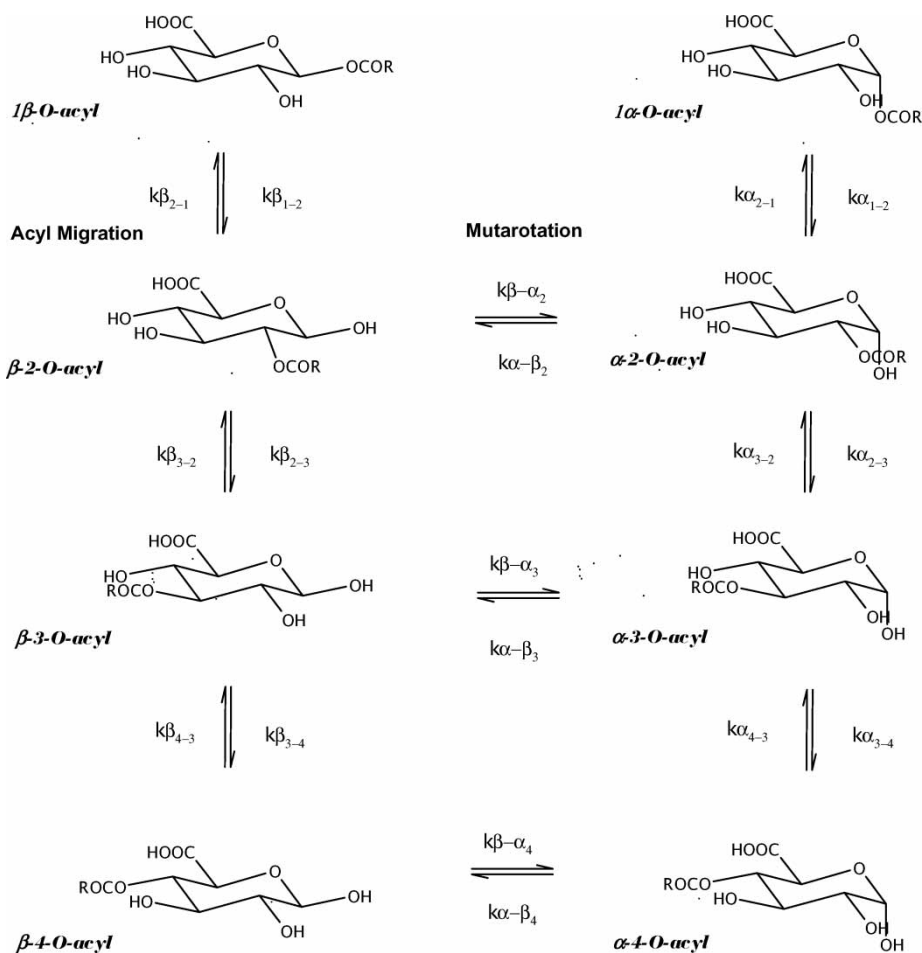


Figure 2. Transacylation reaction scheme for 1β-O-acyl glucuronides. (Note: each isomer in this scheme can also hydrolyse.)

Irreversible binding of acyl glucuronides to proteins was first reported for bilirubin (Gautam *et al.* 1984). Since that first communication, many instances describing covalent binding of proteins to acyl glucuronide conjugates have been reported. Significant covalent binding of the 1β-O-acyl glucuronides of flufenamic acid, clofibric acid, indomethacin and benoxaprofen to human serum albumin *in vitro* has been shown (Van Breeman and Fenselau 1985).

Glucuronide conjugates with rapid acyl migration rates are more likely to undergo irreversible protein binding via the imine mechanism. The likelihood of protein binding increases in going from the 2-O-acyl to the 4-O-acyl glucuronide (2-O < 3-O < 4-O) due to both steric and electronic effects Sidelmann *et al.* (1996). For the simplest situation in this reaction scheme, the degradation of the 1β-O-acyl glucuronide, it is necessary to consider two competing parallel reactions (Sidelmann *et al.* 1995, Logan 1996):

1. [1β-O-acyl glucuronide] $\xrightarrow{k_1}$ [β-2-O-acylglucuronide]
2. [1β-O-acyl glucuronide] + [H₂O] $\xrightarrow{k_2}$ [Aglycone] + [Glucuronic acid]

Acyl migration is a unimolecular first-order process. Hydrolysis is a second-order bimolecular reaction. The rate at which the 1 β -O-acyl component is depleted — the degradation rate (k_d) — is therefore equivalent to that of a single reaction whose rate constant is the sum of these two reactions and because $[H_2O] \gg [1\beta\text{-O-acyl}]$ this is essentially a first-order process. This rate can be easily measured by following the disappearance of the ^1H -NMR resonance arising from the anomeric proton of the 1 β -O-acyl isomer as the reaction progresses in an NMR tube at pH 7.4.

Owing to the ongoing interest in ester glucuronides, the development of quantitative structure–activity relationships (QSARs) for this group of compounds could be of potential benefit when designing new drugs. As part of this development, the degradation rates of a series of model glucuronides based on substituted benzoic acids have been determined and the results have been used to build several promising QSARs based on both experimental and calculated properties, e.g. ^{13}C chemical shifts, atomic charges on ester carbonyl carbons and σ_p values. The purpose of the current paper is to outline the selection of suitable compounds for constructing a QSAR and subsequent synthesis and measurement of the degradation rate for those compounds selected. The methods used to build the QSAR models and results from their analysis will be described in a subsequent paper.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich Ltd (Dorset, UK) and used as received.

SCX cation exchange mega Bond-ElutTM SPEC columns (12 ml reservoir volume/2 g sorbent mass) were obtained from Varian Ltd (Walton-on-Thames, UK).

General method used for preparation of 1- β -O-acyl glucuronides

Each reaction was carried using a 4-substituted benzoic acid as aglycone (substituents X = NO₂, CN, Br, H, I, F, nPr, Et, OMe, Ph, O-nPr, CF₃, OEt, OPh). Aglycone (0.5 mmol) was stirred with carbonyl diimidazole (100 mg, 0.6 mmol, 1.2 equivalent) in dimethylformamide (anhydrous, 1 ml) for 2 h at room temperature in a sealed 25 ml screw-top vial. The tetrabutylammonium glucuronate salt (220 mg, 0.5 mmol, 1 equivalent) and sodium hydride (5 mg, about 0.2 mmol 60% dispersion in mineral oil) were dissolved in dimethylformamide (anhydrous, 1 ml) with gentle warming. Once dissolved, the salt solution was quickly added to the imidazole adduct mixture and the vial resealed. This final reaction mixture was then stirred for 30 min to 6 h at room temperature depending on the aglycone being used.

The reaction was quenched with dilute formic acid (20%, 1 ml) and the pH was adjusted to pH 6.0–6.5 using solutions of 10% ammonium hydroxide and/or 1% formic acid. The solution was then made up to about 7 ml with water. An aliquot of the pH-adjusted solution (3 ml) was evaporated under vacuum at 60°C to remove as much DMF as possible (10–15 min). It was found that too high a temperature or more acidic pH at this stage resulted in the complete degradation of the product. The remainder of the unused pH-adjusted solution from the reaction mixture (about 4 ml) was stored at –40°C until required. The resulting residue was then dissolved in water (10 ml) and applied to a Varian SCX Mega Bond-ElutTM column (12 ml/2 g), which had been conditioned by elution with methanol (1 column-vol.) followed by water (3 column-vols). The eluent resulting after addition of the sample was saved and more water (10 ml) was washed through the column and also collected. The two fractions were combined and adjusted to pH 6.0–6.5 using solutions of 10% ammonium hydroxide and 1% formic acid. The resulting solution (about 20 ml) was then stored at –40°C until required. Estimated yields (based on NMR peak integral comparison using a known amount of trimethylsilyl[$^2H_4$]propionic acid, sodium salt (TSP), an NMR chemical shift and quantitation reference compound) were in the range of 4–14%.

Degradation rate measurements

All NMR measurements were carried out using a Bruker Avance 600 spectrometer operating at 600.13 MHz for proton detection and equipped with either a broadband inverse (BB1) or inverse triple resonance (TXI) 5 mm probe.

In order to obtain results quickly on glucuronide samples, a blank sample was measured first. This contained pH 7.4 buffer (0.5 ml) plus D₂O/TSP (0.05 ml 0.1 mg ml⁻¹ solution) with a total volume equal to that of the test sample (0.55 ml). Carrying out this procedure ensured that the initial field homogeneity settings (shims) were optimized on a sample as similar as possible to the glucuronide sample being analysed subsequently, thereby allowing faster measurement of the first spectrum in the series.

A 5-ml aliquot of the SCX SPEC eluent obtained from the synthetic reaction mixtures at pH 6.0 was evaporated under reduced pressure for exactly 10 min at 60°C on a rotary evaporator. This was the most critical stage of the rate measurement and reproducible results were not obtained if care was not taken to treat each sample in the same manner.

A buffer solution (0.5 ml of pH 7.4 buffer–100 mM phosphate in H₂O) was quickly added to the prepared residue and the time noted using the spectrometer software (this time was used as the starting point for the rate measurement and compared with starting times of subsequent NMR acquisitions). The pH was checked using a Camlab pH Boy 501 pH meter.

The buffered solution (0.5 ml) was transferred to a 5-mm NMR tube containing 0.05 ml D₂O/TSP (0.05 ml 10 mg ml⁻¹ solution). This served as a reference/internal standard for both chemical shift and measurement of the integrated peak area of the 1 β H1 anomeric proton resonance.

The prepared NMR sample was quickly placed in the spectrometer magnet and allowed to reach thermal equilibrium at 310 K for 10 min. The sample was then locked and shimmed and a test spectrum acquired using a standard presaturation solvent suppression pulse sequence (NOESYPR1D) into 64 K data points over a spectral width of 10 ppm. Once the shimming and solvent suppression were satisfactory (usually taking between 15 and 20 min after addition of buffer), automated acquisition of a series of 1D spectra was carried out using a Bruker 'au' program. This program acquired 16 scans every 3 min for the first 60 experiments after which time a spectrum was acquired every 15 min until the initial concentration of the 1 β anomeric proton, as measured by the integral, had approximately halved. During acquisition and measurement, the first-order axial shims (z1 and z2) were monitored and automatically adjusted by the Bruker software. This was necessary to maintain magnet homogeneity during overnight experiments.

Following acquisition the first experiment was phase-corrected, zero-filled once to 64 K data points and treated with an exponential window function equivalent to a line broadening of 0.1 Hz. The same phase parameters (and data treatment) were then used on all subsequent experiments for the rate measurement. Analysis of the resulting spectra and measurement of the rate of decrease in intensity of the 1 β anomeric proton doublet peak area allowed the degradation rate constant (k_d) and half-life ($t_{1/2}$) to be calculated from a plot of $\log_e$ [H1 1 β -O-acyl integral value] versus NMR data set acquisition start time (h) using Microsoft Excel, where k_d was given by the slope of the line and $t_{1/2} = \log_e(2)/k_d$. It was found to be sufficient to analyse every 10th spectrum, up to and including the last spectrum acquired, in order to describe the degradation curve adequately. As each sample contained the same amount of TSP, this was used as an internal standard for both chemical shift and peak area. The TSP chemical shift was set to 0 ppm and the integral set to 1 unit at the beginning of each analysis. This, in combination with keeping both the NMR acquisition and processing parameters identical for each analysis, allowed an easy comparison of the results from each experiment within this dataset. Extraction of the acquisition start-time from the appropriate Bruker data file was achieved for each experiment by use of a Unix c-shell script and the degradation rate calculated using Microsoft Excel to plot the data.

Results

Compound selection and experimental design

Quantifying chemical diversity is a problem that is of special interest when considering which compounds should be selected for a QSAR study, especially if the target compounds must first be synthesized. It is necessary to consider as wide a spectrum of compounds, based on as diverse a set of molecular parameters as possible, as is feasible within the constraints of experimental practicality. Various methods can be used to quantify the extent of diversity between pairs of members of a group of compounds. Clustering algorithms and pattern recognition techniques, for example, enable similar objects within a wider set to be grouped together: this then makes possible the selection of a representative from each group or cluster. Cluster analysis was carried out for the present study and four parameters were used to describe the main features of each of 58 mono-substituted benzenes. In broad terms, these were based upon size,

lipophilicity, σ and π electronegativity–polarity and each parameter was assigned relative to hydrogen, which itself is set as having all values as zero. The actual parameters used were as follows.

Molar refractivity (MR) is a measure of the polarizability of a group. This is strongly correlated with the size of a group and is therefore often taken as a measure of steric bulk. However, as it is a charge-related property, it can have an effect in electrostatic phenomena as well. π is a measure of the lipophilicity of the group, similar to a $\log P$ contribution. F and R are the Swain & Lupton ‘field’ and ‘resonance’ effect parameters respectively (Swain and Lupton 1968). They measure the ability of a group to attract or pull electrons towards itself through σ and π bonds, respectively.

The clusters were calculated using ‘K-means clustering’ (Hartigan 1975, Hartigan and Wong 1978). This statistical method initially makes the number of components equal to the final required number of clusters and the number of clusters is chosen such that the points are mutually farthest apart. Next, the algorithm examines each component in the population and assigns it to one of the clusters depending on the minimum distance. The cluster centroid position is recalculated each time a component is added to the cluster and this process continues until all the components are grouped into the final required number of clusters.

These data were first range-scaled so that each parameter had a value between 0 and 1, thereby ensuring that each parameter had equal weight. The membership of each cluster and the mean coordinates (parameters) of each cluster were then determined by K-means clustering. Analysis resulted in the compounds being grouped into seventeen clusters that could be best represented as the non-linear map shown in figure 3. By using non-linear mapping, the ‘4-space’ dimensionality associated with the four selected parameters is reduced to two and so becomes readily re-presentable in graphical form. Ideally one compound from each cluster should have been synthesized but, since time and synthesis constraints meant that this was impractical, compounds for synthesis were selected from the map based on the commercial availability of the 4-substituted benzoic acid aglycone, and whether the glucuronides ester was likely to be involved in unwanted side-reactions.

Fourteen compounds were selected from 11 of the 17 clusters as possible candidates for synthesis. Using the methods outlined below, 10 glucuronides representing selections from eight of the 17 clusters were successfully synthesized and their degradation rates measured. The eight clusters involved are shown as squares in figure 3. They represent a reasonable spread of the available property space. The triangles represent clusters of compounds for which synthesis and/or degradation rate measurement was unsuccessful and the circles those substituents rejected for the reasons given above.

Synthesis

The synthetic route selected had been developed for the synthesis of retinoic acid glucuronides (Barua *et al.* 1985, Becker *et al.* 1996). This method, using a reactive imidazole adduct of the aglycone (an imidazolide) which is added to the tetrabutyl ammonium salt of glucuronic acid in pyridine, takes advantage of the higher reactivity of the anomeric hydroxyl group relative to the 2-, 3- and

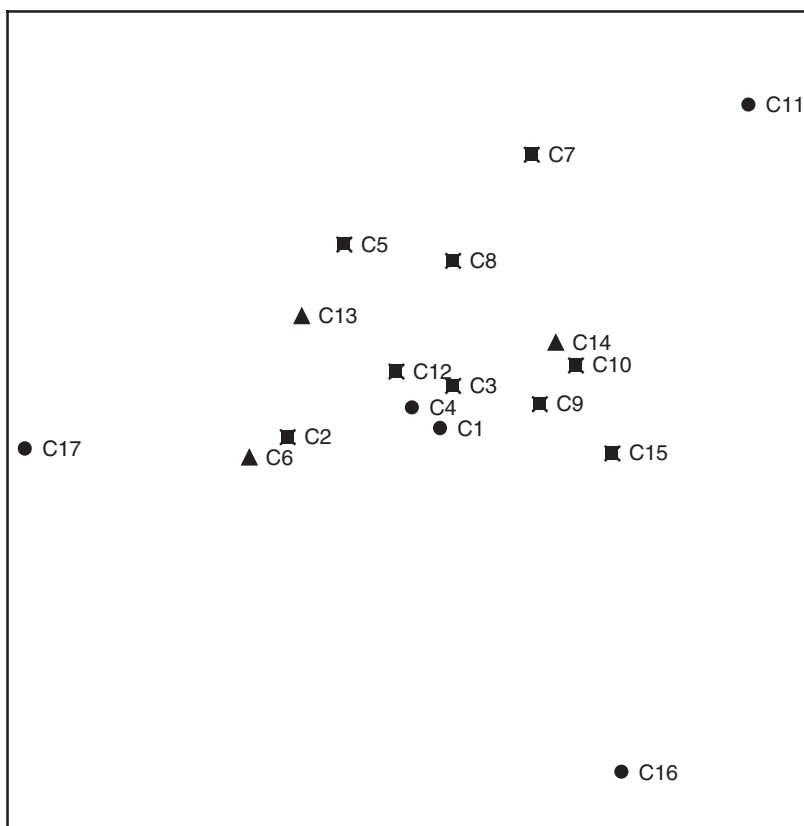
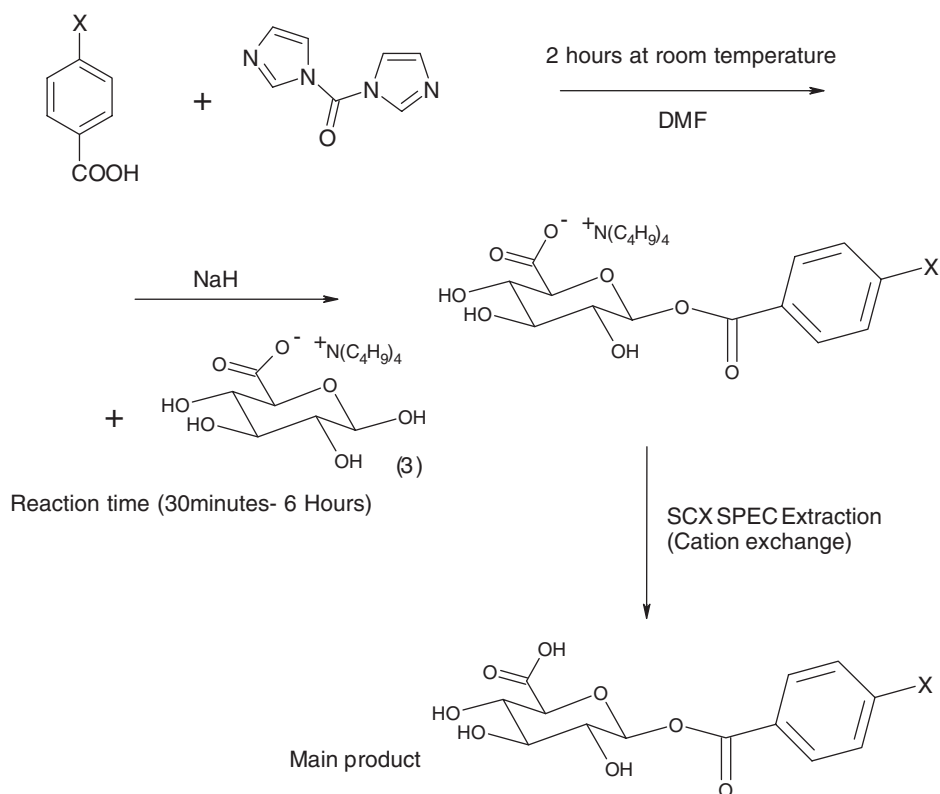


Figure 3. Non-linear map for 58 selected substituted benzoic acids based on their values for molar refractivity (MR), lipophilicity (π), Swain and Lupton field, and resonance effects (F and R). The clusters C1–17 represent functional groups having similar overall properties from the set of 58 benzoic acids, as described by the four parameters MR, π , F and R . Underlined functional groups denote a successful synthesis using the benzoic acid aglycone (10 compounds) with a cluster shown as a square on the plot. Italicized functional groups denote an unsuccessful synthesis or degradation rate measurement using the benzoic acid aglycone (four compounds) with a cluster shown as a triangle on the plot. The syntheses of the remaining compounds (44) were not attempted for reasons outlined in the text and clusters for these are shown as circles on the plot. C1: $\text{trCH}=\text{CHNO}_2$, $\text{CH}=\text{CHCN}$, OCOMe , NHCOMe , NHSO_2Me ; C2: *OEt*, *OnPr*, *OiPr*; C3: N_3 , SH , SMe , *Br*, *I*; C4: $\text{trCH}=\text{CHBr}$, COOEt , 1-Pyrryl; C5: *nPr*, *iPr*, *nBu*, *tBu*, $\text{csCH}=\text{CHMe}$, $\text{trCH}=\text{CHMe}$, $\text{CH}_2\text{CH}_2\text{Cl}$; C6: *OnBu*, *OPh*; C7: *H*, *Me*, CH_2OH ; C8: *Et*, $\text{CH}=\text{CH}_2$, CCH , CCMe , CH_2OMe ; C9: *E*, *Cl*; C10: CHO , COMe , CONH_2 , COOH , COOMe , SO_2NH_2 ; C11: COO^- ; C12: OH , *OMe*, NH_2 , NHOH ; C13: *Ph*, *benzyl*, 2-thienyl, 3-thienyl; C14: *CF*₃, *OCF*₃; C15: *NO*₂, *CN*, SO_2Me ; C16: NMe_3^+ ; C17: NMe_2 , NEt_2 .

4-hydroxyls. The method was modified for use with 4-substituted benzoic acids and the isolation of the glucuronides was improved by use of a cation exchange column (SCX) which removed the tetrabutylammonium counterion. Poly-substituted glucuronides (i.e. poly-esters with multiple aglycone molecules attached the carbohydrate ring) were also removed by this method via fortuitous secondary polar interactions with the SCX stationary phase that retained these less polar isomers. The general reaction scheme for the route used is shown in figure 4.

The salt is prepared by addition of a methanolic solution of tetrabutyl ammonium hydroxide to glucuronic acid and is readily isolated as a crystalline solid. The tetra-butyl ammonium group acts as an ion-pair reagent allowing



X = NO₂, CN, Br, H, I, F, nPr, Et, OMe, OnPr

Figure 4. Reaction scheme for the synthesis of 1-O-acyl glucuronides via an imidazolidine intermediate.

the reaction to take place in an anhydrous solvent. Yields of 1 β -O-acyl glucuronides were estimated by measuring proton NMR peak integral ratios between the glucuronide 1 β -anomeric protons and the methyl singlet of the internal standard (TSP). The yields were low but acceptable for use in determining degradation rates. Despite their yield inefficiency, the reactions had the great advantage of being very quick to carry out when compared with methods involving protection/de-protection of the ring hydroxyls (Gavaghan 1997). For the purposes of this study the measured degradation rates were considered to depend only on the initial concentration of 1 β anomer present; the amount of 1 β isomer formed from the reverse reaction (2 β to 1 β isomer) was assumed to be negligible, a reasonable assumption based on literature evidence (Corcoran *et al.* 2001). The results from the experiments outlined below also support this assumption: when allowed to react to completion, no 1 β -anomer could be detected in the ¹H-NMR spectra (the resulting solutions contained only the six isomers/anomers of the 2-, 3- and 4-position plus hydrolysis products).

Eleven ester glucuronides were successfully synthesized and the results are shown in table 1. The reaction failed to work for three of the aglycones (substituents CF₃, OEt, OPh).

Table 1. Summary of yields for 11 4-substituted benzoic acid *O*-acyl glucuronides synthesized using an imidazolidine.

1 β - <i>O</i> -acyl glucuronide	Yield (%)	Reaction time (h)
4-Nitrobenzoyl	4	0.5
4-Cyanobenzoyl	4	0.5
4-Iodobenzoyl	4	1
4-Bromobenzoyl	12	1
4-Fluorobenzoyl	6	1
Benzoyl	12	1
4-Phenylbenzoyl	< 1	1
4-Propylbenzoyl	6	6
4-Ethylbenzoyl	12	6
4-Methoxybenzoyl	14	6
4-Propoxybenzoyl	4	6

Table 2. Summary of degradation rate measurements for 10 4-substituted benzoic acid *O*-acyl glucuronides.

Substituent	k_d (h ⁻¹)	r^2 *	Half-life (h)
4-Nitro	0.762	0.995	0.9
	0.658	0.995	1.1
4-Cyano	0.261	0.993	2.7
4-Iodo	0.096	0.984	7.2
	0.079	0.997	8.1
4-Bromo	0.068	0.999	10.2
4-Fluoro	0.067	0.992	10.4
	0.047	0.986	14.8
4-H	0.039	0.998	17.6
	0.038	0.986	18.2
4-Propyl	0.024	0.979	29.5
4-Ethyl	0.022	0.981	30.9
	0.020	0.941	35.4
4-Methoxy	0.013	0.996	55.5
4-Propoxy	0.007	0.993	106.6

*Correlation coefficient for the best fit of the NMR intensity data to a straight line defined by first-order kinetics.

Degradation rates

The degradation rates were calculated from plots of $\log_e$ [H1 1 β -*O*-acyl integral value] versus NMR data set acquisition start time (h). These data are summarized in table 2 for the glucuronides synthesized and in general duplicate measurements were made. A reliable result for the 4-phenyl substituted compound could not be calculated due to the low yield obtained for this member of the series. Accurate measurement of the integral of the anomeric proton for this compound proved difficult because of the poor signal-to-noise ratio present in the recorded spectra. This resulted in a poor correlation coefficient ($r^2 < 0.8$) for the rate plot and the results are not included in table 2.

Discussion

The present work describes the first systematic attempt to study the effects of substituents on the acyl migration reaction for glucuronides. The method described has proved successful as a procedure for rapidly synthesizing and measuring the degradation rate of ester glucuronides. The results shown in table 2

show a clear trend indicating the effect of substituent on the degradation rate for each of the 4-substituted benzoic acid *O*-acyl glucuronides synthesized. Electron-withdrawing groups clearly increase the rate whereas electron-donating groups have the opposite effect and slow down the degradation rate. More detailed studies on the QSAR aspects will be more fully explored in a subsequent publication. The use of mixtures with varying amounts of isomers/anomers in the initial solution has meant that measurement could be carried out without time-consuming and often difficult isolation of the pure 1 β -*O*-acyl isomer.

The validity of the method is based on the assumption that the rate of formation of the 1 β -glucuronide from the 2 β isomer present in the mixture is negligible (Gavaghan 1997). This assumption was supported for the compounds measured here in as much as the final equilibrium mixtures never contained any of the 1 β -*O*-acyl isomer. It is also assumed that the presence of the isomeric glucuronides does not affect the degradation rate of the 1 β isomer in more subtle ways. To check this, a direct comparison of degradation rates for unpurified and purified glucuronide would need to be made. However, in as far as this approach is used for observing trends across a series of compounds, such effects should not excessively affect the construction of structure–reactivity relationships. Additionally, it is necessary to investigate whether simply measuring the loss of the 1 β isomer represents a good estimation of the transacylation rate since hydrolysis from this isomer, and potentially from the other isomers, can occur concurrently. In the present case, it was noted that as the degradation proceeded, there was no marked increase in the concentration of glucuronic acid in the samples, and hence it can be reasonably assumed that the principal reaction under study here is the transacylation. Indeed, a previous study (Sidelmann *et al.* 1996) showed that transacylation of the various positional isomers of 2-fluorobenzoyl glucuronide predominated over hydrolysis.

All of the 4-substituted benzoic acid *O*-acyl glucuronides described in here were made and had their degradation rate measurement started in less than 8 h. Previous measurements have used either pure isomers or combinations of HPLC and/or NMR to measure rates (Sidelmann *et al.* 1995, 1996). The choice of approach will be problem-dependent and, for example in addition, ^{13}C -labelled compounds and ^{13}C -NMR spectroscopy have been used to measure the transacylation rates of several compounds as has the use of ^{19}F -NMR spectroscopy when applicable (Nicholls *et al.* 1996, Akira *et al.* 1997, 1998). However, for the purposes of this study, the simplest ^1H -NMR method proved adequate for the construction of data suitable for QSAR purposes as will be demonstrated in a subsequent paper. The synthetic route used here also greatly increased the speed with which the model compounds could be obtained when compared with methods that use protection/de-protection of all of the ring hydroxyls (Kaspersen and Van Boeckel 1987, Stachulski and Jenkins 1998).

However, although simple, the method used here to measure degradation rates is not without problems and requires great care during sample preparation to ensure reproducible results. The use of a rotary evaporator for concentration of the SCX eluent proved to be the biggest cause of errors as erratic performance between samples (largely due to variations in vacuum obtained) resulted in noticeable differences between samples. This resulted on occasion in a sample that was difficult to buffer effectively and slight differences in pH (0.1–0.2 units) translated into large differences in reaction rate. This is thought to be the cause

of the discrepancy for the duplicate measurement found for 1 β -4-fluorobenzoyl glucuronide (table 2). Although not investigated at length in this study, pH has been shown to cause dramatic changes in rate (Gavagahn 1997). Heating the acidic sample also results in a decrease of the 1 β isomer concentration due to both hydrolysis and transacylation. It is also known that different solvent compositions, such as the presence of D₂O or acetonitrile, can have a marked effect on transacylation rates (Mortensen *et al.* 2001).

Transacylation is often thought to be negligible in acidic solution but for a glucuronide that transacylates rapidly, such as the 1 β -4-nitrobenzoyl glucuronide, a sample at pH 6.5 was seen to deteriorate over several days in the NMR tube at room temperature. The temperature necessary (60°C under the conditions used here) to remove excess solvent simply exacerbates the problem for *all* samples even though exposure to the heat may only be for 10 min. These problems can largely be overcome by careful planning when preparing the sample and could possibly be eliminated by use of freeze-drying in place of rotary evaporation for concentrating the sample. One way to improve the efficiency of degradation rate measurement would be to carry out the measurements at elevated temperatures when necessary. Trial experiments with 1 β -4-methoxybenzoyl glucuronide at four temperatures (300, 310, 320 and 340 K) showed that the Arrhenius equation could be used ($r^2=0.92$ for a plot of $\log_e k_d$ versus $1/T$) and, assuming it holds for the rest of the series of model compounds, the relationship could be used to standardize all results to a set temperature: this is a common practice when measuring rate data for a range of compounds with widely differing rates.

One assumption made here is that measurement of the relative degradation rates of the 1 β -glucuronide across a series of substituted analogues can yield information on the relative reactivity of the transacylated products towards proteins. Given that it is the isomers at the 3- and 4-positions of the glucuronide ring which are most reactive towards proteins, it would be strictly necessary to show that the degradation rates for the 1 β -glucuronide are correlated to the reaction rates for the other transacylation reactions or that the 1 β isomer degradation is the rate-determining step. Previous data on the transacylation of 2-fluorobenzoyl glucuronide and of *S*-naproxen glucuronide showed in fact that the half life of the 1 β isomer was shorter than the half lives of the other positional isomers, based on isolation of each isomer in an on-line HPLC-NMR experiment and thus the initial reaction is not rate-determining (Sidelmann *et al.* 1996, Mortensen *et al.* 2001). The reaction rate constants for interconversion from the 2- to the 3-, and then to the 4-isomers are all similar (Sidelmann *et al.* 1996). Nevertheless, there is a general correlation between the half life of the 1 β isomer and known toxicity (Spahn-Langguth *et al.* 1992, Wade *et al.* 1997). This underlines the need to investigate this whole reaction scheme for a systematically defined set of compounds measured under exactly the same conditions so that meaningful structure–reactivity relationships can be achieved.

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High-throughput screening for the assessment of time-dependent inhibitions of new drug candidates on recombinant CYP2D6 and CYP3A4 using a single concentration method

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1. The inhibitory effects of various test compounds on recombinant human CYP3A4 activity assayed by fluorescent metabolite formation from 7-benzyloxyquinoline (7-BQ) and the effect of pre-incubation on inhibition were evaluated using the microtitre plate assay with multiple concentrations of test compounds (multiple concentration method).

2. Among the test compounds studied, ketoconazole inhibited CYP3A4 activity most extensively, followed by miconazole, troleandomycin, terfenazine and midazolam. The IC_{50} values of other compounds exceeded $10\mu M$, but those of many compounds decreased after pre-incubation. The inhibitory effects of verapamil, amiodarone and diltiazem after pre-incubation were 205, 154 and 833 times greater than those in the case of co-incubation, respectively.

3. To assess the inhibitory effects more readily, the validity of the microtitre plate assay with a single concentration of the test compound (single concentration method) was studied. The accuracy of the automated dispensation and the coefficient of variation on enzyme activity were approximately 3%.

4. The IC_{50} values estimated using the per cent of residual activity from the single concentration method matched closely those from the multiple concentration method. When the IC_{50} value as inhibitor concentration was used for a single concentration method, the method enabled easy estimation of inhibitory patterns (such as competitive or time-dependent inhibition) on cytochromes P450. Therefore, from the ease of the technique, automation of the microtitre plate assay and application of the single concentration method might be useful for inhibitory assessment of cytochromes P450 more than that of current conventional methods.

Introduction

A microtitre plate (MTP) assay that consists of recombinant CYP microsomes and fluorescent probe developed by Crespi *et al.* (1997) has been used as a high-throughput screening (HTS) method for evaluation of inhibition of cytochrome P450s (CYPs) in the pharmaceutical industry (Stresser *et al.* 2000, Bapiro *et al.* 2001, Nomeir *et al.* 2001, Yamamoto *et al.* 2002). The IC_{50} value is an important index to compare the inhibitory potential of drug candidates and can be readily obtained using the MTP assay. Crespi *et al.* (1997) and McGinnity and Riley (2001) have reported that the IC_{50} values obtained from the MTP assay using recombinant CYPs reflect those obtained from conventional assays using human liver microsomes (HLM). It is also reported here that the MTP assay is a useful method as HTS to avoid drug candidates with undesirable pharmacokinetic

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properties such as CYP inhibition and that K_i values of various compounds calculated from the IC_{50} with the MTP assay corresponded closely with the K_i values from HLM reported in the literature (Yamamoto *et al.* 2002).

Using the MTP assay, Palamanda *et al.* (1998) and Favreau *et al.* (1999) reported that the inhibitory effects of some new chemical entities on recombinant CYP2D6 activity change depending on incubation time and that the IC_{50} values obtained at early time points in the MTP assay predict the IC_{50} values obtained in HLM more closely. Their suggestions are based on the speculation that the metabolism of new drug candidates proceeds depending on the longer incubation time or that more potent inhibitors are produced with prolonged incubation, in recombinant CYP2D6 microsomes. It was stated that pre-incubation with test compound and enzyme affected the inhibitory effect of test compounds on recombinant CYPs activities (Yamamoto *et al.* 2002, 2003). The IC_{50} value of a typical substrate such as dextromethorphan and metoprolol for CYPs after pre-incubation increased but those of a mechanism-based inhibitor such as troleandomycin (TAO) or furafylline substantially decreased (Yamamoto *et al.* 2002, 2003). These results suggest that the concentration of the test compound relating to CYP inhibition in the incubation medium is decreasing before the substrate is added to the reaction medium and that the concentrations of the enzyme–metabolite complex increase after pre-incubation. Enzyme inactivation could also account for increased inhibition. To identify the inhibitory effects of drug candidates and their metabolites on recombinant CYPs activities is most important at early stages of drug discovery. However, this information can be obtained only after using the MTP assay with multiple concentrations of the test compound (multiple concentration method) and examining the effects of pre-incubation. In previous studies (Yamamoto *et al.* 2002, 2003), inhibition assessments using the MTP assay were undertaken by manual handling. Therefore, automation of the MTP assay and inhibition assessment using a new plate format are needed to decrease the time and labour spent and to increase the throughput of the evaluation of CYP inhibition. HTS methods based on modification of the plate format have been reported (Moody *et al.* 1999, Gao *et al.* 2002, Zhou *et al.* 2002) and these methods are called single or two-point IC_{50} assays, using one or two concentrations of the inhibitor. The IC_{50} values from this assay correlate with those from conventional assays using multiple concentrations of the inhibitor, but for these assays, radiometric and high-performance liquid chromatography (HPLC) analyses are needed.

The authors tried to use a single-point IC_{50} assay to evaluate the CYPs' inhibition using the MTP assay in this present study. It was asked if the MTP assay with a single concentration of the test compound (single concentration method) is suitable to evaluate the inhibitory effects of drug candidates on recombinant CYP activities and their inhibition patterns (particularly; competitive inhibition as substrate metabolism or time-dependent inhibition) instead of multiple concentrations were evaluated.

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-methyl-4-trifluoromethylcoumarin (MFC), 3-[2-*N,N*-diethyl-*N*-methylammonium]ethyl]-7-methyl-4-methylcoumarin (AMMC), 3-[2-(*N,N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC),

7-benzyloxyquinoline (7-BQ) and 7-hydroxyquinoline (7-HQ) were purchased from BD Gentest Co. (Woburn, MA, USA). Furaflavine, quinidine, bufuralol and ketoconazole were purchased from Ultrafine Chemicals Co. (Manchester, UK). Amitriptyline, clomipramine, clozapine, imipramine, haloperidol, desipramine, nortriptyline, trimipramine, temazepam, mianserin, amoxizapine, perphenazine, risperidone, thioridazine, chlorpromazine, alprenolol, timolol, propafenone, dextromethorphan, S-metoprolol, propranolol, terfenadine, erythromycin, midazolam, cyclosporine A, verapamil, amiodaron, diazepam, diltiazem, triazolam, troleandomycin (TAO), temazepam and metyrapone were purchased from Sigma-Aldrich (St Louis, MO, USA). Phenacetin, nifedipine, testosterone, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺ were purchased from Wako Pure Chemical Industries (Osaka, Japan). Clarithromycin (CAM), and compounds A and B were synthesized in Taisho Pharmaceutical Co. Ltd (Saitama, Japan). ¹⁴C-labelled compound A (97.5%, radiochemical purity, 8.53 MBq mg⁻¹) was synthesized at Amersham Biosciences UK, Ltd (Little Chalfont, UK). 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, CYP2D6 and CYP3A4 (Supersomes) were purchased from BD Gentest Co.

Inhibitory effects of various test compounds on recombinant CYP3A4 using the multiple concentration method

The inhibitory effects of various compounds on recombinant CYP3A4 activity were evaluated according to the MTP assay developed by Crespi *et al.* (1997). The MTP assay was conducted in a 96-well microtitre plate (Corning Coster, Cambridge, MA, USA) as described (Yamamoto *et al.* 2002). According to the data sheet provided by the manufacturer, the P450 contents in the incubation medium were 15 pmol P450 ml⁻¹, which corresponded to a protein concentration of 0.095 mg protein ml⁻¹. The concentration of 7-BQ used as a fluorescent probe was 40 µM, which is similar to the *K_m* of 7-BQ for CYP3A4 in recombinant CYP3A4 (Miller *et al.* 2000). The test compounds examined included ketoconazole (inhibitor as a positive control), terfenadine, nifedipine, erythromycin, midazolam, cyclosporine A, verapamil, metyrapone, amiodaron, diazepam, miconazole, temazepam, diltiazem, testosterone, triazolam, CAM, TAO, desipramine, nortriptyline, amitriptyline, clomipramine, clozapine, imipramine, and compounds A and B. All test compounds except CAM and diazepam were dissolved in acetonitrile. CAM and diazepam were dissolved in de-ionized water and diluted to 50:50 with acetonitrile. In the present study, test compounds (except for ketoconazole) were examined in the range of eight concentrations from 0.05 to 100 or from 0.09 to 200 µM as a final concentration. Ketoconazole was tested in a range of 0.002–5 µM. The final acetonitrile concentration was 2%. Serial dilution of the test compound and addition of all reagents to the 96-well plates were done by manual and automated handling. Incubation mixtures containing the serially diluted inhibitors and NADPH-generating solution were prewarmed at 37°C for 10 min. The reactions were initiated by adding 100 µl of enzyme/substrate mixtures on a plate shaker, PS-52M (TAITEC, Saitama, Japan), and were terminated by adding a stop solution (4:1 v/v of acetonitrile/0.5 M Tris base) after incubation for 30 min. In the automated handling procedure, a Genesis workstation (Tecan, Hombrechikon, Switzerland) was used for serial dilution of the test compound and addition of all reagents to the 96-well plates. The fluorescence in the wells was measured using a multilabel counter, 1420 ARVOsx (Wallace, Finland), with excitation wavelengths of 409 nm and emission wavelengths of 530 nm. Control reactions were performed in wells containing no inhibitor. All reactions were carried out in duplicate.

Effect of pre-incubation on inhibition of CYP3A4 using the multiple concentration method

The effects of pre-incubation on the inhibitory potential of various test compounds (described above) on recombinant CYP3A4 activity were studied using the MTP assay with the pre-incubation method reported (Yamamoto *et al.* 2002, 2003). The range of concentrations of test compounds, the concentration of 7-BQ and P450 contents in the incubation medium used were the same as described above. After NADPH-generating solution and serially diluted test compounds were added to each well, pre-incubation was performed by adding 100 µl CYP3A4 enzyme to each well at 37°C for 30 min. The reactions were initiated by adding 100 µl substrate (7-BQ) at 37°C for 30 min and terminated by adding stop solution. Subsequent experiment procedures were done as described above.

Accuracy of dispensation by automated handling and variation of the detected fluorescence on 96 wells after incubation

CHC and 7-HFC (the metabolites of CYP1A2 and CYP2C9, respectively) were used as fluorescent metabolites for the assessment of the accuracy of dispensation among 96 wells using automated handling. The accuracy of the dispensation was confirmed by adding 100 µl enzyme/fluorescent-metabolite

mixtures to each well containing the NADPH-generating system. Other procedures except for those without inhibitors and without incubation were similar to experiments for the inhibition study. Final concentrations of the fluorescent metabolites were 0.125 μM (CHC) and 0.250 μM (7-HFC). Variation of metabolic activities among 96 wells after incubation on the plate shaker was confirmed by measuring the formation activity of CHC from CEC (fluorescent substrate of CYP1A2) using the MTP assay based on automatic handling. The experimental procedure for determination of the activity was as described (Yamamoto *et al.* 2002) and reactions without inhibitors were automatically done in the 96-well plates. The fluorescence in the well was measured using a multilabel counter with excitation and emission wavelengths of 409 and 460 nm (for CYP1A2) or 530 nm (for CYP2C9). Coefficients of variation (CV) of dispensation and the formation activity of CHC were calculated as the ratio of the standard deviations to the means of the fluorescence in 96 wells.

Inhibitory effect of test compounds on recombinant CYP2D6 and 3A4 activities using the single concentration method

The inhibitory effects of various test compounds on recombinant CYP2D6 or CYP3A4 activities were investigated using the single concentration method. Test compounds used are described in figure 3. A total of 96 μl NADPH-generating solution was added to the wells and each solution of test compound (4 μl) was added. After the incubation mixtures were prewarmed on the plate shaker at 37°C for 10 min, the reaction was initiated by adding 100 μl enzyme/substrate mixtures to all wells at 37°C for 30 min and was terminated by adding the stop solution after 30 min. As a blank sample, 100 μl enzyme/substrate mixtures were added to selected wells. The final concentration of the test compound used was 1 and 10 μM for CYP2D6 and CYP3A4, respectively. P450 contents in the incubation medium were as described above (for CYP3A4) and in a previous report (for CYP2D6) (Yamamoto *et al.* 2003). The fluorescence in the well was measured using a multilabel counter with excitation and emission wavelengths of 390 and 460 nm (for CYP2D6), or 409 and 530 nm (for CYP3A4). Control reactions were done in wells including 4 μl acetonitrile without an inhibitor. All reactions using the single concentration method were carried out at triplicate per a test compound and the inhibitory effects of at least 24 test compounds on recombinant CYP2D6 or CYP3A4 activities were assessed per a 96-well plate, respectively.

Effect of pre-incubation on inhibition of CYP2D6 and CYP3A4 by the single concentration method

Effects of pre-incubation on the inhibitory potential of various test compounds (described above) on recombinant CYP2D6 and CYP3A4 activities were determined using the MTP assay with a single concentration method. Test compounds used are described in figure 6. The final concentration of test compounds used was similar to the IC_{50} ($\text{IC}_{50 \text{ multi}}$) values for CYP3A4 obtained from the MTP assay (multiple concentration method) described above and the $\text{IC}_{50 \text{ multi}}$ values for CYP2D6 described by Yamamoto *et al.* (2003). NADPH-generating solution, the solution of test compound and acetonitrile were added using the same procedure described above. After the incubation mixtures had been prewarmed on the plate shaker at 37°C for 10 min, pre-incubation was initiated by adding 80 μl enzyme to all wells at 37°C for 30 min. After pre-incubation, the reactions were initiated by adding 20 μl substrate to all wells at 37°C for 30 min.

Determination of residual concentration of [^{14}C] compound A after pre-incubation

[^{14}C] compound was used for determination of the residual concentration of [^{14}C] compound A in the incubation medium (which included recombinant CYP2D6 microsomes) after pre-incubation at 37°C for 30 min. [^{14}C] compound A was pre-incubated using a 96-well plate in the range of eight concentrations from 0.002 to 5 μM as a final concentration and was stopped by adding methanol. To detect residual compound A in each well, thin layer chromatography (TLC) was carried out using TLC plates and the solvent system described as follows: NH_2 HPTLC plates (Merck Co. Ltd, Whitehouse Station, NJ, USA) and hexane/chloroform/methanol = 7/2/1. Radioluminography, BAS2000 system (Fuji-Film, Tokyo, Japan), was used for quantification of [^{14}C] compound A.

Data analysis

The formation of the individual fluorescent metabolites was proportional to the incubation time (data not shown). Data were exported and analysed using an Excel spreadsheet (Microsoft, Seattle, WA, USA). The inhibitory effect at each concentration of test compounds on the specific activity was expressed as a percentage of the control value. The IC_{50} ($\text{IC}_{50 \text{ multi}}$) values for the multiple concentration method were calculated by linear interpolation. Inhibitory effects of various test compounds using the single concentration method were expressed as the residual per cent for control activity (per cent of residual activity) and the residual per cent was compared with the $\text{IC}_{50 \text{ multi}}$ values obtained using

the multiple concentration method. In addition, IC_{50} (the $IC_{50 \text{ single}}$) values were calculated using the per cent of residual activity from the single concentration method, assuming a slope factor of 1 and background activity of 0, according to the following equations described by Moody *et al.* (1999):

$$IC_{50\text{-single}} = (I \times R)/(100 - R),$$

where I and R are a concentration of test compounds used with the single concentration method and the per cent of residual activity at I. The effect of pre-incubation on inhibition of CYP2D6 and CYP3A4 with the single concentration method was expressed as a residual per cent of control activity. The residual per cent was compared with the ratio ($IC_{50 \text{ ratio}}$) of the $IC_{50 \text{ multi}}$ value obtained after pre-incubation to the $IC_{50 \text{ multi}}$ value obtained with the co-incubation obtained with the MTP assay with the multiple concentration method. The $IC_{50 \text{ multi}}$ values for CYP2D6 have already been reported (Yamamoto *et al.* 2003).

Results

Inhibitory effects of various test compounds on recombinant CYP3A4 using the multiple concentration method

The inhibitory effects of various test compounds on recombinant CYP3A4 activity are shown in figure 1. Here, control activity of 7-HQ formation (fluorescent metabolite of 7-BQ) was $48.6 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ P450}$. The $IC_{50 \text{ multi}}$ value ($0.15 \mu\text{M}$) of ketoconazole used as a positive control inhibitor was in agreement with reported data (Yamamoto *et al.* 2002). Among the test compounds examined, the inhibitory effects were extensive in the order of miconazole ($1.42 \mu\text{M}$) > TAO (5.44) > terfenadine (7.77) > midazolam (9.36). The $IC_{50 \text{ multi}}$ values of nifedipine, verapamil, clomipramine, clozapine, metyrapone, compound A, nortriptyline and compound B were in the range $20\text{--}100 \mu\text{M}$. The $IC_{50 \text{ multi}}$ values of other compounds, including CAM and erythromycin, were $> 100 \mu\text{M}$.

Effect of pre-incubation on inhibition of CYP3A4 using the multiple concentration method

Figure 2 shows $IC_{50 \text{ multi}}$ values of these compounds on recombinant CYP3A4 activity after co-incubation (0 min) or pre-incubation (30 min). Control activity of 7-HQ formation after pre-incubation was $10.9 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ P450}$. The $IC_{50 \text{ multi}}$ value of TAO after pre-incubation was approximately 60 times lower than that with co-incubation and was the lowest among the test compounds. The inhibitory potential after pre-incubation was extensive in the order of verapamil ($0.23 \mu\text{M}$) = diltiazem (0.23) > amiodaron (0.28). The inhibitory effects of verapamil, diltiazem and amiodaron after pre-incubation were 205-, 833- and 154-fold more extensive than in the case of co-incubation, respectively. The $IC_{50 \text{ multi}}$ values of erythromycin, diazepam, CAM and clomipramine after pre-incubation were also over 10 times smaller than after co-incubation. On the other hand, the $IC_{50 \text{ multi}}$ values of ketoconazole, terfenadine and metyrapone after pre-incubation were 4.7, 2.7 and 3.3 times greater than after co-incubation. The $IC_{50 \text{ multi}}$ values of miconazole, testosterone, compound A, desipramine and imipramine were similar to those in case of co-incubation.

Accuracy of dispensation and variation of the detected fluorescence on 96 wells after incubation by automated handling

Table 1 shows the CVs for dispensation and for metabolite formation after incubation using automated handling with the plate shaker and Genesis

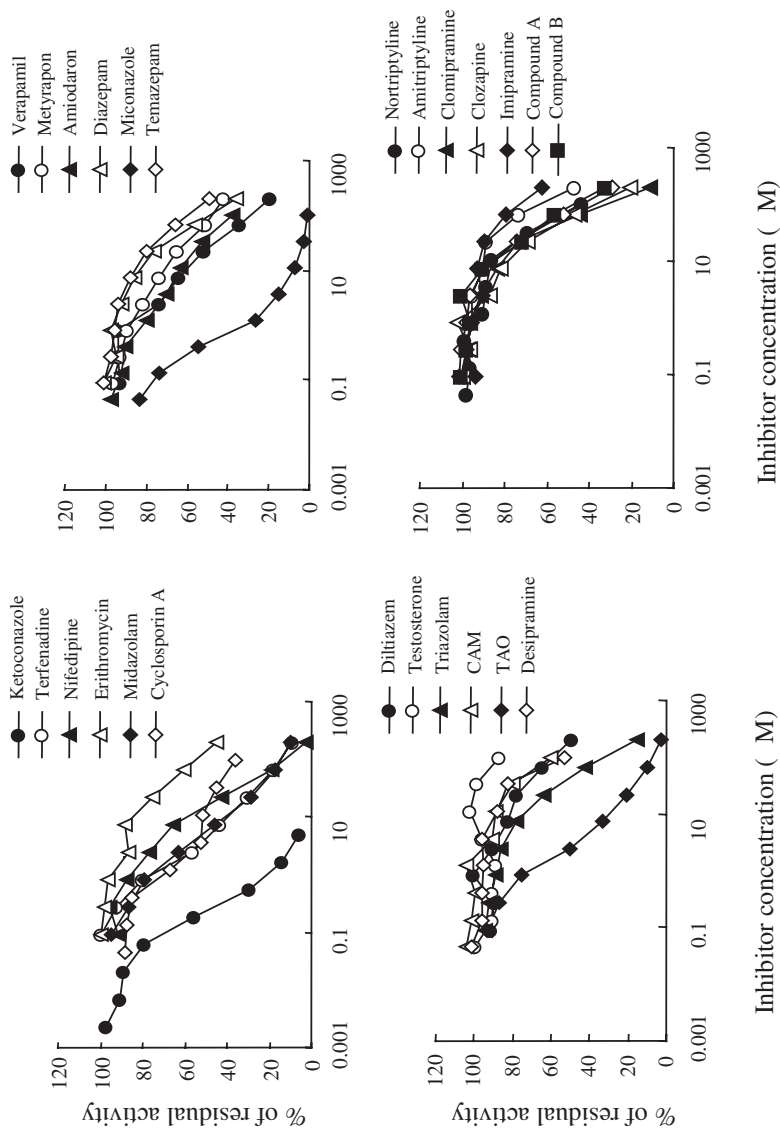


Figure 1. Inhibitory effects of test compounds on recombinant CYP3A4 activity assayed by formation of fluorescent metabolites from 7-benxyloxyquinoline (7-BQ) using the microtitre plate (MTP) assay. The inhibition study using the MTP assay was done using multiple concentrations of test compounds as described in the Materials and methods. Test compounds used as an inhibitor were mainly substrates of CYP3A4. Ketoconazole was the inhibitor as the positive control for CYP3A4. Concentration of 7-BQ as fluorescent probe was 40 μ M, which was similar to the K_m of 7-BQ for CYP3A4 in recombinant CYP3A4 (Miller *et al.* 2000). Control activity of 7-HQ formation (fluorescent metabolite of 7-BQ) was 48.6 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450. Each datum point is the average of duplicate determinations.

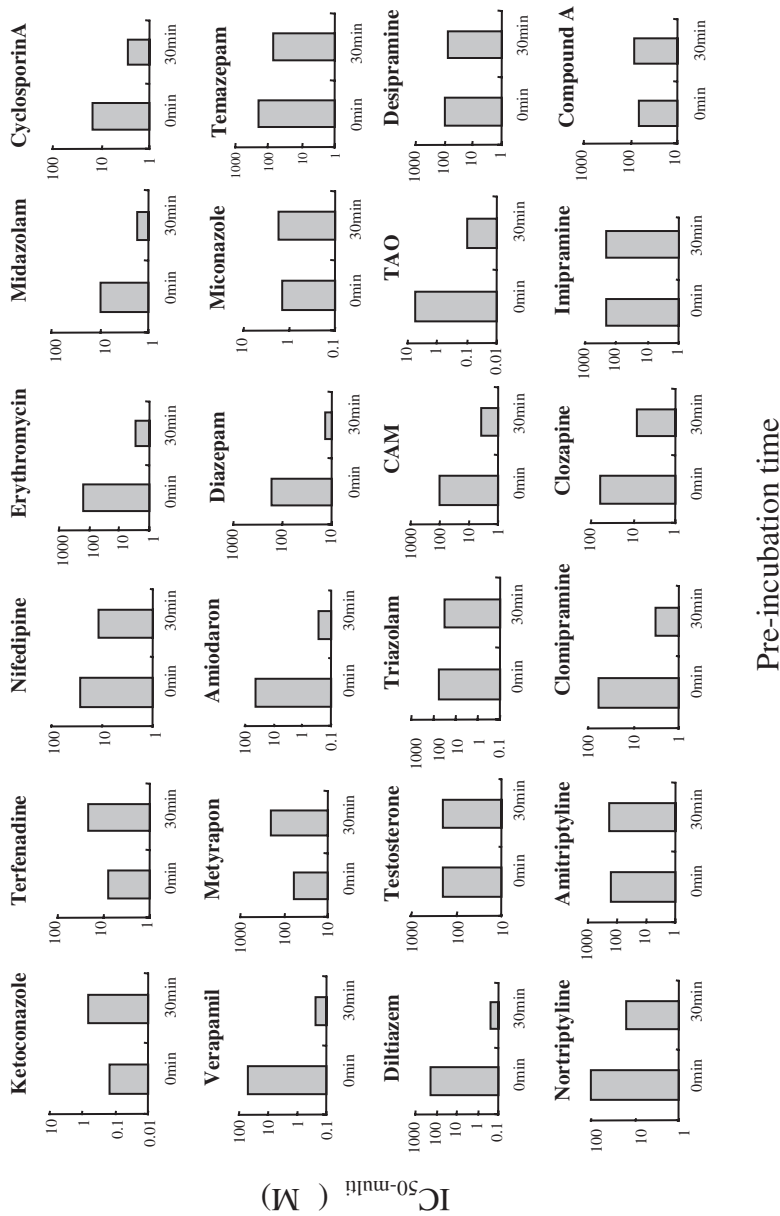


Figure 2. Effect of pre-incubation on the inhibitory potential of test compounds on recombinant CYP3A4 activity. Zero minutes represents the IC_{50} values in the case of co-incubation with the test compound and enzyme, and 30 min represents the IC_{50} values after pre-incubation for 30 min. Concentration of 7-BQ as fluorescent probe was $40 \mu M$. Control activity of 7-HQ formation after pre-incubation was $10.9 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ P450}$. Each datum point is the average of duplicate determinations.

Table 1. Accuracy of dispensation and variance of the detected fluorescence on 96 wells after reaction using automated handling.

CYP forms	Dispensation		After reaction	
	Flourescence	CV (%)	Flourescence	CV (%)
1A2	66 999 $\pm$ 1825	2.7	365 742 $\pm$ 11 973	3.3
2C9	15 995 $\pm$ 498	3.1	–	

Plate shaker heating of the bottom of the plate was used to maintain a constant temperature in the 96 wells and the Genesis workstation was used for automation of dispensation of various reagents. CHC and 7-HFC (the metabolites of CYP1A2 and CYP2C9, respectively) were used as fluorescent metabolites for the detection of fluorescence in each well and the concentrations were 0.125 and 0.250 μ M, respectively. The experimental procedure for determination of the activity was as described (Yamamoto *et al.* 2002) and reactions without inhibitors were automatically determined using CEC of 5 μ M. –, Non experiment.

workstation. The CVs of the fluorescence for CHC and 7-HFC for the assessment of accuracy of dispensation were 2.7 and 3.1%, respectively. Even when CEC (fluorescent probe) was automatically incubated with recombinant CYP1A2 after dispensation of various reagents using the Genesis workstation, the CV of the fluorescence of CHC produced from CEC was only 3.3%.

Inhibitory effects of test compounds on recombinant CYP2D6 and 3A4 activities with the single concentration method

The per cent of residual activity for inhibitory test compounds (1 μ M for CYP2D6, 10 μ M for CYP3A4) in incubation medium containing recombinant CYP2D6 and CYP3A4 after incubation for 30 min was determined using the single concentration method (figure 3A) and were compared with $IC_{50 \text{ multi}}$ values obtained in case of the multiple concentration method (figure 3B). Control activities for AHMC and 7-HQ formation in the single concentration method were similar to activities reported (Yamamoto *et al.* 2003) and described above. When the per cent of residual activity for inhibitory was 50%, it is proposed that the $IC_{50 \text{ multi}}$ value of the compound agrees with the concentration used in the single concentration method. For compounds such as haloperidol, trimipramine and risperidone, when the inhibitory effects of these compounds on recombinant CYP2D6 were assessed using the multiple concentration method, the $IC_{50 \text{ multi}}$ values were approximately 1 μ M. When inhibitory effects of these compounds on recombinant CYP2D6 were assessed using the single concentration method (a concentration of 1 μ M), the per cent of residual activities of these compounds were 53.3, 57.1 and 56.4%, respectively. On the other hand, the per cent of residual activity of the compounds that had very small $IC_{50 \text{ multi}}$ values (such as quinidine, perphenazine, thioridazine, chlorpromazine, propafenone and compound (A)) was <10%. In contrast, compounds that had large $IC_{50 \text{ multi}}$ values showed large a per cent of residual activity with the single concentration method. For CYP3A4, the per cent of residual activities of terfenadine, midazolam and cyclosporin A that showed $IC_{50 \text{ multi}}$ values of approximately 10 μ M with the multiple concentration method were 45.1, 46.9 and 55.0%, respectively, at 10 μ M. As well as the results for CYP2D6, the same pattern of inhibitory effect was observed for CYP3A4 activity between single and multiple concentration methods. Moreover, the $IC_{50 \text{-single}}$ values calculated from the per cent of residual activity from the single concentra-

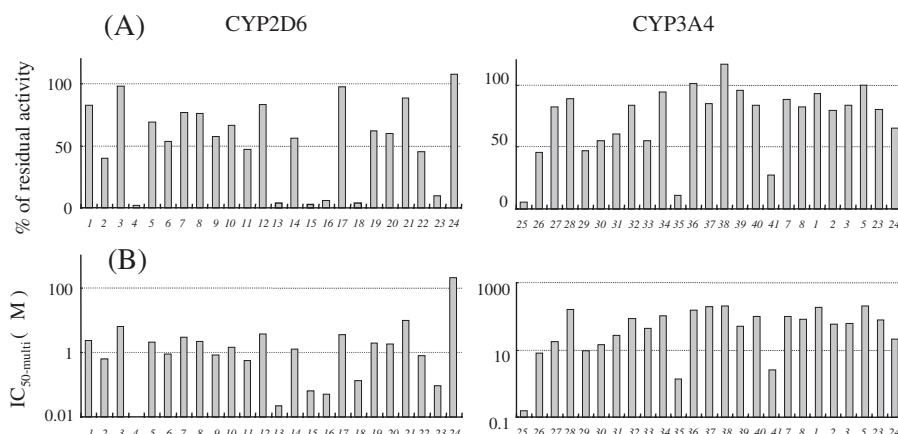


Figure 3. Comparison of the inhibitory effects for CYP2D6 and CYP3A4 activity between the single and multiple concentration methods. (A) Per cent of residual activities of test compounds on recombinant CYP2D6 and CYP3A4 activities obtained from the MTP assay with the single concentration method was 1 and 10 μ M for CYP2D6 and CYP3A4, respectively. (B) $IC_{50\text{ multi}}$ values of test compounds on recombinant CYP2D6 and CYP3A4 activities obtained from the MTP assay with the multiple concentration method. The per cent of residual activity and the $IC_{50\text{ multi}}$ values are the average of triplicate and duplicate determinations. The $IC_{50\text{ multi}}$ values for CYP2D6 have been reported by Yamamoto *et al.* (2003). The test compounds used were as follows: 1, amitriptyline; 2, clomipramine; 3, clozapine; 4, quinidine; 5, imipramine; 6, haloperidol; 7, desipramine; 8, nortriptyline; 9, trimipramine; 10, maprotiline; 11, mianserin; 12, amoxapine; 13, perphenazine; 14, risperidone; 15, thioridazine; 16, chlorpromazine; 17, alprenolol; 18, propafenone; 19, dextromethorphan; 20, timolol; 21, S-metoprolol; 22, propranolol; 23, compound A; 24, compound B; 25, ketoconazole; 26, terfenadine; 27, nifedipine; 28, erythromycin; 29, midazolam; 30, cyclosporine A; 31, verapamil; 32, metyrapone; 33, amiodarone; 34, diazepam; 35, miconazole; 36, temazepam; 37, diltiazem; 38, testosterone; 39, triazolam; 40, CAM; and 41, TAO.

tion method (1 μ M for CYP2D6, 10 μ M for CYP3A4) correlated closely with the $IC_{50\text{ multi}}$ values obtained from the multiple concentration method and the coefficient constant (r^2) was 0.772 (figure 4).

Effect of pre-incubation on inhibition of CYP2D6 and 3A4 with the single concentration method

The effects of pre-incubation on inhibition of CYP2D6 and 3A4 were investigated using the single concentration method and the results were compared with the result from the MTP assay with the pre-incubation method that used serially diluted solutions of compound. Control activities of AHMC and 7-HQ formation after pre-incubation with the single concentration method were similar to activities reported previously and described above (Yamamoto *et al.* 2003). The $IC_{50\text{ multi}}$ values obtained with the multiple concentration method were used as the concentration of test compound in the single concentration method in order to assess the effect of pre-incubation for each test compound, because a large change in the per cent of residual activity was produced at the concentration near the $IC_{50\text{ multi}}$ after pre-incubation (figure 5). Since compound A and propafenone are predominantly metabolized by CYP2D6 (Anzenbacherova *et al.* 2000), the concentrations of compounds in the 96 wells after the pre-incubation seemed to decrease compared with those in the case of co-incubation. For the inhibition of

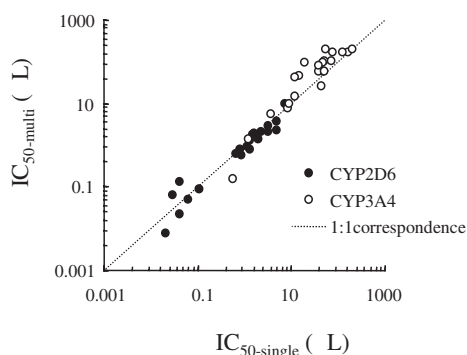


Figure 4. Relationship between $IC_{50\text{ multi}}$ values with the multiple concentration method and $IC_{50\text{ single}}$ values with the single concentration method. The compounds plotted are mainly substrates of CYP2D6 and CYP3A4. Data are based on 43 experiments with 36 test compounds except for clozapine, alprenolol and compound B (for CYP2D6) and temazepam, testosterone and imipramine (for CYP3A4), which showed the per cent of residual activity exceeding 96%. The $IC_{50\text{ single}}$ values were calculated as described in the Materials and methods. The $IC_{50\text{ multi}}$ values and $IC_{50\text{ single}}$ values are the mean of duplicate and triplicate determinations. The dotted line represents a 1:1 correspondence between the $IC_{50\text{ multi}}$ values and $IC_{50\text{ single}}$ values.

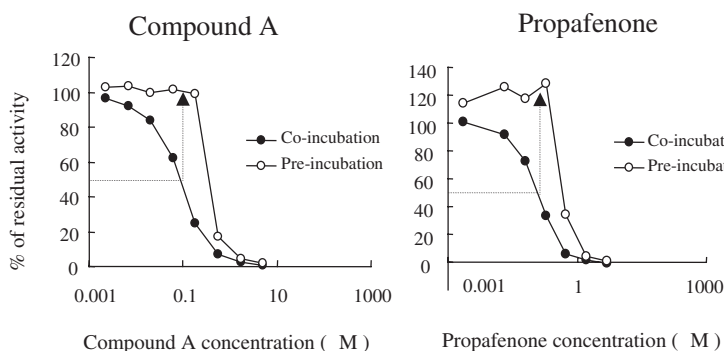


Figure 5. Effect of pre-incubation of compound A and propafenone on recombinant CYP2D6 activity after pre-incubation. Closed circles represent the inhibition curve after co-incubation (0 min) with test compound and CYP2D6/AMMC (1.5 μM) mixtures. Open circles represent inhibition curves after pre-incubation for 30 min. The inhibitory effects of compound A and propafenone were assessed using the MTP assay with the multiple concentration method.

CYP2D6 using the single concentration method, the per cent of residual activity of clozapine, amoxapine, timolol, S-metoprolol and propranolol after pre-incubation was $< 50\%$ but those of mianserin, perphenazine, risperidone, thioridazine, chlorpromazine, alprenolol, propafenone, dextromethorphan and compound A after pre-incubation for 30 min were $> 50\%$ (figure 6A). For the inhibition evaluation of CYP3A4 using the single concentration method, the per cent of residual activity of ketoconazole, terfenadine, metyrapon, testosterone and imipramine $> 50\%$ but those of many compounds studied were $< 50\%$ after pre-incubation for 30 min (figure 6A).

Among the test compounds examined, patterns of the inhibitory effects of these compounds on recombinant CYP2D6 and CYP3A4 activities were similar to the patterns of the IC_{50} ratio expressing the effects of pre-incubation on inhibition of CYPs using the multiple concentration method (figure 6B).

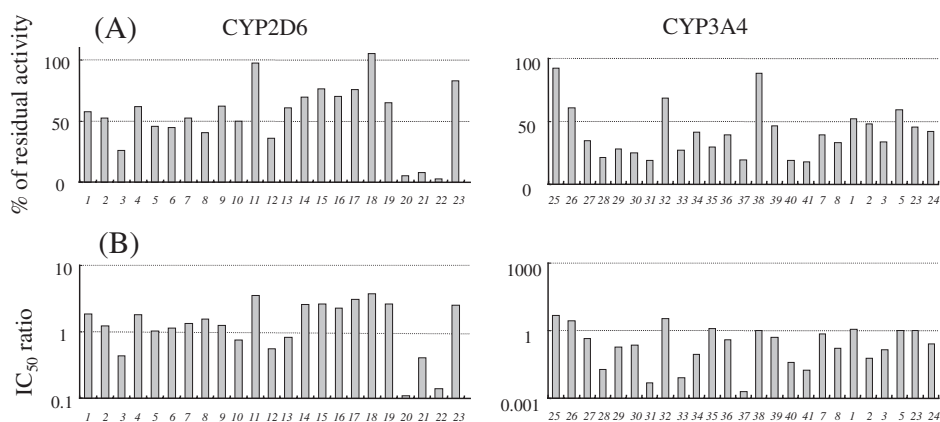


Figure 6. Comparison of the effect of pre-incubation on the inhibition of CYP2D6 and CYP3A4 between the single and the multiple concentration methods. (A) Per cent of residual activities of test compounds on recombinant CYP2D6 and CYP3A4 activities obtained from the MTP assay with the single concentration method. The concentration of test compound used was the IC₅₀_{multi} value obtained from the MTP assay using serially diluted concentrations of the test compound. The reaction was started by adding the enzyme after pre-incubation for 30 min. (B) Ratios (IC₅₀ ratio) of the IC₅₀ of test compounds obtained after pre-incubation with those IC₅₀ values obtained after co-incubation using the multiple concentration method. The IC₅₀ ratio for CYP2D6 was calculated using the reported IC₅₀ (Yamamoto *et al.* 2003). Test compounds used were as follows: 1, amitriptyline; 2, clomipramine; 3, clozapine; 4, quinidine; 5, imipramine; 6, haloperidol; 7, desipramine; 8, nortriptyline; 9, trimipramine; 10, maprotiline; 11, mianserin; 12, amoxapine; 13, perphenazine; 14, risperidone; 15, thioridazine; 16, chlorpromadine; 17, alprenolol; 18, propafenone; 19, dextromethorphan; 20, timolol; 21, S-metoprolol; 22, propranolol; 23, compound A; 24, compound B; 25, ketoconazole; 26, terfenadine; 27, nifedipine; 28, erythromycin; 29, midazolam; 30, cyclosporine A; 31, verapamil; 32, metyrapone; 33, amiodaron; 34, diazepam; 35, miconazole; 36, temazepam; 37, diltiazem; 38, testosterone; 39, triazolam; 40, CAM; and 41, TAO.

Inhibitory effect of compound A on recombinant CYP2D6 after correction for residual concentration of compound A after pre-incubation

The residual concentration of compound A in each well was measured after pre-incubation for 30 min. The concentration was decreased by incubation with recombinant CYP2D6. When the inhibitory curve of compound A on recombinant CYP2D6 after pre-incubation (figure 5, open circle) was corrected based on the residual concentration of compound A in the incubation medium, the inhibitory curve shifted to the left and agreed closely with that in the case of co-incubation (figure 7). This result suggests that compound A is metabolized by CYP2D6 during pre-incubation for 30 min before the addition of a substrate and that the concentration involved in the CYP2D6 inhibition is substantially decreased.

Discussion

Recent studies on CYP inhibition have been done using the MTP assay with recombinant CYPs and fluorescent probes on HTS (Stresser *et al.* 2000, Bapiro *et al.* 2001, Nomeir *et al.* 2001, Yamamoto *et al.* 2002, 2003) and is most useful to avoid compounds inhibiting CYPs at the stage of drug discovery. The IC₅₀ value, which is an important index of enzyme inhibition, can be obtained using the MTP assay but serially diluted concentrations of drug candidates are needed to determine the IC₅₀ values. Therefore, the number of compounds studied in this assay is limited to three or four per 96-well plate. The present study used a single-point

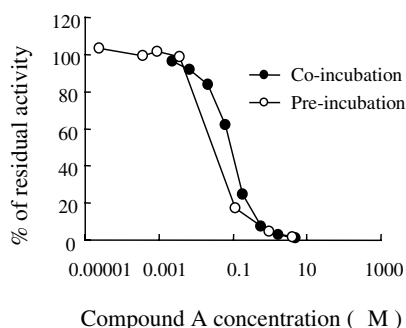


Figure 7. Inhibitory effect of compound A on recombinant CYP2D6 after correction for residual concentration of compound A after pre-incubation for 30 min. Actual concentrations of compound A with co-incubation are a final concentration added to each well and the residual concentration after pre-incubation was measured in each well before addition of the substrate. The inhibitory curve of compound A on recombinant CYP2D6 after pre-incubation was corrected to the inhibitory curve based on the residual concentration of compound A in the incubation medium.

IC₅₀ assay for assessment of the inhibition potential of new drug candidates on recombinant CYPs and for detection of time-dependent inhibition.

First, using the multiple concentration method, the inhibitory effects of various test compounds on recombinant CYP3A4 and effects of pre-incubation were assessed as well as the evaluation for CYP2D6 reported by Yamamoto *et al.* (2003). Among the test compounds assessed, the IC_{50 multi} values of ketoconazole, terfenadine and metyrapon after pre-incubation increased, but those of most of test compounds decreased. The inhibitory effects of TAO, erythromycin and CAM after pre-incubation were larger compared with that after co-incubation. These macrolide antimicrobial agents are known as mechanism-based inhibitors for CYP3A4 and the present results also agree with data in the literature (Yamazaki *et al.* 1996, Greenblatt *et al.* 1998, Kanamitsu *et al.* 2000). In addition, the IC_{50 multi} values of verapamil, amiodarone and diltiazem after pre-incubation were 205, 154 and 833 times lower than those after co-incubation. These results agree with the reports that IC₅₀ values of verapamil and diltiazem on testosterone 6 β -hydroxylase after pre-incubation with NADPH for 30 min greatly decrease compared with those after pre-incubation without NADPH or after co-incubation (Ma *et al.* 2000, Yeo and Yeo 2001). For diltiazem, Sutton *et al.* (1997) reported that the IC₅₀ of diltiazem against CYP3A4-mediated testosterone 6 β -hydroxylase was 120 μ M, but the values of the *N*-desmethyl and *N,N*-didesmethyl metabolites were 11 and 200 times lower than that of diltiazem. For CYP3A4 inhibition by amiodarone, Ohyama *et al.* (2000) reported that amioradone is a weak inhibitor against CYP3A4-mediated testosterone 6 β -hydroxylase but desethylamiodarone (formed by CYP3A4) competitively inhibits the activity 22 times more extensively than does amiodarone.

In the present study, the inhibitory effect of test compounds on recombinant CYP3A4 activity after co-incubation was lower than that for recombinant CYP2D6. However, in the cases of many compounds, the inhibitory effect on CYP3A4 after pre-incubation increased and the time-dependent inhibition is likely to be based on metabolic processes or potent reversible inhibitory metabolites during pre-incubation. For compounds such as diltiazem and amiodarone, therefore, the easy evaluation for the inhibition of CYP3A4 is needed for

metabolites as well as for the unchanged drug. The HTS method for detection of time-dependent inhibition seems important for drug discovery. Consequently, automation of the MTP assay was attempted to decrease both the time and labour involved in the analysis. When the $IC_{50 \text{ multi}}$ values in case of automated handling were compared with the $IC_{50 \text{ multi}}$ values from manual handling, in 52 experiments on 44 test compounds, a good correlation was found between these methods and $r^2 = 0.806$ (data not shown). Subsequently, a single concentration method was adapted for assessment of CYPs inhibition and for detection of time-dependent inhibition. Validity of the inhibitory effect (per cent of residual activity) of compounds on recombinant CYPs using the single concentration method was compared with the results (the $IC_{50 \text{ multi}}$) obtained with the multiple concentration method, regarding 36 test compounds. As shown in figure 3, the degree of per cent of residual activity for test compounds agreed well with the degree of the $IC_{50 \text{ multi}}$ values for these compounds. There were a good correlation between the $IC_{50 \text{ single}}$ values calculated using the per cent of residual activity with the single concentration method and the $IC_{50 \text{ multi}}$ values determined using the multiple concentration method (figure 4). Except for the case that test compound induces too much or too little inhibition at the test concentration, these results show that the single concentration method is useful for evaluation of CYP inhibition and will substantially decrease the time and labour involved.

The effects of pre-incubation were assessed using a single concentration method (the IC_{50} value as a concentration) and were compared with the ratios (IC_{50} ratio) of the IC_{50} of test compounds obtained after pre-incubation to those IC_{50} values obtained after co-incubation, using the multiple concentration method (figure 6). When the IC_{50} ratio < 1 , the inhibitory potentials of those compounds probably increase after the pre-incubation and the inhibition pattern seems to be based on the formation of potent reversible inhibitory metabolites or binding to CYPs. On the other hand, when the IC_{50} ratio > 1 , the compounds seem to be a substrate of the CYPs being studied. As shown in figure 6, the degree in the per cent of residual activity for test compounds from a single concentration method agreed well with the degree in the $IC_{50 \text{ multi}}$ values of these compounds with the multiconcentration method. If the per cent of residual activities is $< 50\%$ after pre-incubation, then the compound induces time-dependent inhibition, and if the per cent $> 50\%$, the compound is probably metabolized by the CYPs being assessed (figure 8). However, despite change of three orders of magnitude in the IC_{50} values of some compounds, which were detected as time-dependent inhibitors using the single concentration method, residual activities of these compounds were of same response.

In conclusion, the single concentration method is applicable to estimations of IC_{50} values of test compounds on recombinant CYPs activities instead of using the multiple concentration method. The inhibitory patterns (particularly; time-dependent inhibition and competitive inhibition) of many drug candidates for CYP forms can be readily estimated by studying the effect of pre-incubation on inhibition of CYP forms, using the MTP assay with the IC_{50} value calculated from the per cent of residual activity obtained from a single concentration method. As shown in figure 8, however, even if the single concentration method is used for identification of time-dependent inhibition, there are limitations for estimating of the degree of time-dependent inhibition. When the possibility of time-dependent inhibition is being estimated using the single concentration method, determination

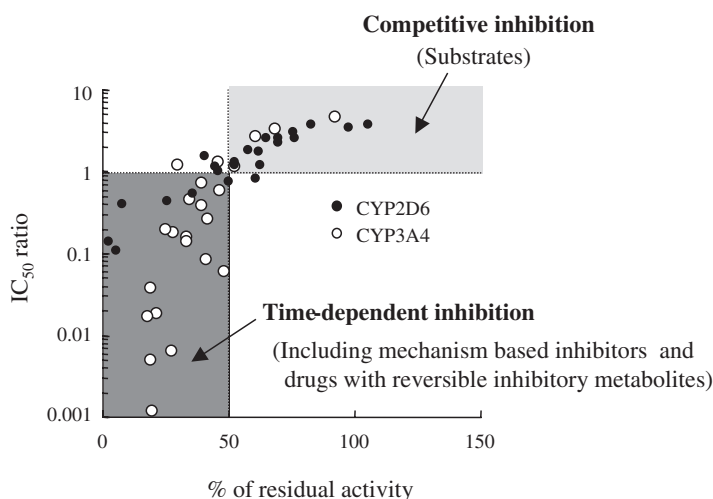


Figure 8. Relationship between the IC_{50} ratio from the multiple concentration method and the per cent of residual activity after pre-incubation using the single concentration method. The IC_{50} ratio represents the ratio of the IC_{50} of test compounds obtained after pre-incubation with those IC_{50} values obtained after co-incubation using the multiple concentration method. The compounds plotted are mainly substrates of CYP2D6 and CYP3A4. Data are based on 47 experiments with 40 test compounds, except for testosterone and imipramine. Since the IC_{50} values of testosterone and imipramine with co-incubation and after pre-incubation exceeded $200\mu M$, the effect of pre-incubation was not assessed for the multiple concentration method. The IC_{50} ratio and per cent of residual activity are the mean of duplicate and triplicate determinations. Zones of dark grey and light grey suggest that test compounds are drugs with potent reversible inhibitory metabolites or mechanism-based inhibitors (based on time-dependent inhibition) and are substrates of the CYP studied (competitive inhibition), respectively.

of the IC_{50} ratio using the multiple concentration method might be effective to estimate the degree of time-dependent inhibition as a following approach at the stage of drug discovery.

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Disposition of a novel and potent $\alpha_v\beta_3$ antagonist in animals, and extrapolation to man

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1. The disposition of 3-{2-oxo-3-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)propyl]-imidazolidin-1-yl}-3(S)-(6-methoxy-pyridin-3-yl)propionic acid (compound A), a potent and selective $\alpha_v\beta_3$ antagonist, was characterized in several animal species in support of its selection for preclinical safety studies and potential clinical development.

2. Compound A exhibited marked species differences in pharmacokinetics; the plasma clearances and bioavailabilities ranged from 33–47 ml min⁻¹ kg⁻¹ in rats and mice to 4–9 ml min⁻¹ kg⁻¹ in dogs and monkeys, and about 20% in rats to 70–80% in dogs and monkeys, respectively. Both the intravenous (i.v.) and oral kinetics of compound A were linear over the dose range studied in dogs (0.1–5 mg kg⁻¹ i.v. and 0.25–20 mg kg⁻¹ orally [p.o.]) and rats (1–30 mg kg⁻¹ i.v. and 4–160 mg kg⁻¹ p.o.).

3. Compound A was eliminated substantially by urinary excretion; the urinary recovery of the unchanged drug was 67% in rhesus, 48% in dogs and about 30% in rats. In these animal species, biotransformation was modest.

4. Following i.v. administration of [¹⁴C]-compound A to rats, the radioactivity rapidly distributed to all tissues investigated, with high levels of the radioactivity detected in liver, kidney and intestine soon after the drug administration. The radioactivity declined rapidly, with less than 1% of the i.v. dose remaining at 30-h post-dose.

5. Compound A was moderately bound to plasma proteins, with unbound fractions of 26, 20, 14 and 5% for rats, dogs, monkeys and humans, respectively. It was bound primarily to human α_1 -acid glycoprotein (about 85% binding at 0.1% concentration), as compared with human albumin (< 50% binding at 4% concentration).

6. Using simple allometry, compound A was predicted to exhibit relatively low clearance (1–3 ml min⁻¹ kg⁻¹) and low volume of distribution (0.1–0.3 l kg⁻¹) in humans. Based on the predicted values, compound A was projected to exhibit a favourable oral pharmacokinetic profile in humans, with good bioavailability (50–80%). These predicted values provided a basis for compound selection for further development.

Introduction

Osteoporosis poses a significant health problem for the elderly population, particularly, post-menopausal women. This disease is characterized by a decrease in bone mass, resulting in an increased risk to fractures. $\alpha_v\beta_3$, also known as the vitronectin receptor, is a member of a family of membrane-bound adhesion receptors called integrins that mediate cell–cell and cell–matrix interactions. The $\alpha_v\beta_3$ integrin is highly expressed in osteoclasts (the bone cells that resorb or destroy bone) and shown to play a key role in the bone resorption process (Miller *et al.* 2000, Hartman and Duggan 2002). $\alpha_v\beta_3$ binds to Arg-Gly-Asp (RGD)-containing

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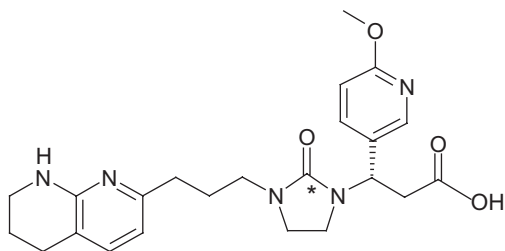


Figure 1. Chemical structure of compound A. *Position of the [^{14}C] label.

proteins such as vitronectin, osteopontin, bone sialoprotein and fibrinogen. RGD containing peptides such as echistatin and antibodies to $\alpha_v\beta_3$ have been shown to inhibit bone resorption *in vitro* and *in vivo* (Engleman *et al.* 1997, Crippes *et al.* 1998, Yamamoto *et al.* 1998). The identification of potent non-peptide vitronectin receptor antagonists has been described by Keenan *et al.* (1997, 1998); however, these molecules have generally possessed poor pharmacokinetic properties and low oral bioavailability. Recently, several low molecular weight RGD mimetics that bind with high affinity to the $\alpha_v\beta_3$ receptor have been reported to possess good orally bioavailable in animals (Lark *et al.* 1999, Miller *et al.* 1999, Coleman *et al.* 2002, Hutchinson *et al.* 2002). Of these, compound A, 3-{2-oxo-3-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)propyl]-imidazolidin-1-yl}-3(S)-(6-methoxy-pyridin-3-yl) propionic acid (figure 1), was shown to be a potent and selective $\alpha_v\beta_3$ antagonist, both *in vitro* and *in vivo* (Rodan *et al.* 2002). Compound A binds to purified human, rat, dog and rhesus monkey $\alpha_v\beta_3$ with high affinity ($k_d=0.3\text{--}0.9$ nM). In cell-based assays, compound A is a potent inhibitor of osteoclast formation and bone resorption *in vitro*, with IC_{50} values of about 10 nM, and exhibits minimal activity on $\alpha_{\text{IIb}}\beta_3$ -dependent human platelet aggregation ($\text{IC}_{50}=35\text{ }\mu\text{M}$). In addition, compound A has also been shown to increase bone mineral density significantly in growing male rats following minipump administration, and in 6-month old ovariectomized rats following oral administration (Rodan *et al.* 2002).

In order to evaluate the potential for further development, preclinical pharmacokinetics and disposition of compound A were characterized in several laboratory animal species, including mice, rats, dogs and monkeys. Due to the marked species differences observed, pharmacokinetic scaling of compound A across these animal species was conducted to help project its pharmacokinetics in humans. Results from these studies, which are reported here, suggest potentially favourable pharmacokinetic properties of compound A in humans, and thus, support its selection for toxicity/safety assessment studies and possible investigations in humans.

Materials and methods

Materials

Compound A and its ^{14}C -label (figure 1) (chemical purity >98% and isomeric purity >99%) were synthesized at Merck Research Laboratories (West Point, PA, and Rahway, NJ, USA, respectively). Human albumin (99% fatty acid free) and human α_1 -acid glycoprotein were obtained from Sigma Chemical Co. (St Louis, MO, USA). Solvents used for analysis were of analytical or HPLC grade (Fisher Scientific, Pittsburgh, PA, USA).

Animals

Male and female Sprague–Dawley rats (220–320 g), male CD-1 mice (about 30–40 g), male beagle dogs ($n=4$, 9–13 kg) and male rhesus monkeys ($n=3$, 4–6 kg) were used for these studies. Except for mice, the animals were housed individually in metabolism cages during the study for 48 h for urine collection. All animals were fasted overnight before dosing.

In vivo studies

All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

Compound A was administered at 1 mg kg⁻¹ i.v. and 4 mg kg⁻¹ p.o. in rats, 1 mg kg⁻¹ i.v. in mice, 0.5 mg kg⁻¹ i.v. and 1 mg kg⁻¹ p.o. in dogs, and 0.2 mg kg⁻¹ i.v. and 1 mg kg⁻¹ p.o. in monkeys. For i.v. dosing, compound A was administered via the jugular vein (rats) or the cephalic vein (dogs and monkeys). In rats and dogs, compound A was also given intravenously at 2.5, 10 and 30 mg kg⁻¹ (male rats) and 0.1, 2 and 5 mg kg⁻¹ (dogs), as well as orally at 2, 10, 40, and 160 mg kg⁻¹ (male rats), 40 and 160 mg kg⁻¹ (female rats), and 0.25, 5 and 20 mg kg⁻¹ (dogs). For all the i.v. and most of p.o. studies, dosing solutions of compound A were prepared in normal saline. At the 40 and 160 mg kg⁻¹ oral doses, compound A was given as suspensions in 0.5% methylcellulose. Pharmacokinetic studies in dogs and monkeys were conducted in a cross-over fashion. For mice, four animals were used for each sampling time point. For all other species, blood samples were taken, via the jugular vein (rats and dogs) or femoral vein (monkeys), in series at appropriate time intervals for up to 48 h. Urine samples were collected during 24- and 48-h periods. All samples were kept frozen until analysis.

Tissue distribution of [¹⁴C]-compound A was studied following 5 mg kg⁻¹ i.v. to male rats. The animals were sacrificed at 0.5, 2, 7 and 30 h post-dose, and tissues were removed for analysis of radioactivity levels.

Plasma protein binding

The binding of compound A to human, dog, monkey and rat plasma proteins was determined using an ultrafiltration method. Plasma from animals or humans, or a phosphate-buffered solution of human α_1 -acid glycoprotein (0.1%) or human albumin (4%) was spiked with compound A to yield final concentrations of 0.2, 2 and 20 μ M. The concentrations of α_1 -acid glycoprotein and albumin used were within the physiological range. The concentrations of compound A studied are pharmacologically relevant based on *in vivo* efficacy studies in animals. Following incubation at 37°C for 15 min, an aliquot of plasma or protein solutions (3 $\times$ 1 ml) was transferred immediately to a centrifuge tube (Amicon Co., Danvers, MA, USA). The tube was centrifuged at 1500g for 15–30 min at 37°C to obtain about 0.2 ml filtrate. The filtrate and original samples were analysed for compound A by HPLC. The unbound fraction was estimated directly from the ratio of the intact drug in the filtrate to that in the original plasma samples or protein solutions before centrifugation. Parallel studies using a phosphate-buffered solution (pH 7.4) instead of plasma indicated that compound A was bound minimally to the ultrafiltrate device.

Analytical procedures

Concentrations of compound A in plasma were analysed using liquid chromatography coupled with tandem mass spectrometry detection (LC/MS/MS). Compound A was isolated from plasma by solid-phase extraction using the TOMTEC QUADRATM pipetting station (Tomtec, Hamden, CT, USA). Briefly, 100–250- μ l aliquots of acidified plasma samples, spiked with an internal standard, which was a close analogue of compound A, were loaded onto a conditioned extraction plate (Waters OASISTM 96-well plate, Waters Corporation, Milford, MA, USA). Following a series of wash steps using water and 5% methanol, the samples were eluted from the plate with 70% acetonitrile. The eluant was evaporated to dryness under N₂ and reconstituted with 20% acetonitrile/water. The analytes were quantitated by LC-MS/MS in the positive ionization selected reaction monitoring (SRM) mode using an AB/MDS SCIEX API 300 tandem mass spectrometer (Concord, Ontario, Canada) interfaced with a SCIEX Turbo IonSpray source to a Perkin Elmer Series 200 liquid chromatography system. Chromatography was effected with a Zorbax Eclipse XDB-C8 column (2.1 mm $\times$ 15 cm, 5 μ m, Agilent Technologies) using a mobile phase consisting of 30% 90:10 acetonitrile:water and 70% 10:90 methanol:water, both containing 0.1% formic acid and 10 mM ammonium acetate, at a flow rate of 0.35 ml min⁻¹. The SRM transitions monitored (parent $\rightarrow$ product) were m/z 440 $\rightarrow$ 261 for compound A, and m/z 425 $\rightarrow$ 175 for the internal standard. Under these conditions, compound A and the internal standard eluted with retention times of approximately 1.9 and 1.7 min, respectively. Calibration curves (0.2–500 ng ml⁻¹ compound A) were prepared by plotting the appropriate peak area ratios against the concentrations of analyte in plasma using a weighted (concentration⁻²) linear regression. The concentration of the analyte in the unknown samples was determined by interpolation from the standard curve. The intra- and interday assay precision (%CV) and accuracy throughout the calibration range was <15% for compound A.

An HPLC method with fluorescence detection as described (Prueksaritanont *et al.* 2002) with minor modifications was also used for the protein binding determination and urinary analysis. In brief, plasma and urine samples, after protein precipitation with acetonitrile (1:1.5 v/v), and filtrates with or without appropriate dilutions were analysed. The supernatant was chromatographed on a Spherisorb SCX column (4.6 × 250 mm, 5 µm), with the mobile phase (A = acetonitrile, B = 25 mM phosphate buffer, pH 6) delivered at a flow rate of 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. The assay precision (%CV) and accuracy throughout the calibration range was <15% for compound A in both matrices examined.

Additionally, qualitative analysis was performed on bile and urine samples to determine metabolites of compound A. The analysis was conducted on a Finnigan MAT LCQ ion-trap mass spectrometer using electrospray ionization (ESI) in the positive mode. Samples were injected onto a BDS Hypersil C₁₈ column (2 × 150 mm, 5 µm) with a mobile phase consisting of 0.2% acetic acid (pH adjusted to 4.5 with ammonium hydroxide, solvent A) and acetonitrile (solvent B), delivered at a constant flow rate of 0.2 ml min⁻¹. The initial mobile phase consisted of 10% of solvent B, which increased linearly to 35% over 20 min.

Levels of total radioactivity in rat plasma and urine samples were measured directly by counting aliquots in a liquid scintillation counter (Tri-carb 2900TR, Perkin-Elmer, Downers Grove, IL, USA). Tissues and faecal homogenates were combusted in a biological sample oxidizer (Oximate 80, Perkin-Elmer, Downers Grove, IL, USA).

Pharmacokinetic analysis

Plasma clearance (CL_p) of compound A was calculated as the i.v. dose divided by the area under the plasma concentration–time curve (AUC) from zero to infinity. The apparent half-life (*t*_{1/2}) was estimated from the slope of the terminal phase of the log plasma concentration–time data. The steady-state volume of distribution (*V*_{dss}) was determined using non-compartmental method (Benet and Galeazzi 1971) as follows:

$$V_{dss} = (\text{dose i.v.} \times \text{AUMC})/(\text{AUC})^2,$$

where AUMC is the total area under the first moment of the drug concentration–time curve from time zero to infinity.

Oral bioavailability (*F*) was estimated as the AUC ratio following oral and i.v. administration normalized for differences in dose.

Pharmacokinetic scaling

Mean values for CL_p and unbound CL_p (CL_{p,u}) obtained from each animal species (mice, rats, dogs and monkeys) were plotted against their respective mean body weights (BW) on a log–log scale. Linear least-squares regression analysis was performed on the plot based on the following relationship (Boxenbaum 1982):

$$\text{CL}_p(\text{or } \text{CL}_{p,u}) = a\text{BW}^x,$$

where *a* and *x* are the allometric coefficient and exponent, respectively, and CL_{p,u} = CL_p/fraction unbound in plasma. The resulting *a* and *x* values were used, along with a standard value for human weight (70 kg), to predict CL_p or CL_{p,u} in humans. To convert predicted CL_{p,u} to predicted CL_p, predicted CL_p was multiplied by fraction unbound in human plasma.

Similarly, prediction of volume of distribution of compound A also was accomplished using the allometric scaling method by plotting the mean body weights versus means for *V*_{dss} or free *V*_{dss} corrected for unbound fraction (*V*_{d,u} = *V*_{dss}/fraction unbound) from animals on a log–log scale. Predicted *V*_{dss} or *V*_{d,u} values for humans were calculated using the respective allometric coefficient and exponent values and based on the 70-kg BW for humans.

Assuming insignificant gut metabolism, *F*, of compound A in humans was predicted based on the following relationship:

$$F = F_a \times (1 - E_h),$$

where *F*_a is the fraction absorbed of compound A estimated from animal data using the above relationship and based on the observed *F* and estimated *E*_h values in the corresponding species; and *E*_h is the fraction eliminated during hepatic first pass, which was obtained by dividing hepatic blood clearance (CL_h) by hepatic blood flow (*Q*_h). CL_h was estimated by multiplying CL_p by the fraction eliminated via non-renal route (1 – fraction eliminated unchanged in urine, *f*_e), and assuming blood/plasma partition ratio of 1. Values for *f*_e in animals were obtained experimentally (table 1), while those for humans were assumed to fall in a range obtained in animals (0.3–0.7) (table 1). *Q*_h values were obtained from literature (69, 41, 52 and 24 ml min⁻¹ kg⁻¹ in rats, dogs, monkeys and humans, respectively) (Boxenbaum 1979).

Table 1. Pharmacokinetic parameters of compound A following i.v. administration to animals.

Species	Gender	<i>n</i>	Dose (mg kg ⁻¹)	CL _p (ml min ⁻¹ kg ⁻¹)	<i>V</i> _{dss} (l kg ⁻¹)	<i>t</i> _{1/2} (h)	<i>f</i> _e (% of dose)
Mice*	male	4	1	33	2.8	3.5	—
Rat	male	4	1	47 ± 9	1.5 ± 0.2	3.0 ± 0.8	27 ± 3
		4	2.5	43 ± 6	N/A	N/A	—
		4	10	47 ± 7	1.3 ± 0.3	5.3 ± 1.6	—
		4	30	37 ± 5	1.0 ± 0.3	4.9 ± 0.5	—
Rat	female	4	1	35 ± 11	1.8 ± 0.6	3.2 ± 0.3	30 ± 6
Dog	male	4	0.5	4.0 ± 0.7	0.6 ± 0.03	4.7 ± 1.0	48 ± 12
		4	0.1	6.8 ± 3.0	1.2 ± 0.2	3.8 ± 0.9	—
		4	2	5.4 ± 1.8	0.9 ± 0.3	3.4 ± 0.2	—
Monkey	male	4	5	4.0 ± 1.1	0.6 ± 0.1	4.5 ± 0.6	—
		3	0.2	9.0 ± 2.1	0.3 ± 0.1	1.6 ± 0.3	67 ± 10

Data are mean ± SD.

N/A, Not determined due to an ill-defined terminal phase.

*Pharmacokinetic parameters were calculated from a set of mean plasma concentrations. Four animals were used for each sampling time.

Results

Pharmacokinetic studies

Following i.v. administration, plasma levels of compound A declined in a biphasic manner in all animal species, with terminal *t*_{1/2} ranging from about 2–5 h (figures 2A–D). Compound A was a relatively high clearance compound in rats (36–47 ml min⁻¹ kg⁻¹) and mice (34 ml min⁻¹ kg⁻¹), but was a low–moderate clearance compound in dogs (6 ml min⁻¹ kg⁻¹) and monkeys (9 ml min⁻¹ kg⁻¹) (table 1). It exhibited moderate volume of distribution (*V*_{dss} about 1.5–2.8 l kg⁻¹) in rats and mice, but relatively low volume of distribution (*V*_{dss} about 0.3–0.6 l kg⁻¹) in dogs and monkeys. Significant levels of unchanged drug were observed in animal urine; urinary recoveries were approximately 30, 50 and 70% in rats, dogs and monkeys, respectively (table 1). LC/MS/MS analysis of urine samples suggested relatively low levels of a glucuronide conjugate (*M* + 176) and few oxidative products of compound A (data not shown). Following oral administration, the bioavailability of compound A ranged from approximately 20% in rats to about 75% in dogs and monkeys, and the oral AUC values varied from 0.8 μM h at 4 mg kg⁻¹ in rats to > 3 μM h at 1 mg kg⁻¹ in dogs and monkeys (table 2). In rats, no marked gender differences were observed in the pharmacokinetic parameters of compound A following i.v. or oral administration, except for the slightly lower CL_p observed in female rats (table 1).

Over the intravenous dose range of 1–30 mg kg⁻¹ in male rats, and 0.1–5 mg kg⁻¹ in dogs, the pharmacokinetics of compound A appeared to be linear (table 1). Following oral administration (table 2), plasma levels of compound A increased with increasing doses, and its absorption kinetics in both animal species appeared to be more or less linear, over the dose range studied (4–60 mg kg⁻¹ in rats, and 0.25–20 mg kg⁻¹ in dogs). In male rats, the oral AUC values were slightly (less than about 2-fold) increased when compound A was given in a 0.5% methylcellulose suspension *vs.* a saline solution (table 2). Comparable or slightly higher AUC values were observed following oral administration of compound A (40 and 160 mg kg⁻¹) in female rats, as compared with male rats (table 2).

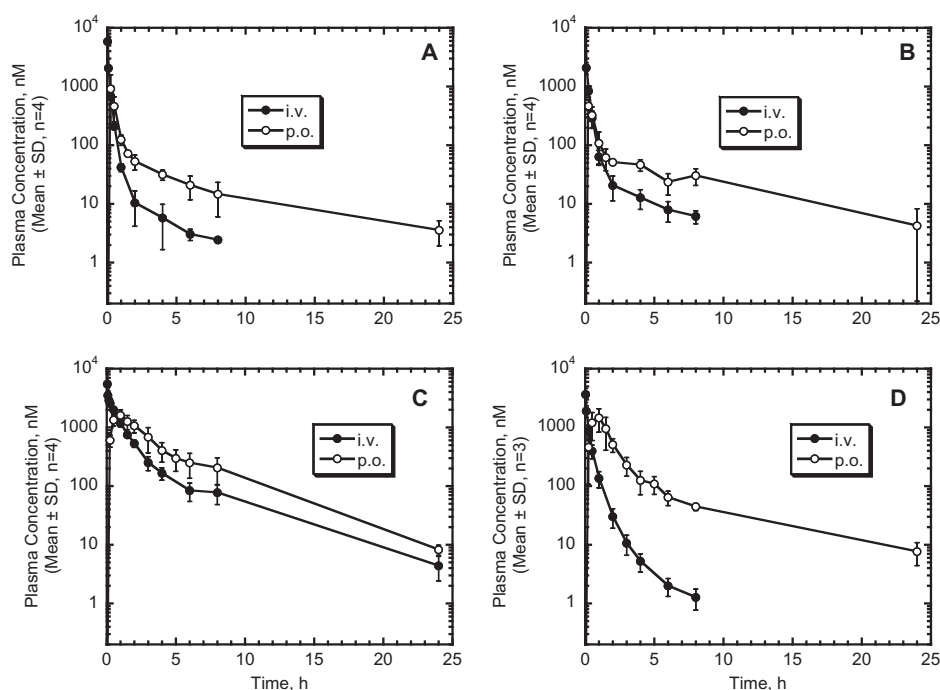


Figure 2. Plasma concentration–time profiles of compound A following i.v. and p.o. administration to male rats (A), female rats (B), male dogs (C) and male rhesus monkeys (D). Results are mean $\pm$ SD, $n=3-4$. Compound A was given at 1 mg kg^{-1} i.v. and 4 mg kg^{-1} p.o. in rats, 0.5 mg kg^{-1} i.v. and 1 mg kg^{-1} p.o. in dogs, and 0.2 mg kg^{-1} i.v. and 1 mg kg^{-1} p.o. in monkeys.

Table 2. Pharmacokinetic parameters of compound A following p.o. administration to animals.

Species	Gender	n	Dose (mg kg^{-1})	C_{max} (μM)	T_{max} (h)	AUC ($\mu\text{M h}$)	F (%)
Rat	male	4	4	0.9 ± 0.7	0.3 ± 0.0	0.8 ± 0.1	25 ± 5
			10	1.3 ± 0.4	0.3 ± 0.0	1.5 ± 0.2	
			40	6.3 ± 5.2	0.3 ± 0.0	8.4 ± 4.4	
			40*	7.8 ± 2.8	0.3 ± 0.1	11.2 ± 4.2	
			160*	39.7 ± 14	0.3 ± 0.1	61.7 ± 16	
Rat	female	4	4	0.5 ± 0.1	0.3 ± 0.0	0.8 ± 0.2	17 ± 4
			40*	7.3 ± 4.5	0.3 ± 0.0	9.3 ± 3.3	
			160*	47.6 ± 24	0.4 ± 0.1	105 ± 45	
Dog	male	4	1	1.6 ± 0.4	0.9 ± 0.3	6.6 ± 2.1	76 ± 8
			0.25	0.3 ± 0.1	1.0 ± 0.4	1.5 ± 0.5	
			5	13.7 ± 3	0.4 ± 0.1	31.6 ± 0.9	
			20	47.1 ± 9	0.6 ± 0.3	117 ± 52	
Monkey	male	3	1	1.8 ± 0.3	0.8 ± 0.3	3.2 ± 0.5	74 ± 6

Data are mean $\pm$ SD.

Except where indicated, dosing solutions of compound A were prepared in normal saline.

*Compound A was given as suspension in 0.5% methylcellulose.

Tissue distribution studies

Following i.v. administration of [^{14}C]-compound A to male rats, radioactivity was distributed rapidly into most tissues, including bone (femur, tibia and vertebra), which express relatively high levels of $\alpha_v\beta_3$ receptor. Among tissues examined, high levels were observed in liver, kidney, intestine (tissues and

Table 3. Tissue distribution of radioactivity following i.v. administration of ^{14}C -compound A at 5 mg kg^{-1} to rats.

Tissue	0.5 h		7 h		30 h	
	$\mu\text{g eq. g}^{-1}$	% of dose	$\mu\text{g eq. g}^{-1}$	% of dose	$\mu\text{g eq. g}^{-1}$	% of dose
Plasma	0.50 ± 0.07	0.45 ± 0.06	0.010 ± 0.003	0.01 ± 0.00	0.002 ± 0.001	0.00 ± 0.00
Liver	3.86 ± 0.93	3.5 ± 0.80	0.12 ± 0.04	0.11 ± 0.03	0.03 ± 0.02	0.02 ± 0.01
Kidney	2.34 ± 0.14	0.41 ± 0.03	0.09 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
Muscle	0.31 ± 0.06	3.1 ± 0.60	0.012 ± 0.002	0.12 ± 0.02	0.003 ± 0.003	0.03 ± 0.02
Brain	0.011 ± 0.002	0.00 ± 0.00	0.002 ± 0.001	0.00 ± 0.00	0.001 ± 0.001	0.00 ± 0.00
Lung	0.35 ± 0.03	0.04 ± 0.01	0.03 ± 0.02	0.00 ± 0.00	0.002 ± 0.001	0.00 ± 0.00
Skin	0.41 ± 0.13	1.44 ± 0.45	0.07 ± 0.06	0.24 ± 0.20	0.01 ± 0.004	0.04 ± 0.01
Fat	0.42 ± 0.28	0.34 ± 0.23	0.06 ± 0.09	0.05 ± 0.07	0.004 ± 0.003	0.00 ± 0.00
Femur	0.30 ± 0.07	0.01 ± 0.00	0.13 ± 0.10	0.01 ± 0.00	0.01 ± 0.003	0.00 ± 0.00
Tibia	0.22 ± 0.08	0.01 ± 0.01	0.09 ± 0.04	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
Vertebra	0.38 ± 0.05	0.01 ± 0.00	0.09 ± 0.04	0.00 ± 0.00	0.03 ± 0.004	0.00 ± 0.00
Large intestine	0.81 ± 0.19	0.11 ± 0.03	8.37 ± 7.00	0.96 ± 0.74	0.41 ± 0.53	0.05 ± 0.06
Small intestine	19.4 ± 4.4	10.4 ± 2.4	0.57 ± 0.22	0.31 ± 0.09	0.12 ± 0.18	0.06 ± 0.08
Bladder	18.2 ± 7.0	0.13 ± 0.06	0.14 ± 0.09	0.00 ± 0.00	0.10 ± 0.11	0.00 ± 0.00
Intestinal content		51 ± 5		61 ± 10		5 ± 7
Urine		N/A		N/A		30 ± 2
Faeces		N/A		N/A		56 ± 9

Values are mean $\pm$ SD, $n = 3$.

N/A, Not available.

content) and bladder shortly after dosing (table 3), indicating rapid biliary and renal excretion. These results were consistent with the relatively high CL_p value observed in this species. Also, consistent with its relatively high polarity, low levels of compound A were observed in rat brain. The total radioactivity level was the lowest in the brain ($< 0.02\mu\text{g}$ equivalent g^{-1} tissue) at all sampling times, indicating poor penetration of the compound through the blood brain barrier (table 3). Over time, the radioactivity declined rapidly in the well-perfused organs, such as liver and kidney, but declined gradually in bone or fat tissues (table 3). At the end of the experiment (30-h post-dose), very low levels of radioactivity (less than 1% of the dose) remained in the body, and $> 90\%$ of the radioactivity was recovered in urine, faeces and intestinal content (table 3).

Plasma protein binding

Compound A was moderately to highly bound to plasma proteins from rats, dogs, monkeys and humans. Over the concentration range ($0.2\text{--}20\mu\text{M}$) studied, the binding appeared to be independent of compound concentrations. The values for the unbound fractions in plasma were 26, 20, 14 and 5% for rats, dogs, monkeys and humans, respectively (table 4). Of the two human binding proteins examined, the binding of compound A was more extensive to α_1 -acid glycoprotein than to albumin (table 4). Unlike the observation with human albumin, concentration-dependent binding was observed with human α_1 -acid glycoprotein at $20\mu\text{M}$ concentration of compound A (table 4), presumably due to a saturation in protein binding site. The finding of lower binding with α_1 -acid glycoprotein and albumin than with whole plasma suggests a possibility that compound A might also bind to other proteins in human plasma.

Table 4. Protein binding (expressed as unbound fraction) of compound A to plasma proteins.

	Species	Concentration (μM)		
		0.2	2	20
Whole plasma	rat	29 ± 0.8	23 ± 0.6	27 ± 1.4
	dog	18 ± 0.8	18 ± 0.6	24 ± 1.0
	monkey	13 ± 0.7	12 ± 0.5	15 ± 0.7
	human	4 ± 0.3	4 ± 0.4	6 ± 0.5
α ₁ -Acid glycoprotein (0.1%)	human	15 ± 1	15 ± 2	46 ± 2
Albumin (4%)	human	67 ± 2	69 ± 2	68 ± 2

Values are means ± SD of triplicate determinations.

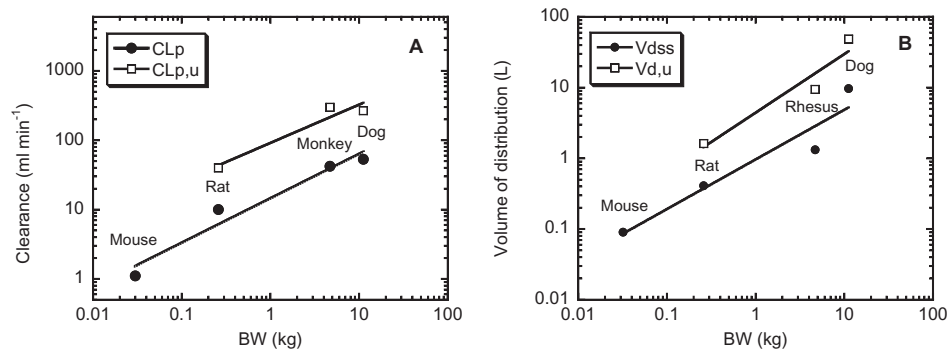


Figure 3. Allometric scaling plots for CL_p and CL_{p,u} (A), and V_d and V_{d,u} (B) of compound A in animals. Each symbol and bar represents the means obtained from studies shown in table 1.

Pharmacokinetic scaling

Figure 3A demonstrates the standard log–log allometric plots of CL_p and CL_{p,u} of compound A across animal species; both CL_p and CL_{p,u} scaled well, with *r*² of 0.97 and 0.85, respectively. The calculated allometric exponents were 0.64 and 0.55, and the allometric coefficients were 14.4 and 90.9 for the CL_p and CL_{p,u} plots, respectively. Using this simple allometric approach, the CL_p value for compound A was predicted to be relatively low in humans, with values of 0.7 and 3 ml min⁻¹ kg⁻¹, with (CL_{p,u}) or without (CL_p) protein binding correction, respectively.

The allometric plots of V_{dss} and V_{d,u} yielded *r*² of 0.83 and 0.90, with the allometric exponents of 0.70 and 0.83 and allometric coefficients of 0.96 and 4.39, respectively (figure 3B). Based on these relationships, values for V_{dss} in humans were predicted to be relatively low (0.1–0.3 l kg⁻¹). Similar to the clearance prediction, the predicted V_{dss} value was lower (0.1 l kg⁻¹) with protein binding correction or based on allometric scaling of V_{d,u} than that without protein binding correction (V_{dss}).

Estimation of *E*_h and *F*_a in human is required to predict *F* in humans. Based on the low CL_p predicted and assuming the fraction eliminated unchanged in humans would fall in the observed range in animals (0.3–0.7, table 1), low hepatic first-pass elimination (< 10%) in humans is expected (table 5). In animals, *F*_a of compound A was estimated based on the observed *F* and CL_h to be 0.5 in rats and

Table 5. Prediction of human pharmacokinetics of compound A.

Predicted CL_p ($ml\ min^{-1}\ kg^{-1}$)	Predicted E_h	Estimated F_a	Predicted F
1	0.01–0.03	0.5–0.8	0.5–0.8
3	0.04–0.09	0.5–0.8	0.5–0.7

CL_p was predicted based on allometric scaling (see text).

$E_h = CL_h/Q_h$; $Q_h = 24\ ml\ min^{-1}\ kg^{-1}$ in humans.

$CL_h = CL_p \times (1-f_e)$, assuming a blood/plasma concentration ratio of 1.

f_e was estimated from animal data ($f_e = 0.3$ – 0.7 ; see table 1).

F_a was estimated from animal data based on $F = F_a \times (1-E_h)$.

F was calculated based on $F = F_a \times (1-E_h)$.

0.8 in dogs and monkeys. Assuming that absorption of compound A in humans would be as good as in animals, F was predicted to range from 50 to 80% in humans (table 5).

Discussion

Compound A is a novel $\alpha_v\beta_3$ antagonist. In support of its selection for potential clinical development, the disposition of compound A was characterized in the animal species commonly employed in early non-clinical pharmacokinetic and safety/toxicology studies.

In animals, compound A exhibited marked species differences in CL_p values, with the highest and lowest values observed in rats and dogs, respectively. The elimination of compound A in these animal species appeared to be mediated by different clearance mechanisms. In monkeys, approximately 70% of CL_p was due to renal elimination of the unchanged drug, which suggests that biotransformation is not a major elimination pathway for compound A. In dogs and rats, the renal elimination of compound A constituted about 50 and 30%, respectively, of CL_p , raising a possibility for substantial biotransformation of compound A in these species. However, in a preliminary study with bile-duct cannulated animals, compound A was a major component in both bile and urine from rats and dogs (data not shown), suggesting that biotransformation is not the major elimination pathway of this compound in rats and dogs. This finding is not unexpected considering the relatively hydrophilic nature of compound A ($\log P = 0.2$), and based on previous observations with other hydrophilic zwitterions (Yano *et al.* 1997, Prueksaritanont *et al.* 2002). Like many compounds that are excreted primarily unchanged, species differences in the hepatic and renal elimination observed with compound A possibly could be due to species differences in transport proteins known to be present in liver and kidney (Ayrton and Morgan 2001). Interestingly, in all animal species studied, the unbound renal clearance of compound A, which was obtained by multiplying CL_p by the fraction excreted unchanged in urine and corrected for unbound fraction in plasma, was much higher than the glomerular filtration rate reported in the respective species. This suggests that the renal elimination of compound A involves significant tubular secretion. In the case of dogs, this active secretion process did not appear to be readily saturated at the concentrations studied since in this species the CL_p , which comprises about 50% renal clearance, was independent of dose over a wide dose range.

In animals, there were also marked species differences in F values of compound A. In contrast to the observation with CL_p value, F was the lowest and highest in rats and dogs, respectively. The relatively high F in dogs and monkeys suggests that compound A is well absorbed in these species, consistent with relatively low CL_p and thus low first-pass elimination. The relatively low F in rats might be attributable to incomplete absorption or high hepatic first-pass elimination. Assuming that gut metabolism of compound A in rats was minimal and considering that 70% of compound A was eliminated by hepatic elimination (based on 30% renal excretion), the E_h of compound A was estimated to be about 60–70% in this species. This estimate suggested that the relatively low F of compound A in rats was due primarily to the extensive first-pass elimination, presumably via a combination of biliary excretion and hepatic metabolism. Consequently, the absorption of compound A in this species was reasonably good (about 50%). The finding of comparable oral AUC values in rats observed following compound A in solution or in 0.5% methyl cellulose suspension was consistent with its relatively high solubility ($>10\text{ mg ml}^{-1}$ in water). Considering the apparently linear kinetics of CL_p and F values observed in both rats and dogs over the wide dose range, the absorption of compound A did not appear to be mediated via saturable process(es) with low affinity.

In addition to the observed species differences in CL_p and F , compound A also exhibited species differences in V_{dss} . Similar to the observation with CL_p , the V_{dss} of compound A was higher in mice and rats than in dogs and monkeys (table 1). As a result, the apparent $t_{1/2}$ of compound A across these animal species was more or less in a comparable range. Marked species differences in plasma protein binding also were observed, especially between the preclinical species investigated and humans (table 4). Plasma protein binding has generally been recognized as an important factor in the pharmacokinetic modelling of compounds. Equations describing the relationship between plasma protein binding and volume of distribution or clearance (Wilkinson and Shane 1975, Oie and Tozer 1979), have been well accepted and successfully utilized. Additionally, it has also been established that only free fraction of a drug is available for transcellular equilibrium and thus for pharmacological effect. In a preliminary study, the addition of α_1 -acid glycoprotein, a major binding protein for compound A in human plasma, increased IC_{50} values for inhibition of osteoclast formation in a cell-based assay (data not shown), thus supporting the latter principle. Therefore, the finding that compound A exhibits higher plasma protein binding in humans than in animals suggests that in humans, higher exposure (based on total drug) would be required, as compared with in animals, to elicit efficacy.

Owing to the aforementioned differences among the preclinical species examined, it was desirable to get an early estimate of human pharmacokinetics of compound A to support its selection for further development. Prediction of CL_p , V_{dss} and F , three primary parameters governing the drug disposition, in humans were of most interest. Considering that biotransformation was not the major route of elimination for compound A in animals, and that compound A was resistant to metabolism in the presence of NADPH or UDPGA when incubated with liver microsomes from animals and humans (data not shown), it is conceivable that compound A may also undergo minimal biotransformation in humans. Therefore, prediction of human clearance of compound A was accomplished by the well-known allometric scaling method. Using this approach, the CL_p value

(and consequently E_h) for compound A was predicted to be relatively low in humans (table 5). Interestingly, in the case of compound A, the projected CL_p value obtained with protein binding correction ($0.7 \text{ ml min}^{-1} \text{ kg}^{-1}$) was lower than that predicted without protein binding correction ($3 \text{ ml min}^{-1} \text{ kg}^{-1}$), and was closer to the observed value ($1 \text{ ml min}^{-1} \text{ kg}^{-1}$) recently obtained in healthy volunteers (unpublished data). It is also noteworthy that simple allometry has been shown to give a reasonable prediction of clearance for compounds with the allometric exponents of <0.7 (Mahmood and Balian 1996) and minimal metabolism (Sawada *et al.* 1984), similar to compound A. Similarly, the allometric scaling method suggested a relatively low volume of distribution of compound A in humans, as compared with the observed values in animals, and the predicted V_{dss} value obtained with protein binding correction (0.1 l kg^{-1}) was lower than that without the correction (0.3 l kg^{-1}). This relatively low V_{dss} observed in humans is consistent with the higher plasma protein binding noted in humans than in animals (table 4).

Ultimately, it is most desirable to develop an oral compound with high bioavailability and high systemic exposure. Since the absorption of compound A in all animal species examined is very efficient, it is reasonable to assume that the absorption in humans would also be efficient. Together with the anticipated low CL_p and thus low E_h , it was projected that compound A would exhibit high F in humans, unlike the observation in rats. The combination of low CL_p (lower than all animals studied) and high F suggest that the oral pharmacokinetic profile of compound A in humans would be excellent, with resulting oral exposure (dose normalized) potentially greater than that obtained in animals. These predicted values were later found to be in good agreement with clinical observations (unpublished data).

To conclude, the present study provides important information about the pharmacokinetic profile of compound A, a novel $\alpha_v\beta_3$ antagonist, in preclinical laboratory animal species, as well as providing a good example of prediction of human pharmacokinetics of a renally excreted compound. The results, which revealed marked species differences in the disposition of compound A in animals and suggested favourable pharmacokinetic properties in humans, provided a basis for the selection of compound A for development as a new and orally bioavailable anti-bone resorptive agent in humans.

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CYP2C subfamily, primarily CYP2C9, catalyses the enantioselective demethylation of the endocrine disruptor pesticide methoxychlor in human liver microsomes: use of inhibitory monoclonal antibodies in P450 identification

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1. The endocrine disruptor pesticide methoxychlor undergoes *O*-demethylation by mammalian liver microsomes forming chiral mono-phenolic (1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane, i.e. mono-OH-M) and achiral bis-phenolic oestrogenic metabolites. Human liver microsomes (HLM) generated primarily the *S*-mono-OH-M.

2. Inhibitory monoclonal antibodies (MAb) identified those P450s catalysing the enantioselective *O*-demethylation of methoxychlor. In HLM, *O*-demethylation was inhibited by MAb anti-CYP1A2 (30–40%), diminishing the per cent of *S*-mono-OH-M from about 80 to 55–60%. MAb anti-CYP1A2, 2A6, 2B6, 2C8, 2C19, 2D6 and 3A4 did not affect the demethylation rate in HLM. Nevertheless, MAb anti-CYP1A2 decreased the formation of *R*-mono-OH-M from 21–23 to 10–17%, indicating that CYP1A2 exhibits a role in generating the *R*-enantiomer.

3. Among cDNA-expressed human P450s (supersomes), CYP2C19 was the most active in demethylation, but in HLM, CYP2C19 appeared inactive (no inhibition by MAb anti-CYP2C19). There was a substantial difference in the per cent inhibition of demethylation by MAb anti-CYP2C9 and anti-rat CYP2C (MAb inhibiting all human CYP2C forms) and in altering the enantioselectivity, suggesting that demethylation by combined CYP2C8, 2C18 and 2C19 was significant (20–30%).

4. Polymorphism of methoxychlor demethylation was examined with supersomes and HLM-expressing CYP2C9 allelic variants. CYP2C9*1 and 2C9*2 were highly active; however, CYP2C9*3 appeared inactive.

Introduction

Methoxychlor (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane) is a pesticide currently used primarily in industrially developed countries as a substitute for the essentially banned DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) (Metcalf *et al.* 1971, Gardner and Bailey 1975, Metcalf 1976). It is relatively non-toxic in mammals, having an oral LD₅₀ in rats approximately 40-fold higher than DDT and exhibiting a much shorter half-life than DDT (Metcalf 1976). Nevertheless, despite the low acute toxicity of methoxychlor, there is concern of subtle or overt long-term endocrine toxicity from inadvertent exposure to it in wild-life and possibly in humans. That concern stems from observations that methoxychlor metabolites are oestrogenic in mammals (Bulger *et al.* 1978a, b, Kupfer and Bulger

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1979, Ousterhout *et al.* 1981, Bulger *et al.* 1985). Indeed, the oestrogenic activity of methoxychlor has been amply demonstrated *in vitro* and *in vivo* (Bulger *et al.* 1978a, b). Surprisingly, certain methoxychlor metabolites interact differently with the two oestrogen receptor (ER) isoforms α and β , and with the androgen receptor (AR), being agonists of ER- α and antagonists of both ER- β and AR (Gaido *et al.* 1999, 2000). These and other findings led to conclusions that methoxychlor is a reproductive toxin (Gray *et al.* 1988, Cummings and Gray 1989, Cummings and Laskey 1993), and consequently, methoxychlor has been classified as a prototype endocrine disruptor (Chapin *et al.* 1997, Cummings 1997, Bigsby *et al.* 1999, You *et al.* 2002). In another series of studies, it was observed that methoxychlor is a cytochrome P450 (CYP) inducer in rats, elevating the levels and activity of CYP2B and 3A enzymes (Li *et al.* 1995, Li and Kupfer 1998). Furthermore, the possible induction of CYP2B6 in humans by methoxychlor was explored with the constitutive androstane receptor (CAR) (Sueyoshi *et al.* 1999, Blizard *et al.* 2001, Sueyoshi and Negishi 2001) and it was concluded that both methoxychlor and its three major phenolic metabolites activate CAR (Blizard *et al.* 2001), indicating that humans exposed to methoxychlor could undergo induction of CYP2B6 and possibly of CYP3A4. However, whether methoxychlor would transactivate the pregnane X receptor (PXR) and hence act as a phenobarbital or rifampin-type inducer of CYP3A enzymes (Lehmann *et al.* 1998, Goodwin *et al.* 2002) in humans has not yet been established. Of additional interest is the recent finding on the differences in the effects of bis-OH-M (1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)-ethane, a methoxychlor metabolite) and oestradiol on gene expression in mouse ovaries (Waters *et al.* 2001), indicating that oestradiol and methoxychlor differ in their oestrogenic activities and that oestradiol is not an appropriate model to study the effects of chlorinated hydrocarbon pesticides on gene expression.

Earlier studies demonstrated that rat liver microsomes and human liver microsomes (HLM) catalyse the mono- and bis-demethylation of methoxychlor forming the phenolic metabolites mono-OH-M and bis-OH-M, respectively. A secondary metabolic step was also observed in which there was *ortho*-hydroxylation of mono-OH-M and bis-OH-M, yielding two catechols, i.e. catechol-M (1,1,1-trichloro-2-(4-methoxyphenyl)-2-(3,4-dihydroxyphenyl)ethane) and Tris-OH-M (1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(3,4-dihydroxyphenyl)ethane) (Bulger *et al.* 1978a, b, 1985, Kupfer *et al.* 1990, Dehal and Kupfer 1994, Hu and Kupfer 2002a). Some of these metabolites are chiral (figure 1) and consequently metabolism may represent formation of unequal amounts of the enantiomers. Indeed, considerable enantioselectivity of demethylation of methoxychlor *in vitro* by certain CYPs was observed in rats (Kishimoto *et al.* 1995, Kishimoto and Kurihara 1996). Moreover, recent studies in humans demonstrated that there is a high degree of enantioselectivity in mono-demethylation of methoxychlor by the various cDNA-expressed P450s (supersomes), with CYP2C9 and CYP2C19 forming primarily the (*S*)-mono-OH-M, whereas CYPs1A2 and 2A6 primarily formed the (*R*)-enantiomer (Hu and Kupfer 2002b). Interestingly, all of the HLM examined by the present authors (including HLM from pooled livers of 25 donors) demonstrated marked enantioselectivity, predominantly forming the (*S*)-mono-OH-M. However, the relative contribution of various CYP enzymes in the HLM, in catalysis of formation of the two mono-OH-M enantiomers, has not been established. The authors' previous studies, based on the use of chemical inhibitors, suggested that CYP2C9 and

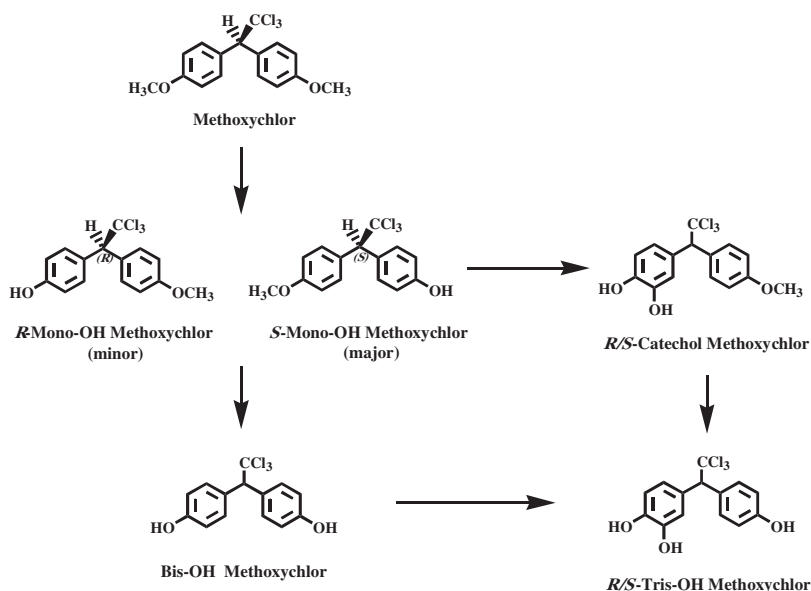


Figure 1. Methoxychlor metabolism by human liver microsomes. Formation of *R*- and *S*-enantiomers of the chiral metabolites.

1A2 and possibly 2C19 are involved in the enantioselective demethylation of methoxychlor in HLM (Hu and Kupfer 2002b, c). Among the cDNA-expressed P450s examined, CYP2C19 was by far the most active P450 in catalysis of mono-demethylation of methoxychlor. However, the lack of specific chemical inhibitors of CYP2C19 precluded the determination of whether 2C19 in HLM is involved in this catalysis.

Since, mono-demethylation of methoxychlor is the first step in the metabolic formation of the oestrogenic chiral methoxychlor metabolite(s) (figure 1), there is a possibility that the preferential formation of the *R*- or *S*-mono-OH-M enantiomers may result in quantitative differences in the oestrogenic activity of mono-OH-M *in vivo* and in qualitative or quantitative differences in their interaction with the ER- α and ER- β . Additionally, potential differences could be envisioned in the interaction of the individual enantiomers of mono-OH-M or of its subsequent chiral metabolites with the CAR or PXR receptors. It was thus of interest to identify the P450 forms in HLM that catalyse selectively the formation of the individual mono-OH-M enantiomers. Consequently, the current study was undertaken to identify the P450s in human liver preparations that are responsible for the enantioselectivity of methoxychlor metabolism. To achieve this aim, monospecific inhibitory anti-P450 monoclonal antibodies (MAbs) were employed to inhibit selectively the various CYPs and to identify their selective participation in catalysis of methoxychlor demethylation.

Materials and methods

Methoxychlor (laboratory grade = 99% + pure) was from Chem Service (West Chester, PA, USA). Mono-OH-M and catechol-M were synthesized in the authors' laboratory (purity > 90%) (Hu and Kupfer 2002a). High-performance liquid chromatography (HPLC)-grade acetonitrile was from Mallinckrodt (St Louis, MO, USA). HPLC columns Prodigy ODS3 (250 $\times$ 4.6 mm) was from

Phenomenex (Torrance, CA, USA) and the Chiral-AGP column was from Chromtech (Apple Valley, MN, USA). NADPH was from Sigma (St Louis, MO, USA). cDNA-expressed recombinant human P450 enzymes co-expressed with NADPH-P450 reductase and, in certain preparations, containing co-expressed cytochrome b₅ (present in an insect cell line, referred to as supersomes) were provided as microsomal preparations; human liver microsomes were from BD-Gentest (Woburn, MA, USA). *S*-(+)-mephenytoin and (±)-4'-hydroxymephenytoin were from Ultrafine (UFC Ltd, Manchester, UK). MAbs were prepared and characterized as described (Gelboin *et al.* 1999, Yang *et al.* 1999, Krausz *et al.* 2001). The antibodies used were monospecific for each respective CYP enzyme and were highly inhibitory of the indicated CYPs.

Incubations

Incubations were conducted in 20-ml vials under an atmosphere of air in a Dubnoff metabolic shaking incubator as follows. cDNA-expressed P450 enzymes (supersomes) 25 pmol P450 or human liver microsomes (150 pmol P450) in sodium phosphate buffer, pH 7.4, total volume 0.5 ml (100 mM) buffer at 37°C for 5 min. Methoxychlor (25 µM added in 10 µl ethanol), MgCl₂ (10 mM), NADPH (1.0 mM) and additional sodium phosphate buffer were added to a final volume of 1 ml. These reaction mixtures were incubated at 37°C for 30 min and the reactions were terminated by the addition of 3 ml diethyl ether (ether) followed by 1 ml water and the products were extracted with ether (2 × 3 ml). In certain experiments, the reactions were terminated by rapid cooling through placing the vials in -78°C (acetone/dry ice) bath and storing frozen until work up. In the latter case, after thawing, 1 ml water was added and the metabolites extracted with ether (2 × 3 ml). In all work-up procedures, the ether phase containing the incubation extracts was evaporated to dryness at room temperature under a stream of nitrogen gas. The residues were dissolved in 0.5 ml methanol, passed through a 20-µm PTFE filter and the filtrate was evaporated to dryness at room temperature under a stream of nitrogen. The resulting residues were dissolved in 50 µl methanol and subjected to HPLC analysis to identify the metabolites and determine the rates of product formation.

Positive control experiments

To determine the competency of the anti-CYP2C19 MAb in its ability to inhibit CYP2C19 catalysis in HLM, experiments were carried out with *S*-mephenytoin (a recognized substrate of CYP2C19). Incubations were conducted with cDNA-expressed CYP2C19 (25 nM P450-supersomes) or with HLM (150 pmol P450s) using a single donor H030 or HLM from pooled 25 livers and included *S*-mephenytoin (25 µM) as the substrate and anti-CYP2C19 (1-7-4-8) MAb (5 µl ascites) or control-MAb (Hy-Hel) in 5 µl ascites fluid; incubations were carried out at 37°C for 30 min. The metabolite (4'-hydroxymephenytoin) was detected and quantified by HPLC, with a Waters 6000 pump and 5 µm Phenomenex Prodigy ODS3 column (250 × 4.6 mm) at a flow rate of 1 ml min⁻¹ using isocratic mobile phase composed of acetonitrile:water (30:70). Retention times were 5.9 min for residual *S*-mephenytoin and 14.5 min for 4'-hydroxymephenytoin. Results exhibiting inhibition of the *S*-mephenytoin hydroxylation by the anti-CYP2C19 MAb in both the supersomes and HLM incubations were indicative of the competency of inhibition of CYP2C19 in both preparations.

Methoxychlor metabolites identification and purification. Methoxychlor metabolites were identified by HPLC, comparing their retention time with authentic compounds (synthesized and identified by the present authors in earlier studies using physicochemical methods) (Hu and Kupfer 2002a, b). An isocratic mobile phase of acetonitrile:water (70:30) was used for analysis of extracts from methoxychlor incubation. Retention times were 15.2 min for methoxychlor, 7.1 min for mono-OH-M, 5.2 min for catechol-M, 4.1 min for bis-OH-M and 3.5 min for Tris-OH-M.

Isolation of methoxychlor metabolites

The distribution of enantiomers of mono-OH-M was analysed on a semipreparative chiral-AGP column, 100 × 10 mm, 5 µm, at λ = 230 nm, with acetonitrile:water (20/80) at a flow rate of 3 ml min⁻¹. The retention time of the *R*-enantiomer was 6.7–6.8 min and of the *S*-enantiomer was 8.6–8.9 min. The enantiomers of mono-OH-M were collected off the HPLC (using λ = 230 nm for detection), and the solvent was evaporated under a stream of nitrogen gas at 40–45°C. The isolated metabolites were stored dry at -20°C for analysis and for subsequent use in metabolic studies (Hu and Kupfer 2002a, b).

Results and discussion

A previous study, employing chemical inhibitors, suggested that CYP2C9 and 1A2 and possibly 2C19 catalyse the enantioselective demethylation of methoxychlor in HLM (Hu and Kupfer 2002a, b). However, the lack of specific chemical

inhibitors precluded the determination of whether in addition to individual CYP2C19 (in supersomes), which were active in methoxychlor demethylation, CYP2C19 in HLM is also involved in this catalysis, or whether merely CYP2C9 and 1A2 are the prime or sole HLM catalysts. Surprisingly, preliminary studies with (+)-*N*-3-benzylnirvanol, a proposed selective inhibitor of CYP2C19 (Suzuki *et al.* 2002), did not demonstrate inhibition of the CYP2C19 (supersomes)-mediated methoxychlor demethylation.

Identification of the P450 forms in catalysis of methoxychlor demethylation by HLM

The availability of inhibitory MAbs (monospecific anti-human P450s) made it feasible to conduct experiments to try to identify the P450 forms in HLM that catalyse the demethylation of methoxychlor. In an initial experiment, individual human P450 isoforms (as supersomes) were used and it was observed that monospecific MAbs inhibited methoxychlor demethylation by the cDNA-expressed CYP1A2, 2A6, 2B6, 2C8, 2C9*1, 2C18, 2C19, 2D6 and 3A4 (supersomes) (table 1). However, whereas the MAbs extensively inhibited the activity of most of their respective cDNA-expressed P450s (by 63–93%), the anti-CYP1A2 and anti-CYP3A4 MAbs inhibited the demethylation of CYP1A2 and 3A4 by only 41 and 58%, respectively. Attempts to increase the inhibition of the CYP1A2-catalysed demethylation by elevating the levels of anti-CYP1A2 from 7.5 to 45 μ l (ascites fluid) were not successful (table 2), indicating saturation of the MAb at about 7.5 μ l ascites, and suggesting that the anti-CYP1A2 MAb does not fully block the active site of CYP1A2, thus permitting some substrate binding and considerable metabolism of methoxychlor. Methoxychlor demethylation in two HLM samples (H030 from a single donor and HLM of pooled livers from 25 donors) was inhibited by anti-CYP2C9 MAb by 38 and 28%, respectively (table 1). This moderate inhibition of demethylation by anti-CYP2C9 did not appear to be due to the use of insufficient MAb, since saturation of inhibition was observed at levels of MAb, as low as 5 μ l ascites, in both the cDNA-expressed CYP2C9 and in HLM samples (table 2). However, the MAb anti-rat CYP2C that inhibit CYP2C8, 2C9, 2C18 and 2C19 activity (Gelboin *et al.* 1997) inhibited H030 and the HLM (pooled from 25 livers) activity by 66 and 57%, respectively, suggesting that the combined contribution of activity from CYP2C8, 2C18 and 2C19 was noteworthy. By contrast, MAb anti-CYP1A2 displayed only minimal, but consistent, inhibition of demethylation by MAb in both HLM samples (10 and 14% inhibition, respectively). These observations were followed by experiments to determine the effects of MAbs on the ratio of the *S/R*-enantiomers of mono-OH-M resulting from the demethylation of methoxychlor by HLM (see below).

Effects of inhibition of CYP2C9 and 1A2 on enantioselectivity of methoxychlor demethylation

The question of whether inhibition of CYP2C9 or 1A2 could significantly alter the ratio of the *S/R*-enantiomers of mono-OH-M resulting from the demethylation of methoxychlor by HLM was addressed. An initial experiment examined whether anti-CYP1A2 and anti-CYP2C9 MAb affect the apparent enantioselectivity of methoxychlor mono-demethylation by cDNA-expressed CYP1A2 and 2C9 in supersomes (data not shown). MAb anti-CYP2C9 had no effect on the ratio of the enantiomers of methoxychlor mono-demethylation yielding the same *S/R*

Table 1. Inhibition by monoclonal antibodies anti-P450s (MAb) of the cDNA-expressed P450s (CYPs) (supersomes) and human liver microsomes (HLM)-mediated catalysis of methoxychlor mono-*O*-demethylation.

	Cytochrome P450										
	1A2	2A6	2B6	2C8	2C9*1	2C18	2C19	2D6	3A4	HLM(H030)	HLM(pooled)
MAb ^a (clone no. of MAb)	Mono-OH-M formation (expressed as per cent inhibition by MAb)										
Anti-1A2 (26-7-5)	41 ± 0.1									10 ± 0	14 ± 0.5
Anti-2A6 (151-45-4)		96 ± 0.2								< 1	< 1
Anti-2B6 (49-10-20)			63 ± 6							6 ± 0.1	< 1
Anti-2C8 (281-1-1)				71 ± 1						3 ± 0.2	2 ± 0
Anti-2C9 (763-15-5)					77 ± 0.2					38 ± 2	28 ± 0.4
Anti-2C19 ^{b,c} (1-7-4-8)							92 ± 0.2			< 1	8 ± 0.2
Anti-rat2C ^d (1-68-11)				64 ± 1	83 ± 3	84 ± 2	86 ± 2			66 ± 2	57 ± 1
Anti-2D6 (512-1-8)								93 ± 0.4		2 ± 0.1	+3 ± 0.2 ^e
Anti-3A4/5 (3-29-9)									58 ± 5	+7 ± 0.1	+15 ± 0.8

^aIncubation with 1 mM NADPH, without regenerating system. A total of 5 µl ascites was used for anti-CYP2C19, 15 µl for anti-CYP1A2, 2A6, 2B6, 2C9, 2C and 2D6; 30 µl for anti-CYP2C8 and anti-CYP3A4/5. Values are the mean ± SD of triplicate incubations.

^bNovel CYP2C19 chemical inhibitor (+)*N*-3-benzylrivanol (Suzuki *et al.*, 2002) yielded only insignificant inhibition (about 10%) of methoxychlor demethylation by cDNA-expressed CYP2C19 (in a reaction involving methoxychlor 5 µM, (*S*)-benzylrivanol 1 µM, 15-min incubation).

^cPositive control experiments were indicated in catalysis of *S*-mephenytoin 4'-hydroxylation by CYP2C19 (supersomes) or by HLM when pronounced inhibition was observed by addition of MAb anti-CYP2C19. Indeed, there was a 97% inhibition of CYP2C19 catalysed hydroxylation, and 98 and 96% inhibition of the HLM (H030) and of pooled HLM catalysis, respectively.

^dAnti-ratCYP2C is against the human CYP2C8/2C9*1/2C9*2/2C9*3/2C18/2C19 (Gelboin *et al.*, 1997).

^ePlus sign before the number signifies increased activity. Non-specific antibody (Hy-Hel-9) representing 100% of activity (no inhibition). Control activity (mono-OH-M pmol/30 min/pmol P450, as mean ± SD for two or three replicates) for H030 (63.5 ± 2.1) and for pooled liver HLM (47.3 ± 4.2).

Table 2. Inhibition of CYP1A2 and 2C9*1 (supersomes) and HLM-catalysed methoxychlor *O*-demethylation by MAbs anti-CYP1A2, anti-CYP2C9 and anti-rat CYP2C.

MAbs anti-CYPs	Ascites fluid (μl)	Mono-OH-M (per cent inhibition by MAbs)			
		CYP1A2	CYP2C9*1	HLM(H030) ^a	HLM(pooled liver) ^b
Anti-1A2	7.5–45	36–42		< 10	< 14
Anti-2C9	5–45		75–78	36–48	28–31
Anti-2C	5–15		84–86	57–59	59

^{a,b}Control activity (mono-OH-M expressed as pmol/30 min/pmol P450, mean ± SD) for H030 is 81.0 ± 2.9 and for pooled sample from 25 livers is 60.8 ± 1.1.

Values are the range of inhibition by the increased amounts (see the range of ascites fluid, μl added) of MAbs; each value is a mean of duplicates.

ratio (5:95) of mono-OH-M as the control, while considerably inhibiting CYP2C9 activity in CYP2C9*1 supersomes by 77% (tables 1 and 2). By contrast, there was a small but consistent alteration in the ratio of *S/R*-enantiomers by MAb anti-CYP1A2 in the CYP1A2-catalysed reaction, from 9:91 (*S/R*) in control to 12:88 in the presence of anti-CYP1A2, independently of the levels of anti-CYP1A2 used (from 7.5 to 45 μl ascites). Apparently, the anti-CYP1A2 only partially blocked the active site (exhibiting approximately 40% inhibition of demethylation in cDNA-expressed CYP1A2); nevertheless, the binding of the MAb anti-CYP1A2 evidently altered the presentation of the prochiral methoxychlor methyls so that the hydroxylation on one of the methyl groups and the formation of the *S*-mono-OH-M was somewhat favoured.

As expected from the above results, both the anti-CYP2C9 and anti-rat CYP2C MAbs markedly altered the ratio of the *S/R*-mono-OH-M enantiomers from methoxychlor mono-demethylation by HLM (figure 2), causing a substantial decrease in the ratio of the *S/R*-enantiomers, apparently due to inhibition of the CYP2C-mediated demethylation that favours primarily the *S*-configuration. Additionally, a relatively small but consistent increase in the *S/R* ratio was observed with MAb anti-CYP1A2, apparently due to the small inhibition of the CYP1A2-mediated demethylation that largely favours the formation of the *R*-mono-OH-M (Hu and Kupfer 2002a, b). The effect of the increased levels of the MAbs on the apparent enantioselectivity (i.e. ratio of *S/R*-enantiomers) of HLM-mediated demethylation of methoxychlor was examined (figure 3). As observed with the inhibitory studies, low amounts of anti-CYP1A2 and anti-CYP2C9 MAbs (7.5 μl) were sufficient to alter maximally the ratio of *S/R*-enantiomers of methoxychlor mono-demethylation by HLM. However, with anti-rat CYP2C, apparently 5 μl was sufficient for saturation and to alter the ratio of *S/R* maximally.

Evidence against a key role of CYP2C19 in the catalysis of methoxychlor demethylation in HLM

Previous attempts with chemical inhibitors of CYP2C19 did not provide unequivocal information of whether CYP2C19 was involved in methoxychlor demethylation by HLM, primarily because the available chemical inhibitors were not sufficiently selective and inhibited CYP2C9 activity as well (Hu and Kupfer 2002b). Additionally, the newly reported potent CYP2C19 inhibitor, (+)*N*-3-benzylnirvanol, only minimally inhibited methoxychlor demethylation by cDNA-expressed CYP2C19 (table 1, footnote b). Therefore, it appeared that

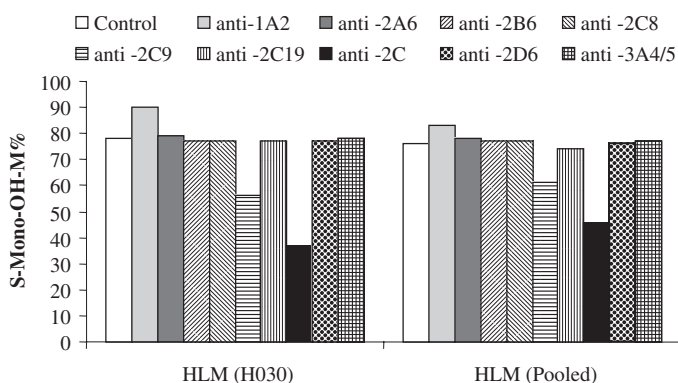


Figure 2. Effect of monoclonal antibodies on the *S/R*-enantiomeric ratio (%) of mono-OH-M resulting from methoxychlor mono-demethylation by human liver microsomes (HLM). Control activity (mono-OH-M pmol/30 min/pmol⁻¹ P450, mean $\pm$ SD of triplicate incubation) for H030 was 63.5 ± 2.1 and for pooled liver HLM was 47.3 ± 4.2 . Anti-CYP2C represents MAb raised against rat CYP2C and is referred to as anti-rat CYP2C (this MAb inhibits CYP2C8, 2C9, 2C18 and 2C19 activity). Values on the ordinate represent the per cent of the *S*-enantiomer, i.e. the value for the *R*-enantiomer is 100% minus the per cent of *S*. Only a single value of each datum point was given because after the isolation and quantification of the mono-OH-M by conventional reverse-phase HPLC from individual triplicate incubations, the mono-OH-M was pooled to obtain sufficient amounts of mono-OH-M for accurate analysis of the *R/S*-enantiomers on a chiral column.

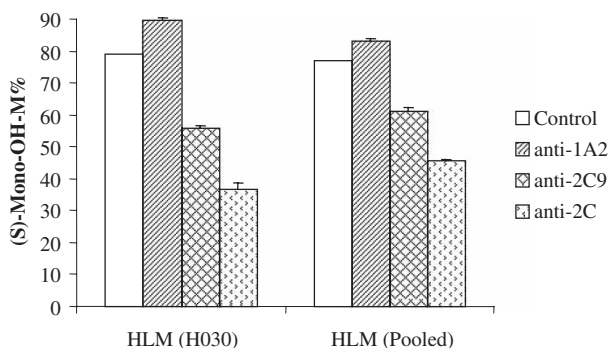


Figure 3. Effect of MAB anti-CYP1A2, anti-CYP2C9 and anti-rat CYP2C on the ratio (%) of formation of *S/R*-enantiomers of mono-OH-M resulting from methoxychlor *O*- demethylation in HLM. MAB was used in incremental concentrations of anti-CYP1A2 (i.e. 7.5–45 μ l, in four concentrations), anti-CYP2C9 (5–45 μ l, in five concentrations) and anti-CYP2C (5–15 μ l, two concentrations). The anti-CYP2C represents MAB raised against rat CYP2C that extensively inhibits methoxychlor *O*-demethylation by CYP 2C8, 2C9, 2C18 and 2C19 (activity assayed when expressed in supersomes). H030 represents HLM from a single donor; HLM pooled is from 25 livers. Control activity (mono-OH-M pmol/30 min/pmol⁻¹ P450, mean $\pm$ SD) in the absence of MAB for H030 was 81.0 ± 2.9 and for the Pooled sample was 60.8 ± 1.1 . Values are the mean $\pm$ SD of the inhibition or stimulation of the per cent of the *S*-mono-OH-M generated using varied amounts of the respective MAB. Each concentration was run in duplicate.

the monospecific inhibitory MABs would provide a more appropriate means to probe the role of CYP2C19 in methoxychlor demethylation in HLM. Surprisingly, MAB anti-CYP2C19 that extensively inhibited CYP2C19 activity in supersomes (when solely CYP2C19 was expressed) did not inhibit methoxychlor mono-demethylation in HLM and had no effect on the enantioselectivity (*S/R* ratio) of methoxychlor *O*-demethylation in HLM. The unlikely possibility that

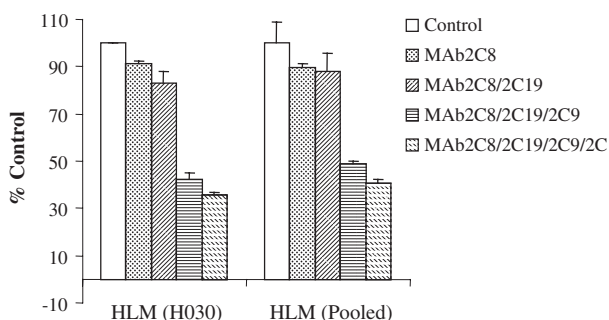


Figure 4. Combinatorial analysis of CYP2Cs in the inhibition of methoxychlor *O*-demethylation (mono-OH-M formation) by antibodies in HLM. Control activity (mono-OH-M pmol/30 min/pmol⁻¹ P450): for H030 (73.1 ± 0.2), for pooled liver (46.7 ± 4.1), values are given as 100%. Control: utilized Hy-Hel (non-specific MAb), 45 µl. Inhibition: total protein represented by 45 µl ascites fluid, including (but not necessarily) individual MAb: anti-CYP2C8, 15 µl, anti-CYP2C9, 10 µl, anti-CYP2C19, 5 µl; and anti-CYP2C, 15 µl (anti-CYP2C was raised against rat CYP2C); Hy-Hel was added to make up to 45 µl total. Each value is the mean of duplicate measurements ± SD, and is presented as the per cent of control activity.

the lack of inhibitory activity of the MAb anti-CYP2C19 in HLM was due to inactivity of these antibodies when exposed to the HLM environment was excluded, since these antibodies (the same lot) were highly effective by strongly inhibiting (by 96–98%) the hydroxylation of *S*-mephenytoin (a positive control for CYP2C19 activity) (table 1, footnote c).

Do other CYP2C enzymes catalyse methoxychlor demethylation?

To explore further whether other P450s of the CYP2C subfamily also catalyse methoxychlor demethylation, a combinatorial MAb analysis was used. Namely, MAbs anti-CYP2C8, anti-CYP2C19, anti-CYP2C9 and anti-rat CYP2C were added in a combinatorial manner (figure 4). Results demonstrate that MAb anti-CYP2C8 and anti-CYP2C19 each had only a minor inhibitory effect; however, the addition of anti-CYP2C9 markedly inhibited the demethylation reaction. Lastly, the addition of anti-rat CYP2C, an apparent 'universal' inhibitor of human CYP2Cs including 2C18, further lowered the demethylation activity. Since MAb anti-rat CYP2C inhibits all the human CYP2C activities (Gelboin *et al.* 1997), this increase in inhibition of demethylation by the addition of anti-rat CYP2C together with anti-CYP2C8/anti-CYP2C9/anti-CYP2C19 may be due to inhibition of CYP2C18 (for which currently there is no monospecific inhibitory MAb). Combinatorial analysis indicates that the major catalyst of the methoxychlor *O*-demethylation was CYP2C9; by contrast, other CYP2Cs (2C8/2C18/2C19), when assessed individually, appeared to be only minor contributors, but when assessed collectively, they represented a significant contribution. The combinatorial MAb analysis of the enantioselectivity of mono-demethylation of methoxychlor in HLM samples yielded similar results (figure 5). As expected, the control (no MAb) exhibited formation of about 80% of the *S*-mono-OH-M enantiomer versus the low formation of the *R*-enantiomer. MAbs anti-CYP2C8 and anti-CYP2C19 exhibited only little or no effect on the 'enantioselectivity' (*S*/*R* ratio of enantiomers). However, the addition of anti-CYP2C9 or of combined anti-CYP2C9 and anti-rat CYP2C markedly altered the *S*/*R* ratio of mono-OH-M apparently by diminishing the formation of the *S*-mono-OH-M.

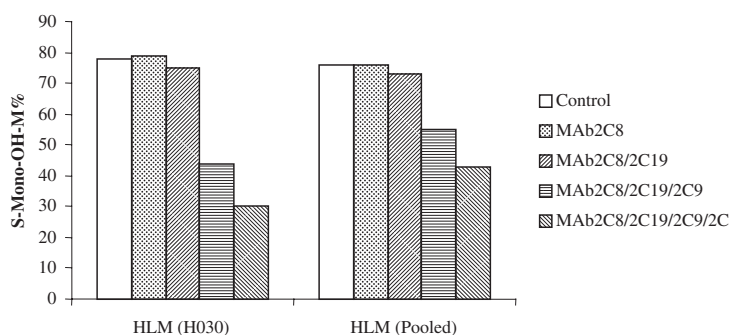


Figure 5. Combinatorial analysis of CYP2Cs affecting the ratio (%) of of *S/R*-enantiomers of mono-OH-M resulting from methoxychlor *O*-demethylation in HLM. Control activity (pmol/30 min pmol⁻¹ P450): for H030 (73.1 ± 0.2), for pooled liver (46.7 ± 4.1). Control: utilized Hy-Hel (non-specific MAb), 45 µl. Inhibition: total protein represented by 45 µl ascites, including (but not necessarily) individual monoclonal antibodies: anti-CYP2C8, 15 µl, anti-CYP2C9, 10 µl, anti-CYP2C19, 5 µl and anti-CYP2C, 15 µl (anti-CYP2C was raised against rat CYP2C). Hy-Hel was used to make up to 45 µl total. Values on the ordinate are the per cent of the *S*-enantiomer, i.e. the value for the *R*-enantiomer would be 100% minus the per cent *S*. Each value represents the isolation of the mono-OH-M metabolite from two incubations followed by the pooling of the metabolite and analysis of the enantiomers with an analytical chiral column (the pooling was done for achieving a sufficient size of sample for accurate analysis). Therefore, only a single value is given for each datum point.

Are the CYP2C9 allelic variants active in methoxychlor demethylation and, if so, do they elicit similar enantiomeric preferences?

CYP2C9 is known to exhibit six genetic allelic variants. However, because the CYP2C9*1 (Arg144, Ile359), 2C9*2 (Cys144) and 2C9*3 (Leu359) alleles were discovered first, those have been more thoroughly investigated than the more recently identified *4, *5 and *6 alleles. Previous findings demonstrated that the CYP2C9*2 and *3 alleles are associated with significant reductions in intrinsic clearance of a variety of CYP2C9 substrates compared with CYP2C9*1. However, the degree of these reductions appears to be substrate dependent (Lee *et al.* 2002 a, b).

The present authors' current findings demonstrated that CYP2C9*1 is the most important enzyme in HLM in catalysing the formation of mono-OH-M. Additionally, mono-OH-M is oestrogenic and chiral and there is a possibility that the mono-OH-M enantiomers differ in their affinity of binding for the ER-α and ER-β isoforms. In addition, the question of whether the induction of the CAR receptor and of CYP2B6 by mono-OH-M (Blizard *et al.* 2001) is equally or differently affected by the presence of individual *R*- or *S*-enantiomers has not been addressed. Similar questions can be raised with respect to the PXR receptor (Lehmann *et al.* 1998, Goodwin *et al.* 2002) that is known to control the induction of the various CYP3A forms. Hence, it was of interest to determine whether the 2C9 allelic variants effectively demethylate methoxychlor and whether their enantioselectivity of demethylation is the same or different from that of the wild-type CYP2C9*1. We observed that the three CYP2C9 variants, in cDNA-expressed P450 supersomes, demethylate methoxychlor (figure 6). Unexpectedly, CYP2C9*2 was about twofold more active than the wild-type CYP2C9*1. However, as expected from earlier observations, CYP2C9*3 was only minimally active. Subsequently, the enantioselective methoxychlor demethylation was examined by three HLM samples from single donors (H030, H103, HK27) with

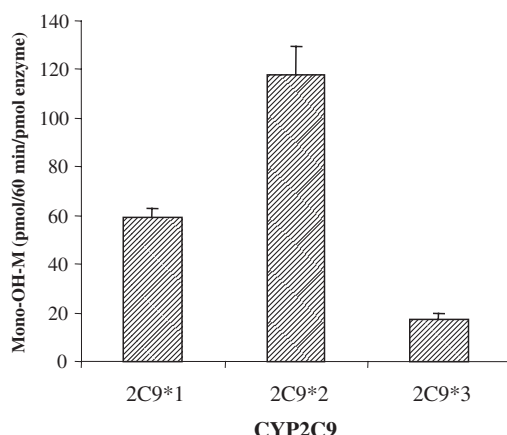


Figure 6. CYP2C9 polymorphism (genetic variations) in catalysis of methoxychlor *O*-demethylation activity (determination of the formation of mono-OH-M) in supersomes; the CYP2C9 variants were expressed without co-expressed cytochrome b₅. Values are the mean of duplicate determinations $\pm$ SD.

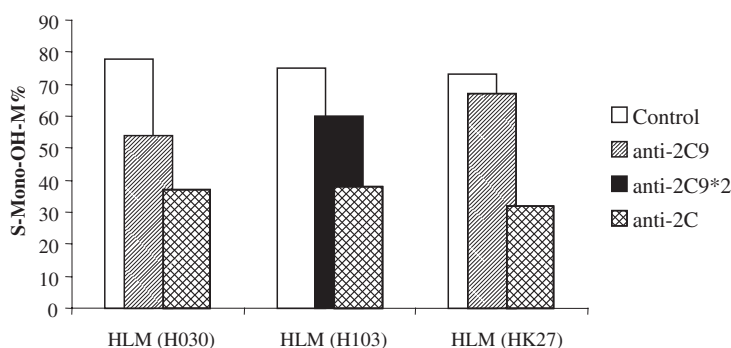


Figure 7. Role of HLM allelic variants of CYP2C9 in enantioselectivity of methoxychlor *O*-demethylation as affected by MAbs. Human liver microsomes (HLM) from donors H030 (CYP2C9*1), H103 (CYP2C9*2) and HK27 (CYP2C9*3); for the catalytic activities of P450s of these HLM, see table 5. Note: Anti-CYP2C represents MAb raised against rat CYP2C (inhibits CYP 2C8, 2C9, 2C18 and 2C19-mediated methoxychlor *O*-demethylation). Values on the ordinate are the per cent of the *S*-enantiomer, i.e. the value for the *R*-enantiomer would be 100% minus the per cent *S*. Each value represents analysis of the pooled metabolite from two incubations (the same rationale for this procedure as in figure 5). Control activity (mono-OH-M pmol/30 min pmol⁻¹ P450) = for H030 (81.0 ± 2.9), for H103 (38.2 ± 1.9) and for HK27 (66.3 ± 3.5). Hy-Hel MAb (non-specific-non-inhibitory MAb) was always used as in the control.

each expressing only one of the CYP2C9 allelic variants (see table 4 for analysis of the enzymatic activity of each of the HLM samples with substrates attributed to these P450 enzymes). Despite differences in demethylation activities, the HLM samples expressing the three allelic CYP2C9 variants exhibited identical enantioselectivity of demethylation (figure 7). It was also noted that the MAb anti-CYP2C9 (active against CYP2C9*1/2C9*2/2C9*3) inhibited catalysis of methoxychlor mono-demethylation of the HLM-H030 sample (expressing CYP2C9*1) by 35%, but did not inhibit that activity in HK27 (an HLM-expressing CYP2C9*3). MAb anti-CYP2C9*2 inhibited the methoxychlor demethylating activity of HLM 103 (expressing CYP2C9*2) by 45%. By contrast, anti-rat CYP2C inhibited the

demethylation catalysis by all three CYP2C9 alleles in HLM; the inhibition of HLM-H030 (CYP2C9*1), H103 (CYP2C9*2) and HK27 (CYP2C9*3) was 66, 65 and 61%, respectively (table 3), indicating that CYP2C9*1 and 2C9*2 are the major P450s catalysing methoxychlor demethylation. Other CYP2Cs (2C8/2C18/2C19) in combination also appeared catalytically significant in the HLM H030 and H103 samples. However, CYP2C9*3 was not important in catalysis of the methoxychlor demethylation reaction (see above under results with cDNA-expressed 2C9*3). Consequently, the combined activity of CYP2C8/2C18/2C19 appeared responsible for methoxychlor demethylation in the HK27 sample expressing CYP2C9*3. Thus, studies with methoxychlor demethylation support the earlier findings that CYP2C9*3 is catalytically inactive or is much less active

Table 3. Effect of CYP2C9 polymorphism on methoxychlor mono-*O*-demethylation by CYP2C9 allelic variants (supersomes) and human liver microsomes (HLM).

Mabs anti-CYPs	Mono-OH-M (per cent Inhibition by MAb) ^a					
	2C9*1	2C9*2	2C9*3	HLM (H030) ^c (CYP2C9*1)	HLM (H103) ^c (CYP2C9*2)	HLM (HK27) ^c (CYP2C9*3)
Anti-2C9	80 ± 1		86 ± 3	35 ± 3		+3.5 ± 0.1 ^b
Anti-2C9*2		96 ± 1			45 ± 0.2	
Anti-rat 2C	83 ± 3	95 ± 1	75 ± 3	66 ± 2	65 ± 2	61 ± 2

Control activity (mono-OH-M pmol/30 min/pmol P450, mean ± SD) = for H030 (81.0 ± 2.9), for H103 (38.2 ± 1.9) and for HK27 (66.3 ± 3.5). Hy-Hel MAb (non specific-non-inhibitory MAb) was always used as a control.

Amounts of MAB used: anti-CYP2C9 (30 µl), anti-CYP2C9*2 (30 µl) or anti-ratCYP2C (15 µl).
^aValues are the per cent inhibition by MABs.
^bPlus sign before the number indicates increased enzymatic activity elicited by MAB's. (i) In H030 and H103, CYP 2C9*1 and 2C9*2 are the major P450s for methoxychlor demethylation; the other CYP2Cs (combined 2C8/2C18/2C19) are also important; (ii) in HK27, CYP2C9*3 is inactive, however, the combined activities of CYP2Cs (2C8/2C18/2C19) appears responsible for the observed demethylation. H103 is not active, except for the CYP2C9 in it, which is active, HK27 has low CYP2C9 activity (because of the presence of the 2C9*3 allele), but the other P450 activity (CYP1A2, 2C8, 2C19) is higher than in H103; unfortunately, data for CYP2C18 are missing for all HLMs.
^cFor the catalytic activity of the various CYPs and especially for mephenytoin-4'-hydroxylase activity (the latter reflecting CYP2C19 activity) in the HLM, see table 4.

Table 4. Characteristics of the human liver microsomes (HLM) from individual donors (H030, H103, HK27) obtained from BD-Gentest Co. (Gentest website).

Cytochrome P450	Assay	H030 (CYP2C9*1)	H103 (CYP2C9*2)	HK27 (CYP2C9*3)
Total P450 ^a	Omura and Sato	520	350	300
1A2 ^b	phenacetin <i>O</i> -deethylase	2600	340	1300
2C8	paclitaxel 6α-hydroxylase	390	60	180
2C9	diclofenac 4'-hydroxylase	5900	2200	480
2C19	(S)-mephenytoin 4'-hydroxylase	310	21	460

^apmol P450mg⁻¹ of total protein.
^bAll cytochrome P450 assays in the HLM were conducted at 0.8mg⁻¹ protein/ml with an NADPH-generating system (1.3mM NADP, 3.3mM glucose 6-phosphate, 0.4U ml⁻¹ glucose 6-phosphate dehydrogenase), 3.3mM MgCl₂, and incubated for 10 or 20 min (CYP2C8, 2C9). Potassium phosphate buffer (0.1 M, pH 7.4) was generally used, except for CYP2C19 (0.05 M buffer) and CYP2C9 in which 0.1 M Tris, pH 7.5, was used instead. Activities are expressed as pmol product mg⁻¹ protein min⁻¹.

than CYP2C9*1. However, the case for CYP2C9*2 is less clear in view of the observation that CYP2C9*2 is more active than CYP2C9*1 in methoxychlor demethylation, an observation contrary to previous findings with other substrates that CYP2C9*2 is substantially less active than CYP2C9*1. Although the intrinsic clearance ($V_{\max}/K_m$) of methoxychlor for the various allelic variants was not determined, it appears that the present findings confirm previous observations that the diminished catalytic activity of the allelic variants largely depends on the substrate used (Van der Weide 2001, Lee *et al.* 2002a, b).

Conclusions

Both the inhibition studies of methoxychlor demethylation and alteration of the ratio of *S/R*-enantiomers of mono-demethylation by HLM, obtained with various anti-CYP MAbs, indicated that CYP2C enzymes were the principal P450s in HLM in the catalysis of mono-demethylation of methoxychlor to form the *S*-mono-OH-M enantiomer. Combinatorial analysis of MAbs demonstrated that CYP2C9 is the most important enzyme in HLM catalysing methoxychlor mono-demethylation. Other CYP2Cs, including CYP2C18, exhibited a minor role in the catalysis of mono-demethylation. However, the collective activities of three CYP2C enzymes (CYP2C8, 2C18, 2C19) appear to contribute significantly to methoxychlor demethylation (figure 8); this is also supported by the difference in inhibition observed between anti-CYP2C9 and anti-rat CYP2C.

Although anti-CYP1A2 MAbs did not appreciably inhibit the CYP1A2-mediated demethylation of methoxychlor in HLM, nevertheless the MAbs altered the *S/R*-enantiomeric ratio of mono-OH-M resulting from methoxychlor monodemethylation, suggesting that CYP1A2 was important in HLM in supporting catalysis of methoxychlor demethylation to form the *R*-mono-OH-M (figure 8). It is speculated that the anti-CYP1A2 MAbs bind to the active site of CYP1A2, but nevertheless permit considerable demethylation by CYP1A2 supersomes (about 60% of control), resulting in a consistent but small change in the *S/R*-enantiomeric ratio of 9:91 (in control) to 12:88 (in the inhibited).

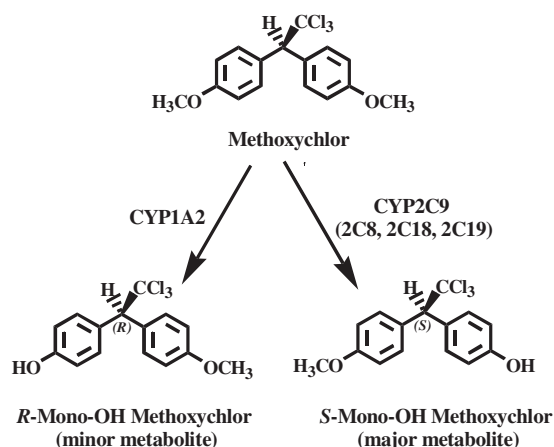


Figure 8. Proposed roles of CYP1A2, CYP2C9 and of other human CYP2C enzymes in enantioselectivity of methoxychlor *O*-demethylation in human liver microsomes.

Of the various P450s examined in methoxychlor mono-demethylation, CYP2C19 was the most active enzyme (when present in supersomes). However, this enzyme appears devoid of that activity when present in HLM. This was shown by the minimal or absent inhibition of methoxychlor mono-demethylation by MAbs anti-CYP2C19 and is supported by a positive control experiment (involving hydroxylation of *S*-mephenytoin in HLM) in which that activity, known to be specific for CYP2C19, was almost totally inhibited by the same clone of MAb anti-CYP2C19, which did not inhibit methoxychlor demethylation.

The examination of polymorphic CYP2C9 allelic variants in the catalysis of methoxychlor demethylation (using single CYP2C9 variants: CYP2C9*1, 2C9*2 and 2C9*3 expressed in supersomes) showed that CYP2C9*2 was the most active (being more active than the wild-type CYP2C9*1) and that CYP2C9*3 was essentially inactive. Similarly, studies with HLM expressing these allelic variants demonstrated that CYP 2C9*1 and 2C9*2 were important in that catalysis, whereas CYP2C9*3 was relatively inactive. With respect to the latter, it appears that in the absence of catalysis by CYP2C9*3 in HLM (HK27), other CYP2C forms collectively catalyse methoxychlor demethylation.

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Ritonavir and dexamethasone induce expression of CYP3A and P-glycoprotein in rats

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1. The consequences of extended exposure to the human immunodeficiency viral protease inhibitor ritonavir (RIT) on the expression and function of CYP3A isoforms in the liver and in enteric mucosal cells, and on the expression of the efflux transport protein P-glycoprotein (P-gp) in enteric mucosa and in brain microvessel endothelial cells, were evaluated in rat. Dexamethasone (DEX), a known inducer of CYP3A and P-gp in rodents, served as a positive control.

2. Male CD-1 rats received RIT (20 mg kg⁻¹), DEX (80 mg kg⁻¹) or vehicle by oral/duodenal gavage once daily for 3 days.

3. Compared with vehicle control, CYP3A activity in liver microsomes (intrinsic clearance for triazolam hydroxylation *in vitro*) was increased by a factor of 2–4 by RIT, and by 10–14-fold by DEX. Similar increases were observed in expression of immunoactive CYP3A protein. Overall, maximum reaction velocity and immunoactive protein were highly intercorrelated ($r^2 = 0.89$). Both RIT and DEX also increased function and expression of enteric CYP3A, although to a more modest extent (about 1.7-fold for RIT, about 3.3-fold for DEX).

4. Enteric P-gp expression was equally induced (by 2.8-fold) by both RIT and DEX. P-gp expressed in brain microvessel endothelial cells was increased by a factor of 1.3 by both compounds.

5. Thus, increased expression of CYP3A isoforms and of P-gp occurs with 3 days of exposure to RIT in rats. Qualitatively similar changes occur in human cell culture models and in clinical studies, and might contribute to drug interactions involving RIT (and other antiretroviral agents) in humans.

Introduction

Treatment of human immunodeficiency virus (HIV) infection with highly active antiretroviral therapies (HAART) is complicated by pharmacokinetic drug interactions involving HAART medications (Tseng and Foisy 1997, Piscitelli and Gallicano 2001). The HIV protease inhibitor ritonavir is among the most complex (Hsu *et al.* 1998b). Initial or short-term exposure to ritonavir causes extensive inhibition of hepatic and enteric CYP3A function, as well as inhibition of efflux transport mediated by P-glycoprotein (P-gp) (von Moltke *et al.* 1998, Perloff *et al.* 2002). This property can be exploited for therapeutic purposes when ritonavir is coadministered with other protease inhibitors (such as lopinavir or saquinavir) for 'pharmacokinetic augmentation' (Merry *et al.* 1997, Hsu *et al.* 1998a, Sham *et al.* 1998, Flexner 2000). However, initial ritonavir co-treatment may also cause large and potentially hazardous increases in plasma levels of other medications, such as

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triazolam, alprazolam or trazodone (Greenblatt *et al.* 1999, 2000a, b, 2003). With extended exposure, ritonavir becomes an inducer of CYP3A and P-gp expression. The net effect of ritonavir on drug disposition, reflecting a combination of inhibition and induction, may be time-dependent and difficult to anticipate or predict (Venkatakrishnan *et al.* 2001).

Because of the complexity of pharmacokinetic drug interactions with ritonavir (and other HAART treatments) in humans, animal models have been used to enhance understanding of such interactions. Rodent models are most commonly used for this purpose (Lin *et al.* 1999, Yamaji *et al.* 1999, Huang *et al.* 2001). Whereas findings cannot be assumed to apply directly to humans, rodent studies may provide insight into the magnitude and time-course of HAART effects on CYP3A and P-gp expression and function, as well as differential effects on protein expression.

The present study evaluated the effects of ritonavir on hepatic and enteric CYP3A expression and function, as well as enteric and blood–brain barrier expression of P-gp in rats. Dexamethasone, known to induce both CYP3A and P-gp in rats (Salphati and Benet 1998, Lin *et al.* 1999, Cotreau *et al.* 2001, Yumoto *et al.* 2001), was used as the positive control.

Materials and methods

Materials

Triazolam (TRZ), α -OH-triazolam (α -OH-TRZ), and 4-OH-triazolam (4-OH-TRZ) were kindly provided by Pharmacia and Upjohn (Kalamazoo, MI, USA) or purchased from Sigma Chemical Co. (St Louis, MO, USA). Ketoconazole (KTZ) was a gift from the Janssen Co. (Beerse, Belgium). Ritonavir (RIT) was kindly provided by Abbott Laboratories (Chicago, IL, USA). Other drugs, chemicals and reaction cofactors (NADP⁺, DL-isocitric acid, magnesium chloride, isocitric dehydrogenase, and potassium phosphate buffer solutions) were obtained from Sigma. A polyclonal antibody to rat CYP3A1 and control serum developed in rabbit were obtained from Xenotech (Phoenix, AZ, USA). As indicated by the manufacturer, this polyclonal antibody immunoblots rat, mouse and human CYP3A proteins with no detectable cross reactivity to other CYP families.

Rat oral gavage with ritonavir and dexamethasone

Male Sprague–Dawley rats, 10–12 weeks old, were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were maintained on a 12-h light/dark cycle and given food (Purina rodent chow, Purina, St Louis, MO, USA) *ad libitum*, for 1 week in on-site animal facilities before beginning experimental paradigms. Pure RIT (50 mg ml⁻¹) and dexamethasone (DEX, 200 mg ml⁻¹) were dissolved in 50% ethanol and 50% polypropylene glycol (PPG). Rats (250–300 g) were given 20 mg kg⁻¹ RIT, 80 mg kg⁻¹ DEX, or vehicle (VEH; 50% ethanol, 50% PPG) daily for 3 days by oral/duodenal gavage of 100–125 μ l drug solution adjusted for animal weight. After a 1 day washout, animals were sacrificed by decapitation. Intestine and liver were immediately removed less than 10 min after death. The liver was immediately frozen on dry ice for later microsomal preparation. Intestine, 30 cm distal from the pyloric sphincter, was isolated and intestinal microsomes were prepared immediately. During intestinal microsome preparation, brains were removed from rats and stored in physiological buffer with 5 mM EDTA at 4°C until later isolation of rat microvessel endothelial cells. Initially each treatment group had eight animals (total of 24 animals). Through the course of study, four animals were lost (2 DEX, 1 RIT, 1 VEH) due to inadvertent death or experimental error.

Liver microsomal preparation

All liver 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 (pH 7.56) containing 20% glycerol and stored at –80°C until use. Total protein concentration was determined by a bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard.

Intestinal microsomal preparation

Rat intestinal microsomes were prepared as described (Cotureau *et al.* 2001, Kotegawa *et al.* 2002, Perloff *et al.* 2003). In brief, all tissue and reagents were kept at 4°C, and 40 µg ml⁻¹ phenylmethylsulfonyl fluoride was used throughout the procedure. Immediately after decapitation, beginning at the pyloric sphincter, 30 cm intestine were 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 to 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 to 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 rat. The cell fraction was then pelleted and resuspended in solution C (5 mM histidine, 0.25 M sucrose, 0.5 M EDTA, pH to 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.

Isolation of rat brain microvessel endothelial cells

Endothelial cells were purified from rat brain using a method developed from previous descriptions (Dallaire *et al.* 1991, Tsuji *et al.* 1992, Jette *et al.* 1993). Rats were decapitated and brains were removed and cleared of superficial blood vessels and the meninges. The cerebellum, hind and mid-brain were removed and the remaining cortex was halved and washed twice in cold (4°C) physiological buffer (147 mM NaCl, 4 mM KCl, 3 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, 15 mM Hepes, pH to 7.4). Beginning with one-half of the cortex, the cortex was homogenized at 6000 rpm for 15 s with a polytron homogenizer in 5 ml buffer (containing 5 mM EDTA to prevent proteolysis) at 4°C. The homogenate was then combined with 2 equal vols 30% dextran T-70 in buffer. The emulsion was then vortexed vigorously and centrifuged at 25 000g for 10 min at 4°C. The pellet of crude microvessels was then resuspended in 5 ml buffer and homogenized in a mini-hand homogenizer and passed through a 180 µm nylon mesh. The filtrate was centrifuged at 25 000g for 10 min at 4°C. The microvessel endothelial cell pellet was then resuspended in lysis buffer (0.5% deoxycholate, 1% triton-X-100 in 50 mM KH₂PO₄) with use of a mini-hand homogenizer. The homogenate was transferred to minicentrifuge tubes and allowed to incubate and shake for 30 min at 4°C. The samples were then vortexed and centrifuged at 8000g for 8 min at room temperature. The supernatant, to be used for Western blotting, was collected and total protein concentration was determined by a BCA protein assay.

Incubation procedures

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). Triazolam (TRZ) in methanol solution was aliquoted into incubation tubes to yield desired final concentrations, and the solvent was evaporated to dryness at 40°C under mild vacuum. Incubations were performed at 0–750 µM TRZ to establish kinetic parameters for metabolite formation in rat liver (Perloff *et al.* 2000, Cotureau *et al.* 2001). Studies of inhibition by ethanol and PPG were performed using 250 µM TRZ, with removal of an equal volume of 50 mM phosphate buffer for the volume of ethanol or PPG tested. Reaction mixtures with both liver and intestinal microsomes were incubated at a protein concentration of 0.1 mg ml⁻¹. Formation of TRZ metabolites with both liver and intestinal microsomes was linear with respect to incubation time (to 30 min) and microsomal protein (to 0.5 mg ml⁻¹) concentration.

Incubations were initiated by the addition of microsomal protein. After 20 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 high-performance liquid chromatography (HPLC) analysis. All kinetic samples were performed 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 TRZ substrate were performed concurrently to validate cytochrome P450 (CYP)-dependent metabolism. The identity of metabolites was verified by comparing HPLC retention time with authentic standards.

Immunoinhibition of metabolism

Serum from rabbits containing polyclonal anti-CYP antibodies has previously been shown to inhibit the activity of human and rat CYPs in a concentration-dependent manner (Kronbach *et al.* 1989, Schmider *et al.* 1996, Perloff *et al.* 2000, 2003). Additionally, TRZ hydroxylation in mouse liver microsomes has been shown to be CYP3A dependent (Perloff *et al.* 2000, Warrington *et al.* 2000).

Antiserum to CYP3A1 was tested for activity against TRZ hydroxylation in rat liver microsomes from each drug treatment group at a single polyclonal serum to microsomal protein ratio (10:1). In preliminary experiments, 20:1 ratios were also tested, demonstrating a minimal increase in inhibition over 10:1 ratios. In each experiment, 250 μ M TRZ and 10 μ g microsomal protein were pre-incubated for 30 min at 37°C with 0 or 100 μ g anti-CYP3A1 serum protein from immunized rabbits, or the same quantities of control pre-immune serum as a negative control. Incubations were initiated by the addition of cofactor solution and stopped after 20 min with cooling and acetonitrile as described above.

HPLC analysis

For TRZ, α -OH-TRZ, and 4-OH-TRZ, 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 (von Moltke *et al.* 1996). 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 Western blotting for CYP3A

Western blot procedures were similar to those described (Perloff *et al.* 2000, 2003, Warrington *et al.* 2000). Microsomal protein was separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% polyacrylamide gels. Sample wells were loaded with 0.05–5 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 (pH 8.3). Samples were then transferred to polyvinylidene difluoride (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.

Quantitative Western blotting for P-glycoprotein

Western blot procedures were similar to those described (Perloff *et al.* 2001). A total of 25–50 μ g rat intestinal microsomes or a 3 μ g microvessel endothelial cell sample were loaded into each well. Highly induced/concentrated LS-180 V cell preparations (Perloff *et al.* 2001) were used to generate a relative standard curve at various known total protein concentrations (band signal was linear over a 20-fold concentration range). The calibration curve allowed for comparison of relative P-gp content among samples. Determination of absolute P-gp content requires a pure P-gp standard that is currently not available. Sample protein was separated by SDS-PAGE (100 V constant) on a 4–15% gradient polyacrylamide gel at 4°C for 2 h. Samples were then transferred to PVDF membrane for 1.5 h at 100 V in 25 mM Tris buffer (pH 7.8)/20% methanol (all buffers were the same as those used for CYP3A Western blotting). Membranes were immunoblotted using monoclonal mouse anti-human MDR1 (C219, 1:1000) for 24 h. Blots were then probed with the secondary antibody, sheep anti-mouse Ig-horseradish peroxidase.

Western blot luminescence and quantification

After incubation with secondary antibody, a Super Signal Cl-HRP Substrate System (Pierce) was used to activate the horseradish peroxidase 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 or μ g LS180 V cell preparation was created and fit to a linear function.

Data analysis

Kinetic parameters for TRZ biotransformation, without addition of inhibitors, were determined by non-linear least-squares regression analysis of untransformed data. Formation of all metabolite formation data was fit to a substrate activation (Hill) model (equation 1) (Segel 1975, Venkatakrishnan *et al.* 2001):

$$V = (V_{\max} \cdot S^a) / (S_{50}^a + S^a), \quad (1)$$

where V is the reaction velocity (nmoles mg protein⁻¹ min⁻¹), $V_{\max}$ is the maximum reaction velocity, S is substrate concentration (μ M), S_{50} is the substrate concentration (μ M) at which $V = 0.5 V_{\max}$ and 'a' is the Hill coefficient.

Values of IC₅₀ (concentration corresponding to a 50% reduction in metabolite formation velocity) for ethanol and PPG were determined by non-linear regression of relative reaction velocities at a single substrate concentration in the presence of varying inhibitor concentrations. These functions (equations 2 and 3) are based on the Hill equation (Segel 1975), and can be used for an empirical determination of

IC₅₀ (Venkatakrishnan *et al.* 1998, 2001):

$$f = 100[1 - I_{\max}I^b/(I^b + IC^b)] \quad (2)$$

$$IC_{50} = (IC)/[(2I_{\max} - 1)^{1/b}], \quad (3)$$

where f is the 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 the $I_{\max}$, IC_{50} is the inhibitor concentration that corresponds to 50% of the uninhibited control ($IC_{50} = IC$, when $I_{\max} = 100\%$), and 'b' is the exponent in IC_{50} determination.

Results

P-gp expression in rat intestine

Both RIT (20 mg kg⁻¹) and DEX (80 mg kg⁻¹) caused a 2.8-fold increase in P-gp intestinal expression (figure 1) following the 3-day drug exposure regimen. DEX treatment, employed as a positive control, caused P-gp induction similar to previous studies (Lin *et al.* 1999).

P-gp expression in rat microvessel endothelial cells

Both RIT and DEX caused an about 30% increase in rat microvessel endothelial cell expression of P-gp (figure 2) following the 3-day drug exposure regimen. DEX treatment, employed as a positive control, caused P-gp induction similar to previous studies (Regina *et al.* 1999).

Metabolism of triazolam in liver and intestinal microsomes

Kinetic parameters for TRZ hydroxylation (figure 3) in pooled liver microsomes from rats following the 3-day drug exposure regimen are presented in table 1. Individually, all liver and intestinal microsomes were screened for activity at 250 μM TRZ, a substrate concentration that corresponds to metabolite formation rates close to the $V_{\max}$. In liver microsomes, α -OH-TRZ formation increased 2.7- and 10.1-fold over VEH controls in RIT- and DEX-treated rats, respectively; and 4-OH-TRZ formation increased 3.7- and 14.6-fold over VEH controls in RIT- and DEX-treated rats, respectively (table 2). In intestinal microsomes, α -OH-TRZ formation increased 1.7- and 1.9-fold over VEH controls in RIT- and DEX-treated rats, respectively; and 4-OH-TRZ formation increased 2.0- and 2.3-fold over VEH controls in RIT- and DEX-treated rats, respectively (table 2). TRZ metabolite formation rate in liver microsomes from VEH treated and naïve rats were very similar (data not shown), however TRZ metabolite formation rate in intestinal microsomes from VEH-treated rats was reduced compared with untreated rats. In previous studies (Cotureau *et al.* 2001, Kotegawa *et al.* 2002) absolute CYP3A-mediated metabolic activity in rat intestinal microsomes was 50–100% of that seen in liver microsomes. In microsomes from rats pretreated with VEH (50% ethanol, 50% PPG), intestinal microsomes only had approximately 20% the CYP3A-mediated metabolic activity of liver microsomes. This effect might be attributable to local inhibitory effects of VEH on CYP3A function in rat intestine.

Antibody and chemical inhibition

CYP3A-mediated TRZ metabolism was verified by co-incubating anti-CYP3A1 polyclonal antibody serum with rat liver microsomes (10:1 protein ratios) from RIT-, DEX- and VEH-treated rats with 250 μM TRZ. Whereas

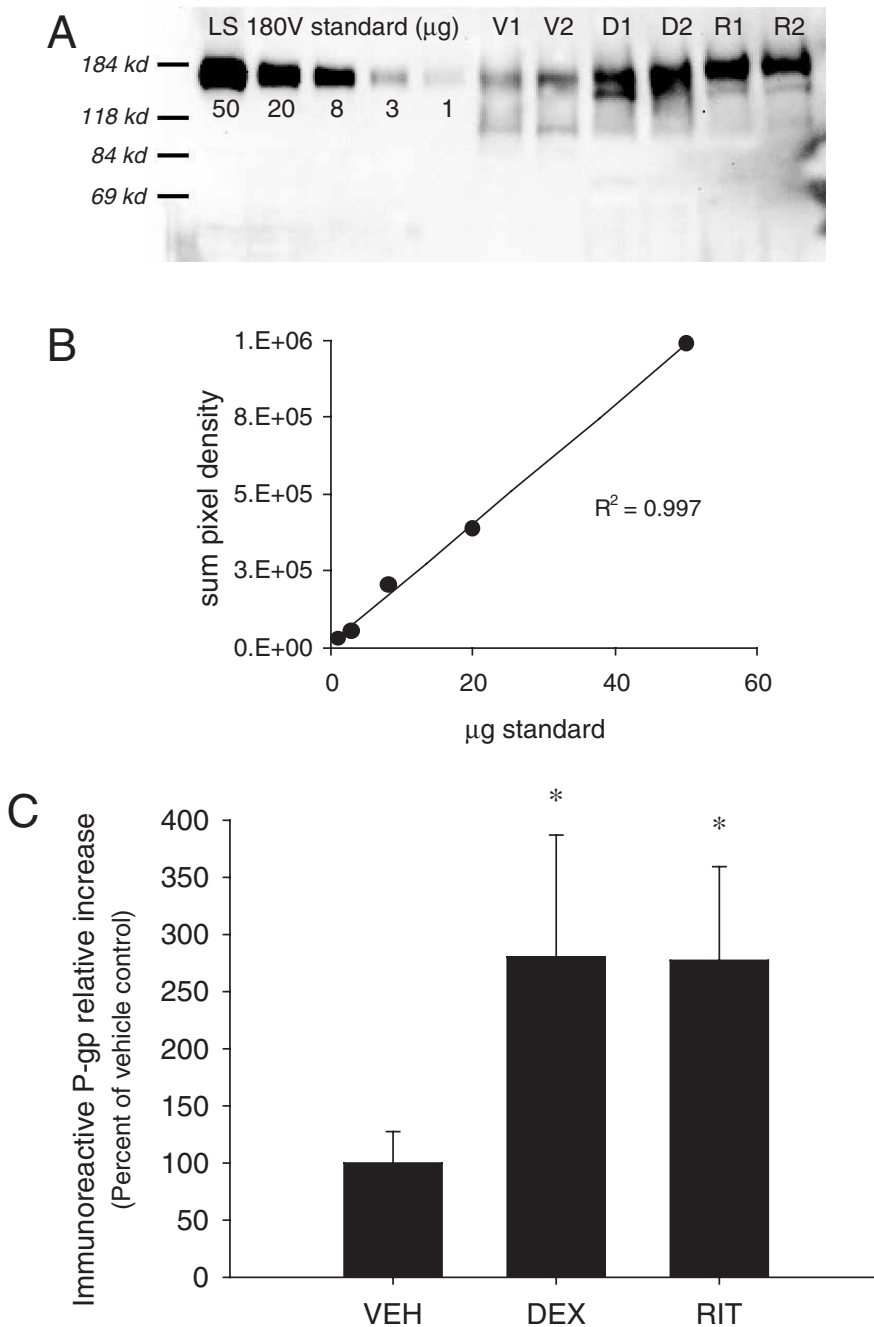


Figure 1. Intestinal P-gp induction. Rats were administered 20 mg kg^{-1} ritonavir, 80 mg kg^{-1} dexamethasone or vehicle alone (50% ethanol, 50% PPG) once daily for 3 days by oral gavage. After 1 day washout, intestinal microsomes were prepared. Immunoreactive protein was detected via Western blotting, loading $50 \mu\text{g}$ of each sample (A: V, vehicle; D, dexamethasone; R, ritonavir), and quantified with computer image analysis by comparison with a standard curve (B). Mean immunoreactive protein detected ($n=5$ or 6 , $\pm$ SD) is expressed as a per cent of vehicle control (C). *Significant differences from the vehicle control ($p < 0.01$) as determined by Student's t -test.

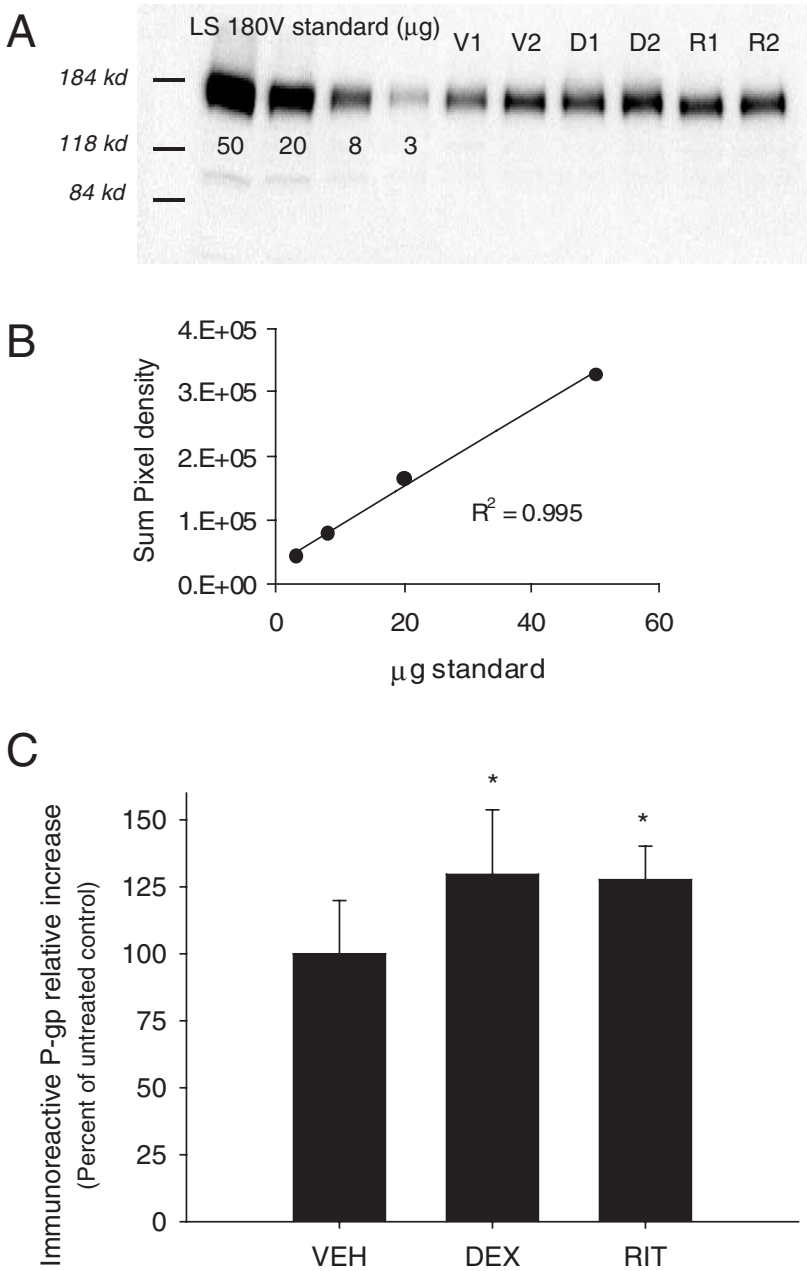


Figure 2. Brain endothelial cell P-gp induction. Rats were administered 20 mg kg^{-1} ritonavir, 80 mg kg^{-1} dexamethasone or vehicle alone (50% ethanol, 50% PPG) once daily for 3 days by oral gavage. After 1 day washout, brain microvessel endothelial cells were prepared. Immunoreactive protein was detected via Western blotting, loading $3 \mu\text{g}$ of each sample (A: V, vehicle; D, dexamethasone; R, ritonavir), and quantified with computer image analysis by comparison with a standard curve (B). Mean immunoreactive protein detected ($n=6$ or 7 , $\pm$ SD) is expressed as a per cent of vehicle control (C). *Significant differences from the vehicle control ($p < 0.05$) as determined by Student's t -test.

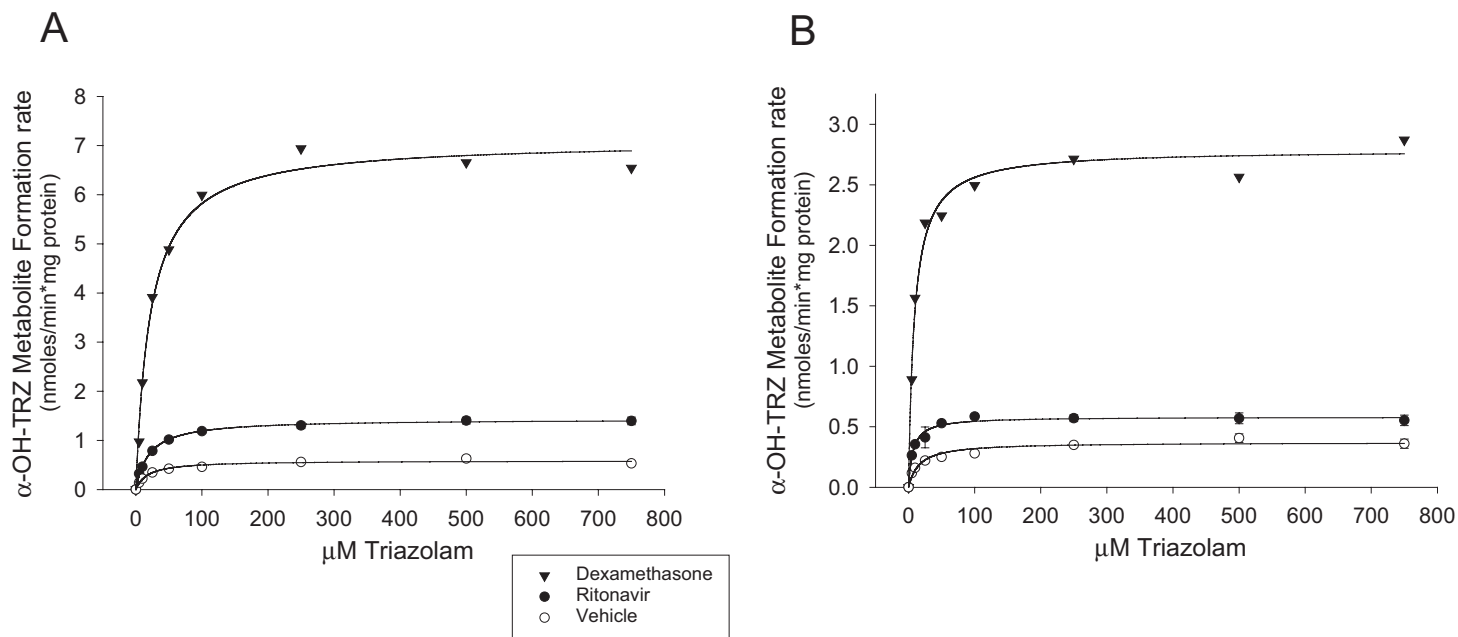


Figure 3. Metabolism of triazolam in liver microsomes. Rates of formation of 4-OH-TRZ (A) and α -OH-TRZ (B) in relation to concentrations of triazolam by liver microsomal preparations from rats previously given 80 mg kg^{-1} dexamethasone (triangles), 20 mg kg^{-1} ritonavir (dark circles), or vehicle alone (open circles, 50% ethanol, 50% PPG) once daily for 3 days by oral gavage. Pooled microsomes of all animals in each group ($n = 6$ or 7) were assayed in triplicate; data points are the mean. Lines represent functions determined by non-linear least-squares regression analysis (equation 1).

Table 1. Biotransformation kinetic parameters of triazolam in liver microsomes from rats pretreated with ritonavir, dexamethasone or vehicle.

	α -OH-TRZ formation	4-OH-TRZ formation
Vehicle (50% ethanol, 50% PPG)		
$V_{\max}^{\dagger}$	0.37	0.59
$S_{50}^{\#}$	16.2	17.9
$V_{\max}/S_{50}$ ratio $^{\phi}$	0.022	0.033
Ritonavir (20 mg kg ⁻¹)		
$V_{\max}$	0.58	1.43
S_{50}	6.6	20.2
$V_{\max}/S_{50}$ ratio	0.088	0.071
Dexamethasone (80 mg kg ⁻¹)		
$V_{\max}$	2.78	7.1
S_{50}	8.9	21.9
$V_{\max}/S_{50}$ ratio	0.312	0.324

Values are the mean of triplicate assays from pooled microsomes.

† nmol min⁻¹ mg protein⁻¹; $^{\#}$ μM; $^{\phi}$ μl min⁻¹ mg protein⁻¹.

Table 2. Biotransformation of triazolam (250 μM) in liver and intestinal microsomes from rats pretreated with ritonavir, dexamethasone, or vehicle.

	α -OH-TRZ formation	4-OH-TRZ formation
Vehicle (50% ethanol, 50% PPG)		
Liver microsomes, n = 7	0.29 ± 0.03	0.47 ± 0.16
Intestinal microsomes, n = 6	0.046 ± 0.040	0.089 ± 0.054
Ritonavir (20 mg ⁻¹ kg ⁻¹)		
Liver microsomes, n = 7	0.79 ± 0.29	1.73 ± 0.40
Intestinal microsomes, n = 6	0.090 ± 0.034	0.155 ± 0.061
Dexamethasone (80 mg ⁻¹ kg ⁻¹)		
Liver microsomes, n = 6	2.92 ± 0.52	6.87 ± 2.23
Intestinal microsomes, n = 5	0.108 ± 0.089	0.170 ± 0.133

Values are the mean (±SD) Units are nmol min⁻¹mg protein⁻¹.

pre-immune serum had a minor inhibitory effect, serum containing anti-CYP3A1 antibodies reduced TRZ metabolite formation to approximately 20% of control values in rat liver microsomes pretreated with VEH and to 10% in rat liver microsomes pretreated with RIT or DEX (figure 4), demonstrating high CYP3A dependence in all cases. As an additional verification of CYP3A-mediated metabolism, KTZ (5 μM) reduced TRZ metabolite formation (tested at 250 μM TRZ) to less than 15% of control values in all rat microsomes (liver and intestinal in the various pretreatment groups, data not shown). To assess whether VEH content (ethanol and PPG) could cause local inhibition of TRZ metabolism, IC₅₀ analysis of ethanol and PPG in rat liver and intestinal microsomes (from naïve rats, tested at 250 μM TRZ) were performed, testing ethanol and PPG at increasing percentages of the incubation volume (figure 5). Ethanol inhibited formation of both metabolites with IC₅₀ values of 3.4 and 2.0% in rat liver and intestinal microsomes, respectively. PPG inhibited TRZ metabolite formation with IC₅₀ values of 5.9 and 1.2% in rat liver and intestinal microsomes, respectively.

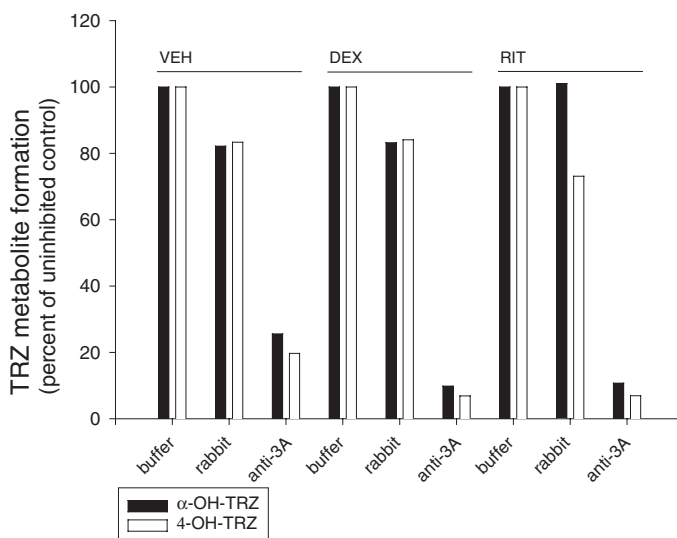


Figure 4. Inhibition of triazolam biotransformation by anti-CYP3A1 antibodies in liver microsomes. Rates of formation of α -OH-TRZ and 4-OH-TRZ in the presence of pre-immune serum and serum containing polyclonal antibody (10:1, antibody serum protein to microsomal protein) against CYP3A1 are expressed as a per cent relative to the rate of formation with no serum present in rat liver microsomes. Each point represents the average of duplicate assays, which did not differ by more than 10%. Similar inhibition with anti-CYP3A1 serum (anti-3A) was seen in each pretreatment group (vehicle, VEH; dexamethasone, DEX; ritonavir, RIT), while treatment with pre-immune rabbit serum (rabbit) caused minimal inhibition.

CYP3A expression in liver and intestinal microsomes

Immunoreactive CYP3A protein content in liver and intestinal microsomes was evaluated in rats following the 3-day drug exposure regimen (sample blot and quantification shown in figure 6). All CYP3A immunoblotting in rat intestinal and liver microsomes detected two immunoreactive bands of approximately 52–54 kDa. In experiments by the manufacturer of the primary anti-CYP3A1/2 antibody (Xenotech, Kansas City, KS, USA) similar results were found in rat liver microsomes. These bands were then excised, and protein sequencing (data not shown) demonstrated that they were rat CYP3A1 (top band) and CYP3A2 (lower band). In liver microsomes, rats pretreated with RIT and DEX showed a 5- and 17.5-fold increase in CYP3A immunoreactive protein compared with VEH controls, respectively (table 3). In intestinal microsomes, rats pretreated with RIT and DEX showed a 1.7- and 3.3-fold increase in CYP3A immunoreactive protein compared with VEH controls, respectively (table 3). Western blots demonstrating induction of immunoreactive CYP3A in rat liver and intestinal microsomes, with RIT and DEX pretreatment, are shown in figure 7. There was a strong correlation (Spearman $r^2=0.89$, $p<0.0001$) between liver microsomal CYP3A-mediated metabolism and CYP3A immunoreactive protein across all rat treatment groups (figure 8).

Discussion

Both RIT and DEX produced substantial induction of enteric P-gp expression in the rat model. DEX is previously identified as an inducer of enteric P-gp in this

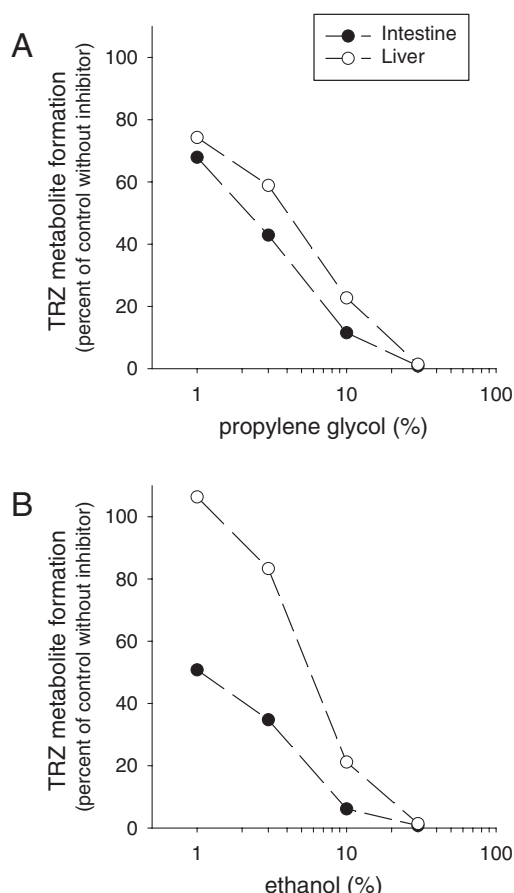


Figure 5. Inhibition of triazolam biotransformation by ethanol and polypropylene glycol. Rates of formation of α -OH-TRZ and 4-OH-TRZ in the presence of increasing concentrations (%) of ethanol (A) and PPG (B) are expressed as a fractional percentage of the rate of formation with no inhibitor present in rat liver and intestinal microsomes (250 μ M TRZ). Each point represents the average per cent inhibition of α -OH- and 4-OH-TRZ formation, which did not differ more than 10% in all cases. Data points are the average of duplicate assays.

species (Lin *et al.* 1999, Huang *et al.* 2001, Yumoto *et al.* 2001). RIT is a strong inducer of P-gp expression and function in studies of human adenocarcinoma cell lines *in vitro* (Perloff *et al.* 2000, 2001, Störmer *et al.* 2002). The present study is the first demonstration of induced enteric P-gp expression by RIT in intact animals. Huang *et al.* (2001) reported that nelfinavir and amprenavir induced enteric P-gp expression in rats. The extent of induction was in the range 1.6–1.8-fold, which is less than the 2.8-fold increase observed with RIT and DEX. It was also observed that both RIT and DEX significantly increased expression of P-gp in blood–brain barrier microvessel endothelial cells. However, the extent of induced P-gp expression (approximately 1.3-fold) was less than the extent of induction of enteric P-gp. Based on the anticipated peak plasma RIT concentrations (about 2 μ M) in rats orally gavaged with 20 mg kg⁻¹ day⁻¹ RIT (Denissen *et al.* 1997), this difference may be attributable to the differing extent of exposure to DEX and RIT between the two sites. Exposure of brain endothelial cells would

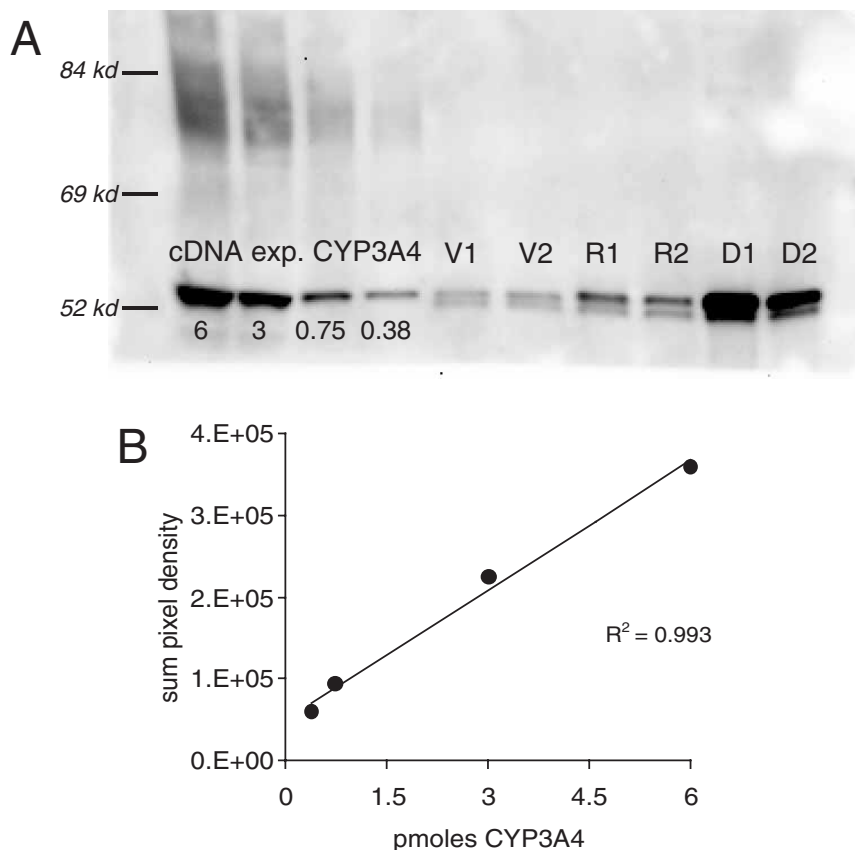


Figure 6. Quantitative Western blotting of CYP3A protein in liver and intestinal microsomes. Rat liver and intestine microsomal CYP3A protein (liver is shown) was quantified by comparison with a standard curve of CYP3A4. Immunoreactive protein was detected via Western blotting (A) and quantified with computer image analysis by comparison with a standard curve (B). For liver microsomal samples shown, 0.5 μ g protein was loaded (V, vehicle; D, 80 mg kg^{-1} dexamethasone; R, 20 mg kg^{-1} ritonavir). All liver and intestinal microsomal samples ranged from 0.05 to 5 μ g protein loaded. Expressed CYP3A4 cDNA (6, 3, 0.75, and 0.375 pmoles) was used for calibration.

Table 3. Expression of immunoreactive CYP3A protein in liver and intestinal microsomes from rats pretreated with ritonavir, dexamethasone or vehicle.

	Immunoreactive CYP3A protein	
	pmoles/ μ g protein	Fold increase (compared with vehicle)
Vehicle (50% ethanol, 50% PPG)		
Liver microsomes, $n = 7$	2.2 ± 0.46	—
Intestinal microsomes, $n = 6$	0.19 ± 0.04	—
Ritonavir (20 mg kg^{-1})		
Liver microsomes, $n = 7$	10.9 ± 7.6	5.0 ± 3.5
Intestinal microsomes, $n = 6$	0.31 ± 0.14	1.7 ± 0.74
Dexamethasone (80 mg kg^{-1})		
Liver microsomes, $n = 6$	38.1 ± 11.2	17.5 ± 5.1
Intestinal microsomes, $n = 5$	0.61 ± 0.08	3.3 ± 0.42

Values are the mean ($\pm$ SD).

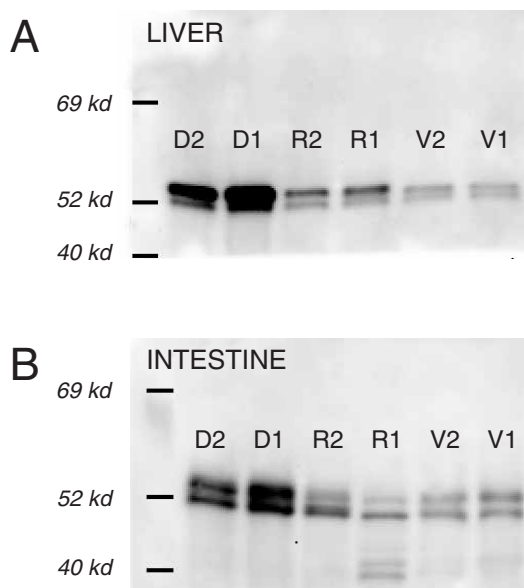


Figure 7. Qualitative Western blotting of CYP3A protein in liver and intestinal microsomes. Rats were administered 20 mg kg^{-1} ritonavir, 80 mg kg^{-1} dexamethasone or vehicle alone (50% ethanol, 50% PPG) once daily for 3 days by oral gavage. After 1 day washout, liver and intestinal microsomes were prepared. Rat liver (A) and intestinal (B) microsomes were immunoblotted for CYP3A protein. Each lane represents a sample (D, dexamethasone; R, ritonavir; V, vehicle) from an individual rat (0.5 and $5.0 \mu\text{g}$ protein loaded for liver and intestinal microsomes, respectively).

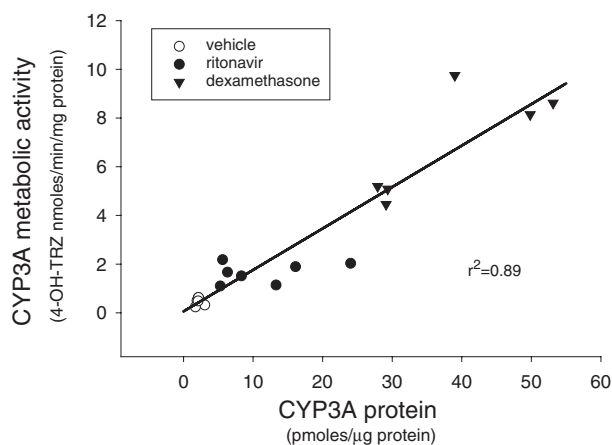


Figure 8. Correlation of CYP3A-mediated metabolism and immunoreactive CYP3A protein in liver microsomes. CYP3A-mediated metabolism (4-OH-TRZ nmoles $\text{min}^{-1} \text{mg}^{-1}$ protein microsomal protein) and immunoreactive CYP3A protein (pmoles immunoreactive protein mg^{-1} microsomal protein) in liver microsomes from rats pretreated with ritonavir, dexamethasone, or vehicle correlated in a linear relationship.

be limited by systemic plasma concentrations, whereas enteric mucosal cells are transiently exposed to very high concentrations of DEX and RIT after oral administration of these drugs. It is also possible that a longer duration of exposure to DEX and RIT would increase the quantitative extent of P-gp induction in microvessel endothelial cells.

Biotransformation of TRZ by rat liver and intestinal microsomal preparations was dependent mainly on CYP3A isoforms, based on a high degree of inhibition produced by anti-CYP3A antibodies as well as the chemical inhibitor, ketoconazole. Consistent with previous reports (Cotureau *et al.* 2001), DEX produced extensive induction of hepatic CYP3A immunoactive protein expression as well as TRZ hydroxylation activity. DEX also induced enteric CYP3A expression, although the extent of increase was not as great as with liver microsomal preparations. RIT induced both hepatic and enteric CYP3A immunoactive protein expression and catalytic activity. In general, DEX induction of hepatic CYP3A was greater than that produced by RIT, whereas induction of enteric CYP3A by DEX was similar to or slightly exceeded that produced by RIT. Overall, there was a high degree of correlation between hepatic immunoactive CYP3A expression and catalytic activity based on hydroxylation of TRZ *in vitro*. It was noted that the ethanol and PPG used as the vehicle to deliver DEX and RIT by gavage were both CYP3A inhibitors. Although preparation of enteric microsomes involved extensive washing, it is possible that residual ethanol and/or PPG not removed by washing, as well as non-specific enteric cell damage produced by the solvents, might have contributed to the relatively low CYP3A catalytic activity in enteric microsomes relative to liver microsomes.

The study demonstrates that the HIV protease inhibitor RIT induces *in vivo* expression of hepatic and enteric CYP3A, and of enteric P-gp, in rats. The positive control, DEX, produced induction of the same systems. Findings from rodent studies of chemical induction cannot be assumed to apply directly to humans. Whereas species differences in molecules that cause CYP3A induction exist, CYP3A induction appears to be mediated through the binding of the pregnane X receptor (PXR) in multiple species (including rats, mice, rabbit, and humans) (LeCluyse 2001). Specifically, RIT is a strong PXR activator in human liver heptacytes (Luo *et al.* 2002). P-gp sequence and substrate specificity is reasonably well conserved across species (Juranka *et al.* 1989, van de Vrie *et al.* 1998). For example, in LLC-PK1 cells, bidirectional transport was very similar in L-MDR1 (human) and L-mdr1a (mouse) transfected cell monolayers for typical P-gp substrates (digoxin, vinblastine, ritonavir, and indinavir); while no differential transport was seen for non-P-gp substrates (progesterone and diazepam) (Yamazaki *et al.* 2001). Differential species induction of P-gp is less extensively studied than that of CYP3A, however induction of P-gp is most likely regulated by PXR, at least in part, across species (Jones *et al.* 2000, Geick *et al.* 2001).

Despite species differences in drug dispositions, issues such as drug absorption, stability and toxicity may be more appropriately addressed through intact animal studies as opposed to *in vitro* models. Data from the rat model, when combined with findings from cell culture systems using human cell lines, provide important preclinical information that can be used to guide the choice of clinical studies of CYP and P-gp induction involving new antiretroviral agents under development, as well as those already in clinical use (Lin *et al.* 1999, Huang *et al.* 2001). In the case of RIT, cell culture data (Perloff *et al.* 2000, 2001, Störmer *et al.* 2002), together with direct or indirect clinical data, strongly indicate significant induction of CYP3A and P-gp expression with extended exposure to RIT, consistent with the findings of the rat model. RIT itself is a substrate for metabolism by CYP3A and for transport by P-gp; extended exposure to RIT in humans is associated with a progressive decline in plasma RIT concentrations, presumably due to induction

of CYP3A and/or P-gp. Initial exposure to RIT in humans generally leads to impaired clearance and elevated plasma levels of other drugs that are substrates for CYP3A and/or P-gp (Merry *et al.* 1997, Hsu *et al.* 1998a, b, Sham *et al.* 1998, Greenblatt *et al.* 1999, 2000a, b, 2003). In some cases, the increased plasma levels of coadministered drugs can be very large and potentially hazardous, leading to warnings or contraindications in product labelling. With extended exposure to RIT, the net change in clearance of a coadministered agent will depend on the balance of induction and inhibition (Venkatakrisnan *et al.* 2001). The outcome could be net induction, net inhibition or little net change. This would depend on the dosage and duration of exposure to RIT as well as the intrinsic clearance of the substrate drug, the overall contribution of CYP3A and P-gp to its disposition, and the extent of presystemic extraction after oral dosage. Another critical factor is likely to be the relative contributions of hepatic and enteric CYP3A to oral clearance of the substrate drug. Whereas initial exposure to RIT is likely to produce extensive inhibition of hepatic and enteric CYP3A, the relative induction of CYP3A at these two sites during extended exposure to RIT in humans is not established. The rat data from the present study suggest that relative induction of enteric CYP3A is less than induction of hepatic CYP3A.

The importance of induction of blood–brain barrier P-gp by RIT remains to be determined. The extent of induction (approximately 30%) was modest. P-gp expressed by blood–brain barrier endothelial cells is known to serve a protective ‘barrier’ function by preventing or reducing exposure of brain tissues to a number of chemicals that are substrates for efflux transport by P-gp (Schinkel 1999, Fromm 2000, Aryton and Morgan 2001). Greatly increased brain uptake of P-gp substrate drugs occurs in experimental animals that do not express P-gp (‘knockout’ mice). In humans, chemical inhibition of P-gp leads to increased central nervous system effects of loperamide, a P-gp substrate inferred to be largely excluded from brain under normal circumstances (Sadeque *et al.* 2000). However, the clinical importance of increased, as opposed to reduced, expression or function of blood–brain barrier P-gp has not been established. It is possible that increased P-gp expression could impair brain uptake of P-gp substrate drugs (such as antiretroviral or antineoplastic agents) intended to treat HIV infection or neoplastic disease in brain tissue. Further experimental and clinical studies are needed to evaluate the potential significance of induced expression of blood–brain barrier P-gp.

Acknowledgments

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Predicting drug clearance from recombinantly expressed CYPs: intersystem extrapolation factors

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1. Recombinantly expressed human cytochromes P450 (rhCYPs) have been under-used for the prediction of human drug clearance (CL).
2. Differences in intrinsic activity (per unit CYP) between rhCYP and human liver enzymes complicate the issue and these discrepancies have not been investigated systematically. We define intersystem extrapolation factors (ISEFs) that allow the use of rhCYP data for the *in vitro-in vivo* extrapolation of human drug CL and the variance that is associated with interindividual variation of CYP abundance due to genetic and environmental effects.
3. A large database ($n = 451$) of metabolic stability data has been compiled and used to derive ISEFs for the most commonly used expression systems and CYP enzymes.
4. Statistical models were constructed for the ISEFs to determine major covariates in order to optimize experimental design to increase prediction accuracy.
5. Suggestions have been made for the conduct of future studies using rhCYP to predict human drug clearance.

Introduction

By providing guidance on dosage and the potential impact of drug–drug interactions, the prediction of the *in vivo* metabolic clearance of drugs and drug candidates from *in vitro* data has become an integral component of drug discovery and development. The methods used for such *in vitro-in vivo* extrapolation (IVIVE) include scaling from animal data by allometry (Lave *et al.* 1999), from data obtained using human liver microsomes or hepatocytes (Houston and Carlile 1997, Iwatsubo *et al.* 1997), or a combination of these procedures (Zuegge *et al.* 2001). Although some success has been achieved in predicting means of metabolic clearance using these approaches, their application has failed to address the important issue of intersubject variability and the identification of individuals at extreme risk of adverse pharmacokinetic-related events. Furthermore, methods based on the use of microsomes and hepatocytes are not readily compatible with a high-throughput approach to defining metabolic clearance because of limitations on the availability and consistency of human tissue. In contrast, the development and commercial availability of recombinantly expressed human cytochromes P450 (rhCYPs) allows this disadvantage to be circumvented. The more fundamental measure of enzyme activity that they provide (activity per unit amount of CYP as opposed to activity per mg protein provided by liver systems), together with

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knowledge of the variability of CYP abundances in different populations (with regard to ethnicity, disease, age, etc.), allows variability in *in vivo* metabolic clearance to be assessed.

Traditionally, two approaches have been taken to scale to *in vivo* metabolic clearance when using data obtained with rhCYPs, as outlined below.

rhCYP/RAF-based in vitro–in vivo extrapolation

Early in the application of rhCYPs, a relative activity factor (RAF) was proposed to allow for differences in the activity of the enzymes per unit of microsomal protein compared with that in human liver microsomes (HLM) (Crespi 1995). Initially, this RAF was defined with respect to $V_{\max}$:

$$\text{RAF}(V_{\max}) = \frac{V_{\max}(\text{HLM})}{V_{\max}(\text{rhCYP})}, \quad (1)$$

where $V_{\max}$ refers to metabolism of a probe substrate by an individual CYP enzyme. The units of $V_{\max}(\text{HLM})$ and $V_{\max}(\text{rhCYP})$ are $\text{pmol min}^{-1} \text{mg}^{-1}$ protein and $\text{pmol min}^{-1} \text{pmol}^{-1}$ CYP enzyme, respectively. Thus, RAF has units of pmol CYP mg^{-1} protein. Hence, the contribution of the activity of each CYP involved in the metabolism of a test compound could be scaled to human liver microsomes. Assuming identical K_m values for the two systems, net intrinsic clearance (CL_{int}) in the liver is then calculated from the sum of $V_{\max}/K_m$ of each isoform and metabolic route multiplied by milligrams of microsomal protein per gram of liver (MPPGL) (Wilson *et al.* 2003) and total liver weight (ICRP 1975):

$$\text{CL}_{\text{int}} = \left[\sum_{j=1}^n \left(\sum_{i=1}^n \frac{V_{\max}(\text{rhCYP}_j)_i \times \text{RAF}_{ij}(V_{\max})}{K_m(\text{rhCYP}_j)_i} \right) \right] \times \text{MPPGL} \times \text{liver weight}, \quad (2)$$

where there are i metabolic pathways for each of j CYPs.

It was recognized subsequently that apparent K_m values obtained using rhCYP systems are not necessarily the same as those determined using HLM. Therefore, Nakajima *et al.* (1999) suggested that a RAF based on intrinsic clearance be used:

$$\text{RAF}(\text{CL}_{\text{int}}) = \frac{\text{CL}_{\text{int}}(\text{HLM})}{\text{CL}_{\text{int}}(\text{rhCYP})}. \quad (3)$$

An important source of difference in apparent K_m , and hence CL_{int} , between HLM and rhCYP is the extent of non-specific microsomal binding (Tucker 1992, Venkatakrishnan *et al.* 2002). Although differences between the systems in such binding per amount of microsomal protein are minimal (Wong 1999, Venkatakrishnan *et al.* 2000b), experiments with rhCYP systems commonly employ lower protein concentrations than those using liver microsomes, which may cause disparity between the extent of non-specific binding in the two systems, especially for lipophilic basic drugs.

The activity per unit amount of CYP (intrinsic activity or turnover number) may also vary between HLM and rhCYP enzymes, reflecting differences in the concentrations of accessory proteins (cytochrome b_5 , NADPH:cytochrome P450 oxidoreductase (OR) and the lipid environment of the enzyme (Venkatakrishnan *et al.* 2000a, Nakajima *et al.* 2002). Thus, RAF factors determined using different

expression systems or even the same expression system for different CYPs are not necessarily interchangeable.

RAF factors suffer from two main disadvantages. First, they must be determined for each individual enzyme/expression system combination in conjunction with microsomes from a single liver, a limited number of livers or from microsomes pooled from several livers that may not be representative of the population. Second, they do not allow prediction of the degree of interindividual variability in *in vivo* metabolic clearance since it is very difficult to differentiate experimental variability from the true interindividual variability in liver samples.

rhCYP/abundance-based in vitro–in vivo extrapolation

This method estimates net *in vivo* intrinsic metabolic clearance from:

$$CL_{\text{int}} = \left[\sum_{j=1}^n \left(\sum_{i=1}^n \frac{V_{\text{max}}(\text{rhCYP}_j)_i \times \text{CYP}_j \text{ abundance}}{K_m(\text{rhCYP}_j)_i} \right) \right] \times \text{MPPGL} \times \text{liver weight}, \quad (4)$$

where CYP_j abundance is the amount of j enzyme per mg microsomal protein. Typically, the average abundance of each CYP has been used (e.g. from Shimada *et al.* 1994). However, by adding estimates of variance to this value (Rowland-Yeo *et al.*, in press) as well as to MPPGL and liver weight, it is possible to predict the population variance in intrinsic metabolic clearance. In turn, by using a model for net hepatic drug clearance and adding variance to liver blood flow and binding of the drug to blood components (Proctor *et al.* 2003), an estimate of the population distribution of net hepatic drug clearance is obtained.

However, this method does not allow for any difference in activity per unit amount of CYP between the expression system and the liver. To obviate the problem of different intrinsic activity (turnover number) in rhCYP systems relative to human liver, we propose incorporating a scaling factor (ISEF) in the abundance method that corrects for this.

As with RAFs, this may be defined with respect to either V_{max} (VISEF) or CL_{int} , (CLISEF), depending on whether or not a possible difference in K_m is allowed for:

$$\text{VISEF} = \frac{V_{\text{max}_{ji}}(\text{HLM})}{V_{\text{max}_i}(\text{rhCYP}_j) \times \text{CYP}_j \text{ abundance}(\text{HLM})} \quad (5)$$

$$\text{CLISEF} = \frac{CL_{\text{int}_{ji}}(\text{HLM})}{CL_{\text{int}_i}(\text{rhCYP}_j) \times \text{CYP}_j \text{ abundance}(\text{HLM})}, \quad (6)$$

where CYP_j abundance (HLM) refers to the abundance of the j th CYP in the liver sample(s) used to determine the ISEF.

Hence, ISEF is inserted into equation (4) as follows:

$$CL_{\text{int}} = \left[\sum_{j=1}^n \left(\sum_{i=1}^n \frac{\text{ISEF}_{ji} \times V_{\text{max}_i}(\text{rhCYP}_j) \times \text{CYP}_j \text{ abundance}}{K_{m_i}(\text{rhCYP}_j)} \right) \right] \times \text{MPPGL} \times \text{liver weight}. \quad (7)$$

ISEF is a dimensionless number used as a direct scaler to convert data obtained with a rhCYP system to an 'HLM environment' (by accounting for differences in

turnover number) that also allows the effect of population variability in CYP abundance to be assessed.

We have estimated values of VISEF and CLISEF from the literature and from in-house studies. Potential covariates affecting these values were explored, namely the probe drug used, the expression system used, the specific CYP and the laboratory in which the experiments were carried out.

Materials and methods

Data sets for enzyme kinetic data (K_m and V_{max}) were compiled for rhCYP systems (194 sets) and HLM (257 sets) (see appendices 1 and 2). Inclusion criteria were as follows:

- K_m and V_{max} were determined simultaneously in the same laboratory.
- Probe substrates and experimental conditions in accordance with consensus criteria (see below) (Tucker *et al.* 2001).
- Use of pooled HLM or individually reported HLM samples.
- V_{max} expressed per amount of CYP (recombinant system) or per milligram of microsomal protein (HLM).

Consensus experimental conditions:

- Metabolite formation experiments should not result in a loss of >20% substrate.
- >10 fold excess of substrate in comparison to CYP.
- <1% organic solvent in incubation matrix (Chauret *et al.* 1998).

Although several sets of kinetic parameters were found for some of the HLM data, the means were used to allow the assignment of median CYP abundance from the literature to these liver samples (table 1). The inclusion of individual sets of HLM data from different sources would have resulted in an additional source of variability that was related to HLM samples not being representative of an average liver (i.e. liver with average abundance of each CYP enzyme).

The data used for the calculation of CLISEF were not corrected for non-specific microsomal binding as this information was only available for a small number of probes. The CYP enzymes investigated were CYP1A2, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. The recombinant systems considered were based on B-lymphoblastoid cells (L), baculovirus transformed insect cells (B), *Escherichia coli* cells (E) and *Saccharomyces cerevisiae* cells (Y).

ISEFs were determined according to equations (5) and (6). Median CYP abundances (table 1) were derived from a systematic review and meta-analysis of more than 20 published and unpublished sources (Rostami-Hodjegan, unpublished data).

The one sample Kolmogorov–Smirnov test was used to investigate the distribution shapes of VISEF and CLISEF and to determine the appropriate statistical analysis. The Wilcoxon Signed Ranks test for two related samples was used to test the similarity of VISEF and CLISEF distribution shapes. Correlations were assessed with Pearson’s two-tailed correlation test for bidirectional relationships, and a univariate analysis of variance (UNIANOVA) was used to determine the significant contributors to the variance of ISEF (assessed by the Akaike Information Criterion). Covariates examined were the

Table 1. CYP enzyme abundances in human liver microsomes (Rostami-Hodjegan, unpublished data).

HLM CYP isoform	Median abundance (pmol CYP mg ⁻¹ HLM)
1A2	42
2C8	50
2C9	32
2C19	5.6
2D6	9.1
2E1	50
3A4	78

Table 2. Summary statistics for VISEF and CLISEF.

Statistic	VISEF	CLISEF
<i>n</i>	194	194
Mean	15.8	18.4
Median	2.7	3.1
Minimum	0.003	0.006
Maximum	408	575
95% Confidence interval of median	0.02–123	0.02–231

Table 3. Correlations between potential covariates and VISEF and CLISEF.

CLISEF SYSTEM ISOFORM PROBE LAB			
VISEF		**	**
SYSTEM		**	**
ISOFORM	+		**
PROBE	++	++	**
LAB		++	

*/+ *p* < 0.05; **/++ *p* < 0.01.

experimental recombinant system (SYSTEM) (*n* = 4), the specific CYP expressed (ISOFORM) (*n* = 7), the probe substrate used (PROBE) (*n* = 64) and the laboratory (LAB) (*n* = 41). All statistical analyses were carried out using SPSS v11.0 (SPSS, Inc., Chicago, IL, USA).

Results

The VISEF and CLISEF values (see appendix 2) were best described by a log-normal distributions. Summary statistics are shown in table 2. There was no significant difference between VISEF and CLISEF (Wilcoxon signed rank test).

Strong covariance was found between VISEF and ISOFORM and PROBE, whereas CLISEF was correlated strongly only with PROBE (table 3).

Median VISEF and CLISEF in relation to recombinant expression system and CYP enzymes are summarized in tables 4 and 5. Cases where the 95% confidence interval (of the median) did not include 1, demonstrating a significant difference (*p* < 0.05) from unity, are shown in **bold**. The distributions of VISEF (figure 1) and CLISEF (figure 2), according to expression system (A) and expressed rhCYP (B), are also represented.

Optimized variance models were constructed for VISEF and CLISEF by selecting those variables that accounted for the greatest proportion of the total variance. The optimal model for VISEF variance included the covariates ISOFORM (11%), PROBE (21%), LAB (12%) and the interaction between each of these (23%), accounting for 68% of the total variance with an unaccounted residual error of 5%. CLISEF variance was best described by SYSTEM (3%), PROBE (32%), LAB (17%) and the SYSTEM*LAB interaction (4%), accounting for 56% of the total variance with a residual error of 34%. Since the only difference between VISEF and CLISEF is the inclusion of *K_m*, the variance of the ratios of rhCYP *K_m* to HLM *K_m* was examined. It was found that 78% of the variance of this ratio could be explained by a model consisting of ISOFORM, PROBE and LAB, with a 2% unexplained error.

Table 4. Median and 95% confidence intervals of VISEF for each recombinant system and CYP enzyme.

rhCYP	Lympho	Baculo	<i>E. coli</i>	Yeast	Median
1A2	11.7 (0.01–1070)	1.37 (0.08–42)	3.62 (0.01–123)	1.35 (0.06–16.7)	2.80 (0.03–282)
2C8	0.8	3.28	–	1.07 (0.05–43.9)	1.07 (0.12–20.5)
2C9	8.6 (0.27–348)	1.72 (0.15–67.2)	2.29 (2.23–2.4)	3.85 (0.09–56.6)	3.85 (0.14–128)
2C19	0.6 (0.00–331)	33.54 (0.20–674)	9.37 (1.77–37.6)	14.2	2.76 (0.02–532)
2D6	1.6 (0.07–75.5)	1.65 (0.04–66)	4.21 (0.43–21.6)	4.19 (0.54–50.5)	3.03 (0.11–67.2)
2E1	92.9 (0.01–17483)	1.11 (0.47–2.4)	0.90	24.8	1.35 (0.05–379)
3A4	3.7 (0.01–177)	0.81 (0.00–54.7)	1.26 (0.12–7.9)	15.3	1.90 (0.00–141)
Median	3.8 (0.02–400)	1.66 (0.01–133)	2.52 (0.06–53.5)	3.52 (0.14–59.84)	

Table 5. Median and 95% confidence intervals of CLISEF for each recombinant system and CYP enzyme.

RhCYP	Lympho	Baculo	<i>E. coli</i>	Yeast	Median
1A2	7.30 (0.10–481)	1.45 (0.05–25.5)	7.31 (0.04–187)	3.38 (0.02–199)	5.46 (0.05–239)
2C8	4.58	0.04	–	0.75 (0.06–18.4)	0.75 (0.01–43.1)
2C9	6.82 (0.53–87.9)	2.64 (0.46–52.7)	3.16 (2.4–2.7)	2.66 (0.20–38.9)	3.35 (0.35–53.5)
2C19	7.69 (0.07–675)	18.3 (0.11–367)	10.0 (0.66–79)	20.2	9.3 (0.12–430)
2D6	1.57 (0.07–75.5)	0.90 (0.00–157)	4.50 (0.43–48.2)	4.63 (0.24–48.7)	1.3 (0.02–109)
2E1	164 (7.68–1612)	142 (2.71–2302)	6.24	50.5	46.9 (2.92–994)
3A4	3.83 (0.03–218)	0.34 (0.01–8.92)	1.92 (1.50–2.43)	12.1	1.79 (0.02–104)
Median	1.8 (0.05–336)	1.09 (0.01–125)	4.49 (0.15–65.0)	3.95 (0.12–80.3)	

Discussion

The estimated ISEF values were not significantly different from 1 at the 95% confidence level, signifying a general concordance between rhCYPs and HLM. However, the means were high (15.8 and 18.4 for VISEF and CLISEF, respectively) and the variances were very large (CV = 275 and 315% for VISEF and CLISEF, respectively) (see appendix 1) suggesting a possible trend for less efficient rhCYP systems when rates are assessed per unit of enzyme. Given that the data were log-normally distributed, the medians (2.7 and 3.1 for VISEF and CLISEF, respectively) provide a more prudent measure of central tendency and correction factor for rhCYP derived prediction of clearances. It would be surprising to find a high degree of agreement between systems considering the many potentially confounding factors such as the NADPH:cytochrome P450

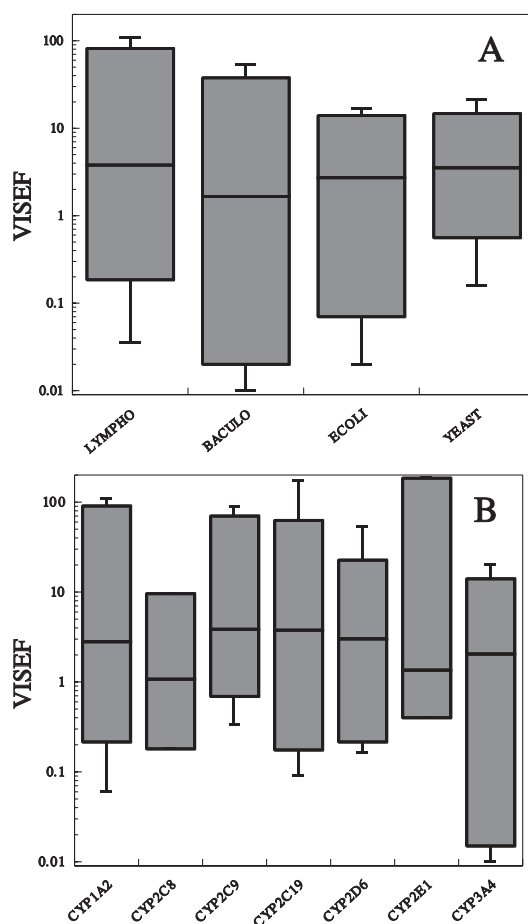


Figure 1. Box and Whisker plots demonstrating the range of values of VISEF by recombinant system (A) and CYP (B).

oxidoreductase ratio (Venkatakrishnan *et al.* 2000a) and the cytochrome b_5 content (Nakajima *et al.* 2002). Although several median ISEFs for individual rhCYPs in certain systems differed from unity (e.g. *E. coli* CYP2C9), when grouped by ISOFORM, neither VISEFs nor CLISEFs were significantly different from unity, with the exception of the CLISEF calculated for rhCYP2E1.

The observed correlation between PROBE and ISOFORM in determining ISEFs was not surprising since, by definition, probe substrates are chosen to be selective for each enzyme. Neither was the correlation between PROBE and LAB, as individual laboratories often have preferences for particular routine assays (Boobis *et al.* 1998). Correlations between ISOFORM and SYSTEM, and LAB and SYSTEM indicate that specific rhCYP systems appear to be used preferentially when examining particular CYP enzymes. The strong correlation between PROBE and SYSTEM was unexpected and may reflect specific laboratory conditions biasing the data set. Correlations between PROBE and both VISEF and CLISEF support previous observations (Venkatakrishnan *et al.* 1998a, Stormer *et al.* 2000) that RAFs demonstrate a degree of substrate specificity. In

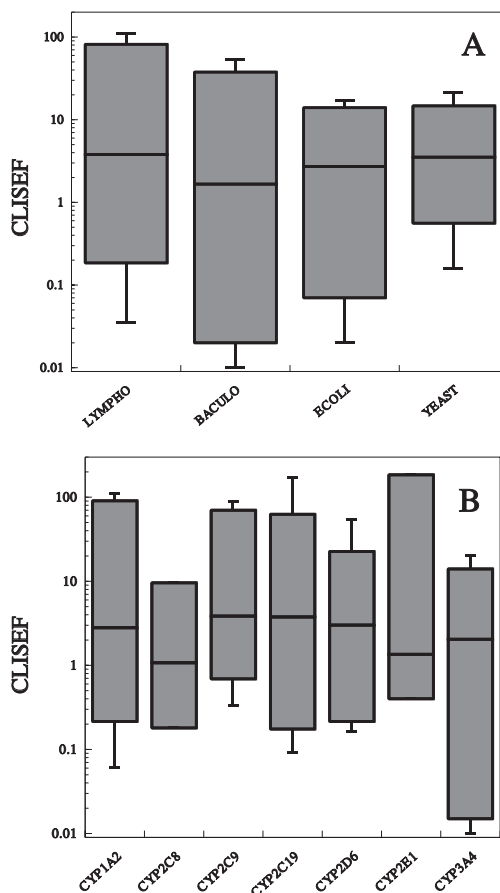


Figure 2. Box and Whisker plots demonstrating the range of values of CLISEF by recombinant system (A) and CYP (B).

the case of CLISEF this may reflect in part the impact of intersystem variability in the extent of non-specific microsomal binding on K_m .

The construction of variance models allowed a more thorough investigation of the sources of the large variability observed in ISEFs. These models again demonstrated that there was a significant probe substrate dependence, accounting for 21% of VISEF variability and 32% of CLISEF variability. Although the overall distributions of VISEF and CLISEF were not significantly different ($p < 0.05$), the factors that contributed to their variability differed from each other. Note that the only difference between the calculation of VISEF and CLISEF is the inclusion of K_m in the latter. Therefore, the relative differences in HLM and rhCYP K_m will influence the covariate model for CLISEF.

The incorporation of ISEFs can improve the accuracy of prediction of *in vivo* drug clearance (Proctor *et al.* 2003). A better outcome using VISEF compared with CLISEF may reflect the fact that no correction of K_m was made for differences in non-specific microsomal binding between HLM and rhCYP systems in the derivation of CLISEF.

The application of ISEFs in IVIVE is envisaged at different levels reflecting a balance between confidence in extrapolation and high-throughput compatibility

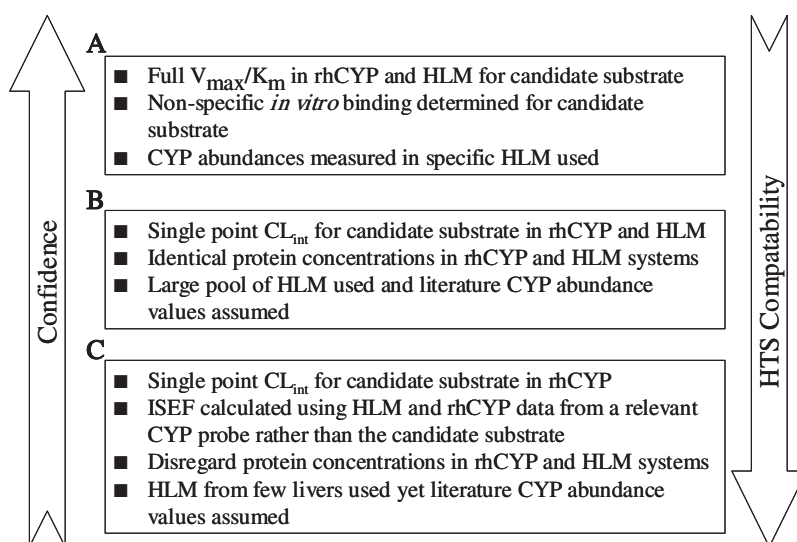


Figure 3. Incorporation of ISEFs into *in vitro-in vivo* extrapolation (see the text for an explanation of levels A–C).

(figure 3). Thus, a three-level approach may be considered when using rhCYP to predict *in vivo* clearance:

- At level A, there is maximum confidence in the calculated ISEFs. Variability associated with choice of probe, laboratory, system and specific CYPs is minimized since the candidate substrate itself is used to derive ISEFs in the same laboratory. Non-specific microsomal binding is also investigated at relevant protein concentrations (to correct the CLISEF) and CYP abundances are measured in the HLM samples used.
- At level B, depletion experiments with single substrate concentrations are used as a higher throughput replacement for the determination of full enzyme kinetics. This assumes that the initial substrate concentration is significantly below the lowest K_m of the metabolic pathways involved and each metabolic pathway is mostly (>90%) cleared by a single CYP. Since a sufficiently large pool of HLM is used, population CYP abundances from the literature may be assumed to represent the likely abundance in the pooled HLM. If the HLM and rhCYP protein concentrations are similar then the effect of non-specific microsomal binding in HLM and rhCYP may be ignored in the calculation of CLISEF.
- At level C, experimental throughput is high but at the expense of a decrease in the accuracy of the estimation of ISEF. Firstly, median ISEFs for the relevant CYP are taken from reported studies using the same rhCYP or from the present report (tables 4 and 5), using probes other than the candidate substrate instead of calculating them based on the candidate substrate. Secondly, any difference in non-specific protein binding between rhCYP (as used by the investigators) and HLM systems (used for calculation of ISEFs) is ignored.

Appendix 1. HLM kinetic parameters.

Drug	Pathway	K_m^1	$V_{\max}^2$	CL_{int}^3	Reference
4-Nitrophenol	hydroxylation	28.8 (26.3–30.0)	1026 (990–1100)	35.6 (33.0–41.8)	Tassaneeyakul <i>et al.</i> (1993b), Iwata <i>et al.</i> (1998)
7-Benzoyloxy-4-trifluoromethyl-coumarin	7-hydroxylation (debenzylation)	8.3	454	54.7	Renwick <i>et al.</i> (2000)
7-Ethoxyresorufin	O-deethylation	0.88 (0.2–2.9)	187 (0.63–400)	213 (0.2–474)	Shimada <i>et al.</i> (1997), Boobis <i>et al.</i> (1998), Iwata <i>et al.</i> (1998), McGinnity <i>et al.</i> (1999), Madan <i>et al.</i> (2002), Nakamura <i>et al.</i> (2002), Venkatakrishnan <i>et al.</i> (2002)
Amiodarone	N-deethylation	31.6	588	18.6	Ohyama <i>et al.</i> (2000)
ATRA (all- <i>trans</i> -retinoic acid)	4-hydroxylation	9.0	98.5	10.9	Nadin and Murray (1999)
Bufuralol	1'-hydroxylation	60.2 (5.4–170)	123 (29.4–273)	3.9 (0.6–12.4)	Minder <i>et al.</i> (1984), Halliday <i>et al.</i> (1995), Boobis <i>et al.</i> (1998), Iwata <i>et al.</i> (1998), Mankowski <i>et al.</i> (2000), Shimada <i>et al.</i> (2001), Nakajima <i>et al.</i> (2002)
Buprenorphine	N-dealkylation	39.3	712	18.1	Kobayashi <i>et al.</i> (1998)
Caffeine	paraxanthine	1314 (180–4126)	250 (72–770)	0.47 (0.02–1.4)	Tassaneeyakul <i>et al.</i> (1992), Boobis <i>et al.</i> (1998), Labedzki <i>et al.</i> (2002)
Caffeine	theobromine	2393 (310–6400)	223 (2.5–120)	0.08 (0.003–0.2)	Tassaneeyakul <i>et al.</i> (1992), Labedzki <i>et al.</i> (2002)
Caffeine	theophylline	510 (130–8900)	320 (0.9–640)	0.054 (0.004–0.1)	Tassaneeyakul <i>et al.</i> (1992), Labedzki <i>et al.</i> (2002)
Chlorzoxazone	6-hydroxylation	77.8 (27–248)	3483 (4.17–16 465)	40.1	Peter <i>et al.</i> (1990), Court <i>et al.</i> (1997), Boobis <i>et al.</i> (1998), Eagling <i>et al.</i> (1998), Hickman <i>et al.</i> (1998), Lucas <i>et al.</i> (1999), Madan <i>et al.</i> (2002)

Coumarin	7-OH coumarin	0.7 (0.6–0.8)	887 (412–1300)	1400 (228–2281)	Draper <i>et al.</i> (1998), Hickman <i>et al.</i> (1998), Madan <i>et al.</i> (2002)
Cyclosporin A	Hydroxylation	1.7	82.5	48.5	Back and Tjia (1991)
Debrisoquine	4-hydroxylation	104 (78–130)	45.9	0.4 (0.3–0.5)	Kahn <i>et al.</i> (1982), Nakajima <i>et al.</i> (2002)
Dextromethorphan	O-demethylation	4.7 (1.3–8.3)	179 (5.9–360)	40.7 (4.5–128)	Broly <i>et al.</i> (1989), Dayer <i>et al.</i> (1989), Jacqz-Aigrain <i>et al.</i> (1993), Kerry <i>et al.</i> (1994), Mautz <i>et al.</i> (1995), Transon <i>et al.</i> (1996), Hickman <i>et al.</i> (1998), Von Moltke <i>et al.</i> (1998), Abdel-Rahman <i>et al.</i> (1999), McGinnity <i>et al.</i> (1999), Mankowski <i>et al.</i> (2000), Madan <i>et al.</i> (2002), Ramamoorthy <i>et al.</i> (2002)
Dextromethorphan	N-demethylation	529 (57–846)	576 (205–1313)	1.8 (0.4–5.1)	Jacqz-Aigrain <i>et al.</i> (1993), Gorski <i>et al.</i> (1994), Kerry <i>et al.</i> (1994), Von Moltke <i>et al.</i> (1998), McGinnity <i>et al.</i> (1999)
Diazepam	N-demethylation	39.6 (19–100)	290 (240–350)	11.3 (3.5–15.6)	Yasumori <i>et al.</i> (1994), McGinnity <i>et al.</i> (1999)
Diclofenac	4-hydroxylation	7.8 (3.4–22)	3021 (715–8575)	519.8 (32.5–973)	Leemann <i>et al.</i> (1993), Transon <i>et al.</i> (1996), Carlile <i>et al.</i> (1998), Mankowski <i>et al.</i> (2000), Tang <i>et al.</i> (2000), Voorman <i>et al.</i> (2001), Madan <i>et al.</i> (2002)
Diltiazem	N-demethylation	53.0	12 500	236	Jones <i>et al.</i> (1999)
Erythromycin	N-demethylation	78.0	2000	25.6	Wang <i>et al.</i> (1997)
Etoposide	3-demethylation	53.9	249	4.6	Kawashiro <i>et al.</i> (1998)
Felodipine	ring oxidation	14.1 (9.2–19)	8658 (945–8185)	267 (945–8185)	Eriksson <i>et al.</i> (1991), Boobis <i>et al.</i> (1998)
Ibuprofen	2-hydroxylation	77.0	171	2.2	Carlile <i>et al.</i> (1998)
Ibuprofen	3-hydroxylation	110	576	5.2	Carlile <i>et al.</i> (1998)

(Continued)

Drug	Pathway	K_m^1	V_{max}^2	CL_{int}^3	Reference
Imipramine	2-hydroxylation	27 (25–31)	76 (53–95)	2.8 (1.7–3.8)	Skjelbo and Brosen (1992)
Imipramine	N-demethylation	975 (625–2000)	6794 (1333–16 667)	2.8 (1.2–4.4)	Skjelbo and Brosen (1992)
Lauric acid	ω -1-hydroxylation	107 (84–130)	1477 (120–2833)	23.2 (1.4–21.8)	Clarke <i>et al.</i> (1994), Amet <i>et al.</i> (1995)
Lornoxicam	5'-hydroxylation	3.6	433	12.0	Bonnabry <i>et al.</i> (1996)
MDMA	demethyleneation	1.5	7.5	6.8	Ramamoorthy <i>et al.</i> (2002)
Melatonin	6-hydroxylation	216	2246	10.4	Facciola <i>et al.</i> (2001)
Melatonin	N-acetylserotonin	99.2	191	1.9	Facciola <i>et al.</i> (2001)
Mexiletine	<i>p</i> -hydroxylation	15.8	17.0	1.1	Nakajima <i>et al.</i> (1998)
Mexiletine	2-hydroxylation	22.5	29.2	1.3	Nakajima <i>et al.</i> (1998)
Midazolam	1'-hydroxylation	6.47 (1.4–14)	8350 (35–29 800)	1690 (11–6143)	Gascon and Dayer (1991), Transon <i>et al.</i> (1996), Boobis <i>et al.</i> (1998), Maenpaa <i>et al.</i> (1998), Hamaoka <i>et al.</i> (1999), Oda <i>et al.</i> (1999), Martinez <i>et al.</i> (2000), Wang <i>et al.</i> (2000), Hamaoka <i>et al.</i> (2001), Nakajima <i>et al.</i> (2002)
Naproxen	O-demethylation	246 (92–400)	444 (350–538)	3.35 (0.9–5.8)	Tracy <i>et al.</i> (1997), McGinnity <i>et al.</i> (1999)
Nifedipine	oxidation	47.0	3360	71.5	Wang <i>et al.</i> (2000)
Omeprazole	5-hydroxylation	5.5 (3.5–9.8)	17.7 (0.1–88)	3.0 (0.01–15)	Chiba <i>et al.</i> (1993), Shu <i>et al.</i> (2000)
Paclitaxel	6-hydroxylation	88 (4–21)	550 (19–930)	60 (1.1–218)	Harris <i>et al.</i> (1994), Kumar <i>et al.</i> (1994), Rahman <i>et al.</i> (1994), Iwata <i>et al.</i> (1998), Madan <i>et al.</i> (2002)
Phenacetin	O-deethylation	23.7	505	71.6	Tassaneeyakul <i>et al.</i> (1993a), Rodrigues <i>et al.</i> (1997), Boobis <i>et al.</i> (1998), Eagling <i>et al.</i> (1998), Kobayashi <i>et al.</i> (1998), Mankowski <i>et al.</i> (2000), Nakajima <i>et al.</i> (2002), Venkatakrishnan <i>et al.</i> (2002)

Phenytoin	4-hydroxylation	20 (4.4–56)	23 (3.3–54)	2.3 (0.1–4.8)	Doecke <i>et al.</i> (1991), Boobis <i>et al.</i> (1998), Carlile <i>et al.</i> (1998), Nelson <i>et al.</i> (2001)
Proguanil	cycloguanil	102	18.3	0.2	Coller <i>et al.</i> (1999)
Rac-metoprolol	a-hydroxylation	33 (14–73)	76 (36–156)	3.4 (0.5–8.2)	Belpaire <i>et al.</i> (1998), Madani <i>et al.</i> (1999)
Rac-metoprolol	O-demethylation	94.5	138	1.5	Belpaire <i>et al.</i> (1998)
R-citalopram	N-demethylation	17.3	25	1.4	Rochat <i>et al.</i> (1997)
R-H259/31	sulfoxidation	735	380	0.5	Abelo <i>et al.</i> (2000)
R-H259/32	6-hydroxylation	196	430	2.2	Abelo <i>et al.</i> (2000)
R-H259/33	O-dealkylation	130	160	1.2	Abelo <i>et al.</i> (2000)
R-metoprolol	a-hydroxylation	33 (22–44)	41 (37–45)	1.4 (1–1.7)	Mautz <i>et al.</i> (1995), Ellis <i>et al.</i> (1996)
R-metoprolol	O-demethylation	23 (17–29)	172 (167–177)	8 (6.1–9.8)	Mautz <i>et al.</i> (1995), Ellis <i>et al.</i> (1996)
R-warfarin	4'-hydroxylation	181 (114–249)	11 (8.3–14)	0.08 (0.03–0.1)	Rettie <i>et al.</i> (1989).
R-warfarin	6-hydroxylation	865 (588–1270)	176 (108–291)	0.2 (0.1–0.4)	Rettie <i>et al.</i> (1989)
R-warfarin	7-hydroxylation	864 (498–1387)	31 (23–42)	0.05 (0.02–0.1)	Rettie <i>et al.</i> (1989)
R-warfarin	8-hydroxylation	469 (405–578)	32 (23–47)	0.08 (0.04–0.1)	Rettie <i>et al.</i> (1989)
R-warfarin	10-hydroxylation	398 (343–428)	50 (28–67)	0.2 (0.1–0.2)	Kunze <i>et al.</i> (1996)
S-citalopram	N-demethylation	33.8	20	0.6	Rochat <i>et al.</i> (1997)
S-H259/31	sulfoxidation	369	1040	2.8	Abelo <i>et al.</i> (2000)
S-H259/32	6-hydroxylation	35.9	300	8.4	Abelo <i>et al.</i> (2000)
S-H259/33	O-dealkylation	72.0	80	1.1	Abelo <i>et al.</i> (2000)
Simvastatin	6-ethoxymethylene SV	21 (5–36)	1075 (857–1293)	101 (36–166)	Prueksaritanont <i>et al.</i> (1997)
Simvastatin	3',5'-dihydrodiol SV	20 (5–35)	1399 (262–2536)	131 (59–73)	Prueksaritanont <i>et al.</i> (1997)
S-mephenytoin	4'-hydroxylation	50 (22–102)	170 (55–380)	4.5 (0.7–11)	Chiba <i>et al.</i> (1993), Hickman <i>et al.</i> (1998), Iwata <i>et al.</i> (1998), Coller <i>et al.</i> (1999), Mankowski <i>et al.</i> (2000), Madan <i>et al.</i> (2002), Venkatakrishnan <i>et al.</i> (2002)
S-metoprolol	a-hydroxylation	32 (26–38)	49 (47–51)	1.6 (1.3–1.8)	Mautz <i>et al.</i> (1995), Ellis <i>et al.</i> (1996)
S-metoprolol	O-demethylation	26 (16–36)	105 (104–107)	4.8 (2.9–6.7)	Mautz <i>et al.</i> (1995), Ellis <i>et al.</i> (1996)

(Continued)

Appendix 1. Continued.

Drug	Pathway	K_m ¹	V_{max} ²	CL _{int} ³	Reference
S-warfarin	7-hydroxylation	4.8 (2.8–11)	6.0 (0.01–11.5)	1.6 (0.002–3.2)	Rettie <i>et al.</i> (1989), Kunze <i>et al.</i> (1996), Hemeryck <i>et al.</i> (1999)
S-warfarin	6-hydroxylation	6.3 (2.6–15)	2.4 (1.8–3.2)	0.6 (0.1–1.0)	Rettie <i>et al.</i> (1989), Kunze <i>et al.</i> (1996)
S-warfarin	8-hydroxylation	360	3.65	0.0	Rettie <i>et al.</i> (1989)
Tacrine	1-OH	16.0	140	8.8	Becquemont <i>et al.</i> (1998)
Tacrine	2-OH	13.0	38	2.9	Becquemont <i>et al.</i> (1998)
Tacrine	4-OH	9.0	24	2.7	Becquemont <i>et al.</i> (1998)
Terfenadine	<i>t</i> -butyl hydroxylation	15 (14–15)	367 (273–460)	25 (20–31)	Jones <i>et al.</i> (1998), Wang <i>et al.</i> (2000)
Testosterone	6β-hydroxylation	84 (23–229)	10 056 (3448–32 340)	142 (21–498)	Boobis <i>et al.</i> (1998), Draper <i>et al.</i> (1998), Eagling <i>et al.</i> (1998), Iwata <i>et al.</i> (1998), Mankowski <i>et al.</i> (2000), Wang <i>et al.</i> (2000), Taguchi <i>et al.</i> (2001), Madan <i>et al.</i> (2002), Nakajima <i>et al.</i> (2002), Row, (2002)
Tolbutamide	4-hydroxylation	194 (74–449)	334 (117–625)	2.3 (0.3–6.1)	Purba <i>et al.</i> (1987), Miners <i>et al.</i> (1988), Doecke <i>et al.</i> (1991), Boobis <i>et al.</i> (1998), Carlile <i>et al.</i> (1998), Eagling <i>et al.</i> (1998), Hickman <i>et al.</i> (1998), Iwata <i>et al.</i> (1998), Lasker <i>et al.</i> (1998), Hemeryck <i>et al.</i> (1999), Venkatakrishnan <i>et al.</i> (2002)
Triazolam	a-hydroxylation	280	860	3.1	Kanamitsu <i>et al.</i> (2000)
Triazolam	4-hydroxylation	988	3350	3.4	Kanamitsu <i>et al.</i> (2000)
Zopiclone	N-oxidation	84	54	0.6	Becquemont <i>et al.</i> (1999)
Zopiclone	N-desmethylation	78	45	0.6	Becquemont <i>et al.</i> (1999)

¹μM, ²pmol metabolite min⁻¹ mg⁻¹ HLM protein, ³μl min⁻¹ mg⁻¹ HLM protein.

Appendix 2. rhCYP kinetic parameters and ISEFs.

Drug	Route	CYP	rhCYP (K_m^1)	rhCYP (V_{max}^2)	rhCYP (CL_{int}^3)	V_{max} (RAF^5)	HLM (CL_{int}^6)	CL_{int} (RAF^5)	ISEF (V_{max})	ISEF (CL_{int})	System	Reference
7-Benzoyloxy-4-trifluoromethyl-coumarin	7-hydroxylation (debenzylation)	1A2	4.1	11.10	2.71	8.9	10.94	4.0	0.2	0.1	L	Renwick <i>et al.</i> (2000)
7-Ethoxyresorufin	O-deethylation	1A2	1.7	1.70	1.00	67.1	231	231	1.6	5.5	E	Parikh <i>et al.</i> (1999)
7-Ethoxyresorufin	O-deethylation	1A2	27.0	0.90	0.03	0.7	0.22	6.5	0.02	0.2	E	Yun <i>et al.</i> (2000)
7-Ethoxyresorufin	O-deethylation	1A2	3.0	0.23	0.08	2.7	0.22	2.8	0.1	0.1	E	Shimada <i>et al.</i> (1997)
7-Ethoxyresorufin	O-deethylation	1A2	1.7	1.00	0.60	114	231	382	2.7	9.1	E	Iwata <i>et al.</i> (1998)
7-Ethoxyresorufin	O-deethylation	1A2	1.0	0.60	0.60	190	231	385	4.5	9.2	E	McGinnity <i>et al.</i> (1999)
7-Ethoxyresorufin	O-deethylation	1A2	0.5	0.40	0.80	285	231	289	6.8	6.9	L	McGinnity <i>et al.</i> (1999)
7-Ethoxyresorufin	O-deethylation	1A2	1.1	0.90	0.86	127	231	270	3.0	6.4	Y	Masimirembwa <i>et al.</i> (1999)
7-Ethoxyresorufin	O-deethylation	1A2	1.4	2.01	1.46	56.7	231	158	1.4	3.8	Y	Ching <i>et al.</i> (2001)
7-Ethoxyresorufin*	O-deethylation	1A2	1.7	2.72	1.63	41.9	231	142	1.0	3.4	Y	Eugster <i>et al.</i> (1993)
Amiodarone	N-deethylation	1A2	41.9	5.00	0.12	76.0	0.52	4.3	1.8	0.1	B	Ohyama <i>et al.</i> (2000)
Bufuralol	1'-hydroxylation	1A2	29.0	0.37	0.01	331	3.87	303	7.9	7.2	B	Shimada <i>et al.</i> (2001)
Bufuralol	6-hydroxylation	1A2	28.0	0.67	0.02	39.4	0.25	10.3	0.9	0.2	B	Shimada <i>et al.</i> (2001)
Caffeine	theobromine (37X)	1A2	160	0.05	0.0003	1483	0.08	256	35.3	6.1	L	Ha <i>et al.</i> (1996)
Caffeine	theophylline (13X)	1A2	250	0.02	0.000 08	17 136	0.04	527	408	12.5	L	Ha <i>et al.</i> (1996)
Caffeine	paraxanthine (17X)	1A2	190	0.51	0.00	491.9	0.47	175	11.7	4.2	L	Ha <i>et al.</i> (1996)
Chlorzoxazone	6-hydroxylation	1A2	17.0	2.20	0.13	1583	40.14	310	37.7	7.4	B	Shimada <i>et al.</i> (1999)
Melatonin	6-hydroxylation	1A2	85.5	977	11.42	2.3	10.41	0.9	0.1	0.02	Y*	Facciola <i>et al.</i> (2001)
Melatonin	N-acetylserotonin	1A2	144	17.40	0.12	10.9	1.92	15.9	0.3	0.4	Y*	Facciola <i>et al.</i> (2001)
Mexiletine	p-hydroxylation	1A2	13.9	0.005	0.0003	3615	1.08	3180	86.0	75.7	L	Nakajima <i>et al.</i> (1998)
Mexiletine	2-hydroxylation	1A2	15.2	0.01	0.0004	4794	1.30	3239	114	77.1	L	Nakajima <i>et al.</i> (1998)
Phenacetin	O-deethylation	1A2	28.0	20.0	0.71	25.3	71.6	100	0.6	2.4	B	Kobayashi <i>et al.</i> (1999)
Phenacetin	O-deethylation	1A2	47.0	43.0	0.91	11.8	71.6	78.3	0.3	1.9	B	Mankowski <i>et al.</i> (2000)
Phenacetin	O-deethylation	1A2	48.0	1.10	0.02	212	4.32	189	5.0	4.5	E	Parikh <i>et al.</i> (1999)
Phenacetin	O-deethylation	1A2	13.0	1.80	0.14	281	71.6	517	6.7	12.3	E	Yun <i>et al.</i> (2000)
Phenacetin	O-deethylation	1A2	16.0	1.80	0.11	281	71.6	636	6.7	15.1	E	Yun <i>et al.</i> (2000)
Phenacetin	O-deethylation	1A2	46.0	0.92	0.02	551	71.6	3593	13.1	85.5	L	Rodrigues <i>et al.</i> (1997)
Phenacetin	O-deethylation	1A2	12.0	2.80	0.23	180.5	71.6	307	4.3	7.3	L	Kobayashi <i>et al.</i> (1998)
Phenacetin	O-deethylation	1A2	22.0	1.86	0.08	272	71.6	847	6.5	20.2	L	Katoh <i>et al.</i> (2000)
Phenacetin	O-deethylation	1A2	116	2.08	0.02	243	71.6	3993	5.8	95.1	Y	Ching <i>et al.</i> (2001)
R-H259/31	sulfoxidation	1A2	47.0	0.28	0.01	1357	0.52	86.8	32.3	2.1	L	Abelo <i>et al.</i> (2000)

(Continued)

Appendix 2. Continued.

Drug	Route	CYP	rhCYP (K_m^{-1})	rhCYP (V_{max}^{-2})	rhCYP (CL_{int}^{-3})	V_{max} (RAF^5)	HLM (CL_{int}^{-6})	CL_{int} (RAF^5)	ISEF (V_{max})	ISEF (CL_{int})	System	Reference
R-H259/33	6-hydroxylation	1A2	652	0.15	0.00	2867	2.19	9536	68.2	227	L	Abelo <i>et al.</i> (2000)
R-H259/35	O-dealkylation	1A2	47.0	0.05	0.00	3200	1.23	1157	76.2	27.5	L	Abelo <i>et al.</i> (2000)
R-warfarin	6-hydroxylation	1A2	1600	1.46	0.00	121	0.23	249	2.9	5.9	L	Zhang <i>et al.</i> (1995)
R-warfarin	8-hydroxylation	1A2	1400	0.29	0.0002	108	0.07	343	2.6	8.2	L	Zhang <i>et al.</i> (1995)
R-warfarin	6-hydroxylation	1A2	1623	17.63	0.01	10.0	0.23	20.8	0.2	0.5	L	Renwick <i>et al.</i> (2000)
R-warfarin	7-hydroxylation	1A2	1754	3.26	0.002	9.4	0.05	24.4	0.2	0.6	L	Renwick <i>et al.</i> (2000)
R-warfarin	8-hydroxylation	1A2	1685	2.64	0.002	11.9	0.07	45.5	0.3	1.1	L	Renwick <i>et al.</i> (2000)
S-H259/31	sulfoxidation	1A2	43.0	0.26	0.01	4000	2.82	466	95.2	11.1	L	Abelo <i>et al.</i> (2000)
S-H259/33	6-hydroxylation	1A2	33.8	0.07	0.002	4286	8.36	4035	102	96.0	L	Abelo <i>et al.</i> (2000)
S-H259/35	O-dealkylation	1A2	34.0	4.00	0.12	20.0	1.11	9.4	0.5	0.2	L	Abelo <i>et al.</i> (2000)
Tacrine	1-OH	1A2	14.0	2.54	0.18	55.1	8.75	48.2	1.3	1.1	Y	Becquemont <i>et al.</i> (1998)
Tacrine	2-OH	1A2	11.0	0.40	0.04	95.0	2.92	80.4	2.3	1.9	Y	Becquemont <i>et al.</i> (1998)
Tacrine	4-OH	1A2	35.0	0.41	0.01	58.5	2.67	228	1.4	5.4	Y	Becquemont <i>et al.</i> (1998)
7-Ethoxyresorufin	O-deethylation	1A2*	0.1	1.47	17.0	77.4	231	13.6	1.8	0.3	B	Nakajima <i>et al.</i> (2002)
Phenacetin	O-deethylation	1A2*	9.2	15.01	1.63	33.7	71.60	43.8	0.8	1.0	B	Nakajima <i>et al.</i> (2002)
Dextromethorphan	O-demethylation	2C19	219	1.30	0.01	85.7	1.29	218	15.3	38.9	B	Yu and Haining, (2001)
Dextromethorphan	N-demethylation	2C19	237	1.90	0.01	303	1.81	225	54.1	40.2	B	Yu and Haining, (2001)
Dextromethorphan	N-demethylation	2C19	49.2	1.45	0.03	397	1.81	61.3	70.9	10.9	L	Venkatakrishnan <i>et al.</i> (1998b)
Dextromethorphan	O-demethylation	2C19	32.9	0.84	0.03	213	40.7	1593	38.1	285	L	Venkatakrishnan <i>et al.</i> (1998b)
Diazepam	N-demethylation	2C19	17.0	1.00	0.06	290	11.28	191.8	51.8	34.2	B	McGinnity <i>et al.</i> (1999)
Diazepam	N-demethylation	2C19	31.0	3.70	0.12	78.4	11.28	94.5	14.0	16.9	E	McGinnity <i>et al.</i> (1999)
Diazepam	N-demethylation	2C19	60.0	0.23	0.004	1522	3.50	913	272	163	L	McGinnity <i>et al.</i> (1999)
Omeprazole	5-hydroxylation	2C19	6.6	8.30	1.26	2.1	2.95	2.3	0.4	0.4	B	Yamazaki <i>et al.</i> (1997)
Proguanil	cycloguanil	2C19	7.7	0.42	0.05	44.0	0.18	3.3	0.03	0.6	L	Coller <i>et al.</i> (1999)
R-citalopram	N-demethylation	2C19	211	0.89	0.004	28.2	1.45	345	0.3	61.5	L	Rochat <i>et al.</i> (1997)
R-H259/33	6-hydroxylation	2C19	2.8	1.97	0.71	218	2.19	3.1	0.4	0.6	L	Abelo <i>et al.</i> (2000)
R-H259/35	O-dealkylation	2C19	5.5	0.40	0.07	400	1.23	17.0	0.2	3.0	L	Abelo <i>et al.</i> (2000)
S-citalopram	N-demethylation	2C19	198	1.30	0.01	118	0.83	126	0.1	22.5	L	Rochat <i>et al.</i> (1997)
S-H259/33	6-hydroxylation	2C19	1.1	1.79	1.67	168	8.36	5.0	1.5	0.9	L	Abelo <i>et al.</i> (2000)
S-H259/35	O-dealkylation	2C19	0.5	0.37	0.76	216	1.11	1.5	0.2	0.3	L	Abelo <i>et al.</i> (2000)
S-mephenytoin	4'-hydroxylation	2C19	32.0	11.0	0.34	15.4	4.49	13.1	2.8	2.3	B	Mankowski <i>et al.</i> (2000)
S-mephenytoin	4'-hydroxylation	2C19	24.8	6.39	0.26	26.6	4.49	17.4	4.7	3.1	E	Iwata <i>et al.</i> (1998)
S-mephenytoin	4'-hydroxylation	2C19	21.7	1.88	0.09	90.4	4.49	51.8	0.8	9.3	L	Katoh <i>et al.</i> (2000).
S-mephenytoin	4'-hydroxylation	2C19	70.0	9.17	0.13	18.5	4.49	34.3	0.8	6.1	L	Coller <i>et al.</i> (1999).
S-mephenytoin	4-hydroxylation	2C19	54.0	2.14	0.04	79.4	4.49	113	14.2	20.2	Y	Masimirembwa <i>et al.</i> (1999)

Amiodarone	N-deethylation	2C19*	17.5	1.30	0.07	292	0.52	7.0	52.2	1.2	B	Ohyama <i>et al.</i> (2000)
ATRA (all- <i>trans</i> -retinoic acid)	4-hydroxylation	2C8	6.1	0.18	0.03	41.7	6.81	231	0.8	4.6	L	Nadin and Murray (1999)
Paclitaxel	6a-hydroxylation	2C8	4.9	1.14	0.23	482	60.4	259	9.6	5.2	Y	Masimirembwa <i>et al.</i> (1999)
Zopiclone	N-oxidation	2C8	59.0	1.00	0.02	54.0	0.64	37.9	1.1	0.8	Y	Bequemont <i>et al.</i> (1999)
Zopiclone	N-desmethylation	2C8	71.0	2.50	0.04	18.0	0.58	16.4	0.4	0.3	Y	Bequemont <i>et al.</i> (1999)
Amiodarone	N-deethylation	2C8*	8.6	2.30	0.27	165	0.52	1.9	3.3	0.0	B	Ohyama <i>et al.</i> (2000)
ATRA (all- <i>trans</i> -retinoic acid)	4-hydroxylation	2C9	100	0.38	0.004	19.7	6.81	1791	0.6	55.9	L	Nadin and Murray (1999)
Dextromethorphan	O-demethylation	2C9	222	3.40	0.02	32.8	1.29	84.5	1.0	2.6	B	Yu and Haining, (2001)
Dextromethorphan	N-demethylation	2C9	343	1.00	0.003	576	1.81	619	18.0	19.3	B	Yu and Haining, (2001)
Dextromethorphan	N-demethylation	2C9	254	2.10	0.01	274	1.81	218	8.6	6.8	L	Von Moltke <i>et al.</i> (1998)
Dextromethorphan	O-demethylation	2C9	229	7.16	0.03	25.0	40.7	1301	0.8	40.6	L	Venkatakrishnan <i>et al.</i> (1998b)
Diclofenac	4-hydroxylation	2C9	8.0	55.0	6.88	54.9	520	75.6	1.7	2.4	B	Mankowski <i>et al.</i> (2000)
Diclofenac	4'-hydroxylation	2C9	1.8	12.5	6.94	242	520	74.9	7.6	2.3	Y	Yamazaki <i>et al.</i> (1998)
Diclofenac	4-hydroxylation	2C9	8.3	15.0	1.81	201	520	288	6.3	9.0	Y	Masimirembwa <i>et al.</i> (1999)
Diclofenac	4-hydroxylation	2C9	15.0	24.0	1.60	126	520	325	3.9	10.2	Y	Mancy <i>et al.</i> (1999)
Diclofenac	4-hydroxylation	2C9	2.0	12.4	6.11	244	520	85.1	7.6	2.7	Y	Ieiri <i>et al.</i> (2000)
Diclofenac	4-hydroxylation	2C9	3.9	35.6	9.13	84.9	520	57.0	2.7	1.8	Y	Takanashi <i>et al.</i> (2000)
Melatonin*	N-acetylserotonin	2C9	282.2	117	0.42	1.6	1.92	4.6	0.1	0.1	Y*	Facciola <i>et al.</i> (2001)
Naproxen	O-demethylation	2C9	307	6.70	0.02	74.0	2.34	107	2.3	3.4	E	McGinnity <i>et al.</i> (1999)
Naproxen	O-demethylation	2C9	116	4.90	0.04	101	2.34	55.4	3.2	1.7	L	McGinnity <i>et al.</i> (1999)
Phenytoin	4'-hydroxylation	2C9	15.0	0.19	0.01	123	2.27	178	3.9	5.6	Y	Takanashi <i>et al.</i> (2000)
R-H259/31	sulfoxidation	2C9	22.5	0.12	0.01	3220	0.52	98.6	101	3.1	L	Abelo <i>et al.</i> (2000)
R-H259/33	6-hydroxylation	2C9	67.0	0.31	0.005	1387	2.19	474	43.3	14.8	L	Abelo <i>et al.</i> (2000)
R-H259/35	O-dealkylation	2C9	14.0	0.39	0.03	410	1.23	44.2	12.8	1.4	L	Abelo <i>et al.</i> (2000)
S-H259/31	sulfoxidation	2C9	38.3	0.42	0.01	2476	2.82	257	77.4	8.0	L	Abelo <i>et al.</i> (2000)
S-H259/33	6-hydroxylation	2C9	39.2	1.66	0.04	181	8.36	197	5.6	6.2	L	Abelo <i>et al.</i> (2000)
S-H259/35	O-dealkylation	2C9	26.3	0.67	0.03	119	1.11	43.6	3.7	1.4	L	Abelo <i>et al.</i> (2000)
S-warfarin	7-hydroxylation	2C9	0.8	0.003	0.004	2007	1.61	418	62.7	13.1	L	Katoh <i>et al.</i> (2000)
S-warfarin	7-hydroxylation	2C9	18.0	0.22	0.01	27.4	1.61	132	0.9	4.1	Y	Yamazaki <i>et al.</i> (1998)
S-warfarin	7-hydroxylation	2C9	5.8	0.25	0.04	24.3	1.61	37.6	0.8	1.2	Y	Takanashi <i>et al.</i> (2000)
Tolbutamide	4-hydroxylation	2C9	192	4.59	0.02	72.7	2.27	95.1	2.3	3.0	E	Iwata <i>et al.</i> (1998)
Tolbutamide	hydroxylation	2C9	151	9.20	0.06	36.3	2.27	37.3	1.1	1.2	Y	Takanashi <i>et al.</i> (2000)
Tolbutamide	hydroxylation	2C9	145	0.61	0.004	547	2.27	540	17.1	16.9	Y	Sullivan-Klose <i>et al.</i> (1996)

(Continued)

Appendix 2. Continued.

Drug	Route	CYP	rhCYP (K_m^{-1})	rhCYP (V_{max}^{-2})	rhCYP (CL_{int}^{-3})	V_{max} (RAF^{-5})	HLM (CL_{int}^{-6})	CL_{int} (RAF^{-5})	ISEF (V_{max})	ISEF (CL_{int})	System	Reference
Bufuralol	1'-hydroxylation	2D6	3.4	128	37.7	1.0	3.87	0.1	0.1	0.01	B	Mankowski <i>et al.</i> (2000)
Bufuralol	1'-hydroxylation	2D6	13.0	5.10	0.39	24.0	3.87	9.9	2.7	1.1	B	Shimada <i>et al.</i> (2001)
Bufuralol	6-hydroxylation	2D6	19.0	2.00	0.11	13.2	0.25	2.3	1.5	0.3	B	Shimada <i>et al.</i> (2001)
Bufuralol	1'-hydroxylation	2D6	11.7	80.9	6.94	1.5	3.87	0.6	0.2	0.1	B	Zanger <i>et al.</i> (2001)
Bufuralol	1'-hydroxylation	2D6	33.5	2.04	0.06	60.0	3.87	63.5	6.6	7.0	E	Iwata <i>et al.</i> (1998)
Bufuralol	1'-hydroxylation	2D6	11.1	13.3	1.20	9.2	3.87	3.2	1.0	0.4	E	Pritchard <i>et al.</i> (1998)
Bufuralol	1'-hydroxylation	2D6	3.1	2.03	0.65	60.3	3.87	5.9	6.7	0.7	L	Katoh <i>et al.</i> (2000)
Bufuralol	1'-hydroxylation	2D6	15.0	8.00	0.53	15.3	3.87	7.3	1.7	0.8	Y	Masimirembwa <i>et al.</i> (1999)
Chlorzoxazone	6-hydroxylation	2D6	700	5.40	0.01	645	40.1	5203	71.3	575	B	Shimada <i>et al.</i> (1999)
Debrisoquine	4-hydroxylation	2D6	12.1	18.2	1.50	2.5	0.41	0.3	0.3	0.03	L	Granvil <i>et al.</i> (2002)
Debrisoquine	4-hydroxylation	2D6	29.0	1.66	0.06	27.7	0.41	7.2	3.1	0.8	Y	S. W. Ellis, personal communication, (2002)
Debrisoquine	4-hydroxylation	2D6	14.9	2.11	0.14	21.8	0.41	2.9	2.4	0.3	Y	Lightfoot <i>et al.</i> (2000)
Dextromethorphan	O-demethylation	2D6	0.7	36.0	51.4	5.0	40.7	0.8	0.6	0.1	B	Mankowski <i>et al.</i> (2000)
Dextromethorphan	O-demethylation	2D6	4.0	1.68	0.42	107	40.7	97.2	11.8	10.7	B	Ramamoorthy <i>et al.</i> (2002)
Dextromethorphan	O-demethylation	2D6	3.7	11.9	3.22	15.1	40.7	12.6	1.7	1.4	B	Yu and Haining, (2001)
Dextromethorphan	N-demethylation	2D6	1290	38.5	0.03	15.0	1.81	60.5	1.7	6.7	B	Yu and Haining, (2001)
Dextromethorphan	O-demethylation	2D6	6.5	6.58	1.01	27.2	40.7	40.2	3.0	4.4	B	Abdel-Rahman <i>et al.</i> (1999)
Dextromethorphan	O-demethylation	2D6	2.3	4.70	2.04	38.1	40.7	19.9	4.2	4.5	E	McGinnity <i>et al.</i> (1999)
Dextromethorphan	O-demethylation	2D6	1.3	4.40	3.38	40.7	40.7	12.0	4.5	1.3	L	McGinnity <i>et al.</i> (1999)
Dextromethorphan	N-demethylation	2D6	838	4.07	0.005	142	1.81	372	15.6	41.1	L	Venkatakrishnan <i>et al.</i> (1998b)
Dextromethorphan	O-demethylation	2D6	5.2	10.8	2.08	16.6	40.7	19.6	1.8	2.2	L	Venkatakrishnan <i>et al.</i> (1998b)
Dextromethorphan	O-demethylation	2D6	1.7	4.85	2.85	37.0	40.7	14.3	4.1	1.6	Y	Ellis <i>et al.</i> (1996)
MDMA	demethylenation	2D6	4.1	3.28	0.80	2.3	6.81	8.5	0.3	0.9	B	Ramamoorthy <i>et al.</i> (2002)
Mexiletine	<i>p</i> -hydroxylation	2D6	22.6	2.25	0.10	7.6	1.08	10.8	0.8	1.2	L	Nakajima <i>et al.</i> (1998)
Mexiletine	2-hydroxylation	2D6	22.1	2.49	0.11	11.7	1.30	11.5	1.3	1.3	L	Nakajima <i>et al.</i> (1998)
<i>R</i> (+)-metoprolol	O-demethylation	2D6	46.0	4.39	0.10	39.1	7.95	83.3	4.3	9.2	Y	Ellis <i>et al.</i> (1996)
<i>R</i> (+)-metoprolol	a-hydroxylation	2D6	31.0	0.75	0.02	54.4	1.34	55.6	6.0	6.1	Y	Ellis <i>et al.</i> (1996)
Rac-metoprolol	O-demethylation	2D6	40.0	5.90	0.15	23.4	1.46	9.9	2.6	1.1	Y	S. W. Ellis, personal communication, (2002)

Rac-metoprolol	a-hydroxylation	2D6	40.0	1.70	0.04	44.6	3.43	80.7	4.9	8.9	Y	S. W. Ellis, personal communication, (2002)
R-metoprolol	a-hydroxylation	2D6	10.0	11.7	1.17	3.5	1.34	1.2	0.4	0.1	L	Mautz <i>et al.</i> (1995)
R-metoprolol	O-demethylation	2D6	7.0	50.0	7.14	3.4	7.95	1.1	0.4	0.1	L	Mautz <i>et al.</i> (1995)
S-(–)-metoprolol	O-demethylation	2D6	46.0	2.71	0.06	38.9	4.78	81.1	4.3	9.0	Y	Ellis <i>et al.</i> (1996)
S-(–)-metoprolol	a-hydroxylation	2D6	42.0	1.12	0.03	43.6	1.57	58.8	4.8	6.5	Y	Ellis <i>et al.</i> (1996)
S-metoprolol	a-hydroxylation	2D6	10.0	12.0	1.20	4.1	1.57	1.3	0.4	0.1	L	Mautz <i>et al.</i> (1995)
S-metoprolol	O-demethylation	2D6	7.0	24.0	3.43	4.4	4.78	1.4	0.5	0.2	L	Mautz <i>et al.</i> (1995)
S-metoprolol	a-hydroxylation	2D6	36.0	1.45	0.04	33.7	1.57	38.9	3.7	4.3	Y	S. W. Ellis, personal communication, (2002)
S-metoprolol	O-demethylation	2D6	33.0	3.51	0.11	30.0	4.78	44.9	3.3	5.0	Y	S. W. Ellis, personal communication, (2002)
Amiodarone	N-deethylation	2D6*	21.0	2.20	0.10	173	0.52	4.9	19.1	0.5	B	Ohyama <i>et al.</i> (2000)
Bufuralol	1'-hydroxylation	2D6*	1.8	10.29	5.69	11.9	3.87	0.7	1.3	0.1	B	Nakajima <i>et al.</i> (2002)
Bufuralol	1'-hydroxylation	2D6*	11.5	74.5	6.48	1.6	3.87	0.6	0.2	0.1	B	Zanger <i>et al.</i> (2001)
Bufuralol	1'-hydroxylase	2D6*	7.1	1.00	0.14	122	3.87	27.5	13.5	3.0	Y	Nakamura <i>et al.</i> (2002)
Debrisoquine	4-hydroxylation	2D6*	22.1	1.11	0.05	41.4	0.41	8.1	4.6	0.9	B	Nakajima <i>et al.</i> (2002)
Dextromethorphan	O-demethylation	2D6*	1.2	0.11	0.09	1675	40.7	456	185	50.4	Y	Nakamura <i>et al.</i> (2002)
Caffeine	theobromine (37X)	2D6-met	15 900	0.28	0.000 02	237	0.00	186.4	26.2	20.6	L	Ha <i>et al.</i> (1996)
Caffeine	theophylline (13X)	2D6-met	12 500.0	0.63	0.000 05	133.2	0.00	86.7	14.7	9.6	L	Ha <i>et al.</i> (1996)
Caffeine	paraxanthine (17X)	2D6-met	11 000.0	0.56	0.000 05	328.7	0.01	120.5	36.3	13.3	L	Ha <i>et al.</i> (1996)
4-Nitrophenol	hydroxylation	2E1	197.0	22.60	0.11	45.4	35.94	313.3	0.9	6.2	E	Pritchard <i>et al.</i> (1998)
Caffeine	theobromine (37X)	2E1	1440.0	0.01	0.00	9272.5	0.08	14 382.6	184.6	286.3	L	Ha <i>et al.</i> (1996)
Chlorzoxazone	6-hydroxylation	2E1	660.0	22.00	0.03	158.3	40.14	1204.2	0.8	24.0	B	Shimada <i>et al.</i> (1999)
Chlorzoxazone	6-hydroxylation	2E1	177.0	2.80	0.02	1244.1	40.14	2537.5	24.8	50.5	Y	Masimirembwa <i>et al.</i> (1999)
Phenacetin	O-deethylation	2E1	785.0	4.30	0.01	117.5	71.60	13 071.2	1.4	260.2	B	Kobayashi <i>et al.</i> (1999)
Phenacetin	O-deethylation	2E1	240	7.91	0.03	63.9	71.6	2172	1.3	43.2	L	Snawder <i>et al.</i> (1994)

(Continued)

Appendix 2. Continued.

Drug	Route	CYP	rhCYP (K_m^{-1})	rhCYP (V_{max}^{-2})	rhCYP (CL_{int}^{-3})	V_{max} (RAF^5)	HLM (CL_{int}^{-6})	CL_{int} (RAF^5)	ISEF (V_{max})	ISEF (CL_{int})	System	Reference
7-Benzoyloxy-4-trifluoromethyl-coumarin	7-hydroxylation (debenzylation)	3A4	26.0	61.2	2.35	1.6	10.9	4.6	0.02	0.1	L	Renwick <i>et al.</i> (2000)
ATRA (all- <i>trans</i> -retinoic acid)	4-hydroxylation	3A4	74.0	0.56	0.01	13.4	6.81	899	0.2	11.5	L	Nadin and Murray (1999)
Buprenorphine	N-dealkylation	3A4	23.7	592	24.98	1.2	18.12	0.7	0.02	0.01	L	Kobayashi <i>et al.</i> (1998)
Dextromethorphan	O-demethylation	3A4	18.0	2.50	0.14	36.5	19.0	137	0.5	1.8	E	McGinnity <i>et al.</i> (1999)
Dextromethorphan	N-demethylation	3A4	38.0	1.60	0.04	360	1.81	42.9	4.6	0.6	L	McGinnity <i>et al.</i> (1999)
Dextromethorphan	N-demethylation	3A4	1155	11.9	0.01	48.4	1.81	175	0.6	2.2	L	Venkatakrishnan <i>et al.</i> (1998b)
Diltiazem	N-demethylation	3A4	17.0	121	7.12	103	236	33.1	3.0	0.4	B	Jones <i>et al.</i> (1999)
Erythromycin	N-demethylation	3A4	46.0	2.00	0.04	1000	25.6	590	12.8	7.6	L*	Wang <i>et al.</i> (1997)
Etoposide	3-demethylation	3A4	34.4	1.22	0.04	205	4.62	131	2.6	1.7	L	Kawashiro <i>et al.</i> (1998)
Midazolam	1'-hydroxylation	3A4	4.0	5.00	1.25	1518	1536	1229	19.5	15.8	L	Hamaoka <i>et al.</i> (1999)
Midazolam	1'-hydroxylation	3A4	3.6	4.70	1.31	1615	1536	1177	20.7	15.1	L	Oda <i>et al.</i> (1999)
Midazolam	1'-hydroxylation	3A4	1.7	3.30	1.94	2300	1536	791	29.5	10.2	L	Hamaoka <i>et al.</i> (2001)
Midazolam	1'-hydroxylation	3A4	3.0	8.27	2.76	918	1536	557	11.8	7.1	L*	Wang <i>et al.</i> (2000)
Omeprazole	5-hydroxylation	3A4	60.0	2.40	0.04	9.7	0.22	5.5	0.1	0.1	B	Yamazaki <i>et al.</i> (1997)
Omeprazole	sulfoxidation	3A4	140	13.0	0.09	1.0	1.33	14.3	0.01	0.2	B	Yamazaki <i>et al.</i> (1997)
R-H259/31	sulfoxidation	3A4	508	3.90	0.01	97.4	0.52	67.3	1.2	0.9	L	Abelo <i>et al.</i> (2000)
R-H259/33	6-hydroxylation	3A4	628	1.27	0.002	339	2.19	1085	4.3	13.9	L	Abelo <i>et al.</i> (2000)
R-H259/35	O-dealkylation	3A4	1215	1.00	0.001	160	1.23	1495	2.1	19.2	L	Abelo <i>et al.</i> (2000)
R-warfarin	10-hydroxylation	3A4	369	101	0.27	0.5	0.13	0.5	0.01	0.01	L	Kunze <i>et al.</i> (1996)
S-H259/31	sulfoxidation	3A4	326	17.6	0.05	59.1	2.82	52.2	0.8	0.7	L	Abelo <i>et al.</i> (2000)
S-H259/33	6-hydroxylation	3A4	355	0.70	0.002	429	8.36	4238	5.5	54.4	L	Abelo <i>et al.</i> (2000)
S-H259/35	O-dealkylation	3A4	1000	0.67	0.001	119	1.11	1658	1.5	21.3	L	Abelo <i>et al.</i> (2000)
Terfenadine	hydroxylation	3A4	9.0	1.26	0.14	292	25.1	180	3.7	2.3	L	Jones <i>et al.</i> (1998)
Terfenadine	hydroxylation	3A4	14.0	1.00	0.07	367	25.1	351	4.7	4.5	L*	Wang <i>et al.</i> (2000)
Testosterone	6 β -hydroxylation	3A4	33.0	83.8	2.54	114	137	53.8	1.5	0.7	B	Lee <i>et al.</i> (1995)
Testosterone	6 β -hydroxylation	3A4	60.0	59.0	0.98	161	137	139.0	1.8	1.8	B	Mankowski <i>et al.</i> (2000)
Testosterone	6 β -hydroxylation	3A4	70.8	59.7	0.84	159	137	162	2.0	2.1	E	Iwata <i>et al.</i> (1998)
Testosterone	6 β -hydroxylation	3A4	33.5	15.3	0.46	620	137	298	8.0	3.8	L	Katoh <i>et al.</i> (2000)

Testosterone	6 β -hydroxylation*	3A4	56.0	31.8	0.57	299	137	241	3.8	3.1	L*	Wang <i>et al.</i> (2000)
Testosterone	6 β -hydroxylation*	3A4	66.0	27.3	0.41	349	137	331	4.5	4.2	L*	Wang <i>et al.</i> (1997)
Testosterone	6 β -hydroxylation	3A4	55.0	8.00	0.15	1189	137	939	15.2	12.1	Y	Masimirembwa <i>et al.</i> (1999)
Triazolam	a-hydroxylation	3A4	176	3.84	0.02	224	3.07	141	0.04	1.8	L	Kanamitsu <i>et al.</i> (2000)
Triazolam	4-hydroxylation	3A4	652	20.3	0.03	165	3.39	109	0.04	1.4	L	Kanamitsu <i>et al.</i> (2000)
Amiodarone	N-deethylation	3A4*	54.2	18.9	0.35	20.1	0.52	1.5	0.01	0.02	B	Ohyaama <i>et al.</i> (2000)
Bufuralol	6-hydroxylation	3A4*	300	0.64	0.002	41.3	0.25	116	0.003	1.5	B	Ohyama <i>et al.</i> (2000)
Dextromethorphan	O-demethylation	3A4*	157	38.5	0.25	2.9	1.29	5.3	0.02	0.1	B	Yu and Haining, (2001)
Dextromethorphan	N-demethylation	3A4*	232	21.3	0.09	27.0	1.81	19.7	0.02	0.3	B	Yu and Haining, (2001)
Diltiazem	N-demethylation	3A4*	16.0	585	36.6	21.4	236	6.5	3.0	0.1	B	Jones <i>et al.</i> (1999)
Testosterone	6 β -hydroxylation	3A4*	72.0	27.0	0.38	352	137	364	4.5	4.7	B	Yamazaki <i>et al.</i> (1997).
Midazolam	1'-hydroxylation	3A4**	1.6	17.0	10.4	448	1536	148	5.7	1.9	B	Nakajima <i>et al.</i> (2002)
Testosterone	6 β -hydroxylation	3A4**	44.7	71.7	1.60	133	137	85.2	1.7	1.1	B	Nakajima <i>et al.</i> (2002)
Bufuralol	1'-hydroxylation		11.8	87.2	7.39	1.4	3.87	0.5	0.2	0.1	B	Zanger <i>et al.</i> (2001)

¹ μ M, ²pmol min⁻¹ pmol⁻¹ rhCYP, ³ μ l min⁻¹ pmol⁻¹ rhCYP, ⁴pmol min⁻¹ mg⁻¹ HLM pr-, ⁵pmol rhCYP mg⁻¹ HLM pr-, ⁶ μ l min⁻¹ mg⁻¹ HLM pr-, ⁷pmol hCYP mg⁻¹ HLM pr-.

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***In vivo* metabolism of the new designer drug 1-(4-methoxyphenyl)piperazine (MeOPP) in rat and identification of the human cytochrome P450 enzymes responsible for the major metabolic step**

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1. The *in vivo* metabolism of 1-(4-methoxyphenyl)piperazine (MeOPP), a novel designer drug, was studied in male Wistar rats.

2. MeOPP was mainly *O*-demethylated to 1-(4-hydroxyphenyl)piperazine (4-HO-PP) in addition to degradation of the piperazine moiety.

3. *O*-demethylation, the major metabolic step, was studied with cDNA-expressed human hepatic cytochrome P450 (CYP) enzymes in pooled human liver microsomes (pHLM) and in single donor human liver microsomes with CYP2D6 poor metabolizer genotype (PM HLM).

4. CYP2D6 catalysed *O*-demethylation with apparent K_m and V_{max} values of $48.34 \pm 14.48 \mu\text{M}$ and $5.44 \pm 0.47 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ CYP, respectively. pHLM catalysed the monitored reaction with an apparent $K_m = 204.80 \pm 51.81 \mu\text{M}$ and $V_{max} = 127.50 \pm 13.25 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein.

5. The CYP2D6-specific chemical inhibitor quinidine (1 and $3 \mu\text{M}$) significantly inhibited 4-HO-PP formation by $71.9 \pm 4.8\%$ and by $98.5\% \pm 0.5\%$, respectively, in incubation mixtures with pHLM and $200 \mu\text{M}$ MeOPP.

6. *O*-demethylation was significantly lower in PM HLM compared with pHLM ($70.6\% \pm 7.2\%$).

7. These data suggest that polymorphically expressed CYP2D6 is the enzyme mainly responsible for MeOPP *O*-demethylation.

Introduction

Since the 1990s, the illicit drug market for recreational drugs has changed considerably. Several new types of drugs have appeared and information about these drugs is readily available on the Internet (Wax 2002). Piperazine-derived compounds such as *N*-benzylpiperazine (BZP), 1-(3,4-methylenedioxybenzyl)-piperazine (MDBP), 1-(4-methoxyphenyl)piperazine (MeOPP), 1-(3-trifluoromethylphenyl)piperazine (TFMPP) and 1-(3-chlorophenyl)piperazine (mCPP) belong to one of these newer groups of designer drugs, which are mentioned as being psychoactive chemicals in 'scene books' (Shulgin 1991, Trachsel and Richard 2000) as well as on so-called drug information websites (<http://www>).

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erowid.org; <http://www.lycaeum.org>). Seizures have been made throughout the world (Roesner *et al.* 1999, de Boer *et al.* 2001, Staack *et al.* 2002, Drug Enforcement Administration — Office of Forensic Sciences 2002, 2003) and organizations that check the 'purity' of illegally sold tablets have reported their occurrence more and more often (<http://www.dancesafe.org>). A fatality involving piperazine-derived compounds has already been reported (Balmelli *et al.* 2001). The increasing abuse of BZP and TFMPP in the USA led to the temporary placement of these two compounds into Schedule I of the Controlled Substance Act (Drug Enforcement Administration — Department of Justice 2002).

Introduction of a methoxy moiety is a known structural variation of designer drugs of the amphetamine type. The corresponding methoxy-substituted piperazine derivative is MeOPP. So far, only little information about the pharmacological and toxicological properties of MeOPP is available. However, as other 1-arylpiperazines are known to show central serotonergic effects (Pawlowski 1983, Caccia *et al.* 1985, Lyon *et al.* 1986, Glennon 1987, Murphy *et al.* 1991), it might be assumed that MeOPP shows similar effects.

The metabolism of a new therapeutic drug is usually thoroughly investigated before it can be marketed. Such investigations allow the prediction of possible drug–drug interactions, interindividual variations in pharmacokinetic profiles or an increased appearance of side-effects and serious poisonings (Evans and McLeod 2003). However, such risk assessment is typically performed for substances intended for therapeutic use, but not for drugs of the illicit market (Springer *et al.* 2003c, e). In addition, there is good evidence that genetic variations in drug metabolism have important behavioural consequences that can alter the risk of drug abuse and dependence (Howard *et al.* 2002). Furthermore, knowledge of the metabolites is a prerequisite for developing analytical procedures for their detection in body fluids necessary for expertise in clinical and forensic toxicology (Staack and Maurer 2003b).

The metabolism of MeOPP has not yet been studied. Therefore, the aim of the current study was to identify the MeOPP metabolites in rat urine using gas chromatography-mass spectrometry (GC-MS) in the electron ionization (EI) and positive-ion chemical ionization (PICI) mode.

Furthermore, the human hepatic cytochrome P450 (CYP) enzymes catalysing the major metabolic step of MeOPP should be identified, the kinetic constants for this metabolic reaction should be determined, and the metabolite formation in pooled human liver microsomes (pHLM) and in single donor human liver microsomes with CYP2D6 poor metabolizer genotype (PM HLM) should be compared.

Materials and methods

Animals, treatment and collection of urine samples

Investigations were performed using urine of male Wistar rats, 500 g body weight (Charles River, Sulzflück, Germany), for toxicological diagnostic reasons according to the corresponding German law. Animals were housed in groups under controlled humidity and temperature ($23 \pm 1^\circ\text{C}$) and a 12-h light–dark cycle (lights on 07:00–21:00 hours) with food (standard diet: Altromin 1314; Altromin, Lage, Germany) and water *ad libitum*. A single 50 mg kg^{-1} body mass (BM) dose or a 1 mg kg^{-1} BM dose of MeOPP in aqueous solution was administered to rats by gastric intubation ($n=2$ for each dose). The rats were housed in metabolism cages for 24 h, having water *ad libitum*. Urine was collected separately from the faeces over these 24 h. All samples were analysed directly as described below. Blank rat urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

Materials

MeOPP-2 HCl 97%, 1-(4-hydroxyphenyl)piperazine (4-HO-PP) 98%, 1-(2-hydroxyphenyl)piperazine (2-HO-PP) 98%, 4-methoxyaniline 99%, 4-hydroxyaniline 98%, and paracetamol (acetaminophen) 98% were from Lancaster Synthesis (Mühlheim, Germany), NADP⁺ was from Biomol (Hamburg, Germany), quinidine-HCl, isocitrate and isocitrate dehydrogenase were from Sigma (Taufkirchen, Germany). All other chemicals and reagents were 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), pHLM (20 mg microsomal protein ml⁻¹, 400 pmol total CYP mg⁻¹ protein) and PM HLM (20 mg microsomal protein ml⁻¹). After delivery, the microsomes were thawed at 37°C, aliquoted, shock-frozen in liquid nitrogen and stored at -80°C until use.

Sample preparation for metabolism studies

A 5-ml portion of urine was adjusted to pH 5.2 with acetic acid (1 M) and incubated at 37°C for 12 h with 100 µl of a mixture (100 000 Fishman units ml⁻¹) of glucuronidase (EC 3.2.1.31) and arylsulfatase (EC 3.1.6.1) from *Helix pomatia*, then adjusted to pH 8–9 and extracted with 5 ml of a dichloromethane-isopropanol-ethyl acetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was transferred into pear-shaped flasks, evaporated to dryness and the residue was derivatized as described below (Kraemer *et al.* 2000).

Acetylation was conducted with 100 µl of an acetic anhydride-pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W (Maurer and Bickeboeller-Friedrich 2000). After evaporation, the residue was dissolved in 100 µl methanol and 2 µl of this solution were injected into the GC-MS. Trifluoroacetylation of another extract was conducted with 50 µl trifluoroacetic anhydride and 50 µl ethyl acetate for 5 min under microwave irradiation at about 440 W. After evaporation of the derivatization mixture, the residue was dissolved in 50 µl alcohol- and water-free ethyl acetate and 1–2 µl of this sample were injected into the GC-MS. The same procedure with the exception of enzymatic hydrolysis was used to study whether metabolites of MeOPP were excreted as conjugates.

Another urine sample was worked up as described above, but the pH was adjusted to 4–5. The corresponding extract was analysed after methylation and subsequent acetylation. After reconstitution of the extraction residue in 50 µl methanol, methylation was conducted with 50 µl of a solution of diazomethane in diethyl ether, synthesized according to the procedure of McKay *et al.* (1950). The reaction vials were sealed and left at room temperature for 15 min. Thereafter, the mixture was again gently evaporated to dryness under a stream of nitrogen, acetylated as described above and finally redissolved in 50 µl methanol, and 3 µl of this sample were injected into the GC-MS (Maurer 2000a).

GC-MS

MeOPP metabolites were separated and identified in acetylated, trifluoroacetylated or methylated plus acetylated urine extracts using a Hewlett Packard (Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were: splitless injection mode; column, HP-1 capillary (12 m × 0.2 mm i.d.), cross-linked methylsilicone, 330 nm film thickness; injection port temperature, 280°C; carrier gas, helium; flow-rate, 1 ml min⁻¹; column temperature, programmed from 100 to 310°C at 30°C min⁻¹; initial time, 3 min; final time, 8 min. The MS conditions were: full-scan mode; EI ionization mode: ionization energy, 70 eV; chemical ionization using methane, positive mode (PCI): ionization energy, 230 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C.

Microsomal incubations

Incubation mixtures (final volume 50 µl) 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 the addition of ice-cold microsomes and terminated with 5 µl 60% (w/w) perchloric acid. After addition of 1 µl 0.25 mM 2-HO-PP in methanol as internal standard (IS), the samples were centrifuged and the supernatants transferred to autosampler vials.

Initial screening studies

In order to investigate the involvement of particular CYP enzymes in MeOPP O-demethylation, incubations were performed with 200 µM MeOPP 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

The 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 MeOPP concentrations between 5 and 400 μM for CYP2D6 and between 30 and 800 μM for pHLM ($n=2$ each). pHLM and cDNA-expressed CYP protein contents were 1.0 mg ml^{-1} and 50 pmol CYP ml^{-1} , respectively. Incubation times were 30 and 20 min for pHLM and cDNA-expressed CYPs, respectively. Kinetic profiles and values were estimated first by visual inspection of Eadie–Hofstee plots, since deviations from simple enzyme Michaelis–Menten kinetics could be seen readily (Clarke 1998). Apparent K_m and V_{max} values for single enzymes were estimated by non-linear regression according to the Michaelis–Menten equation:

$$V = (V_{\text{max}} \times [S]) / (K_m + [S]).$$

The kinetic data were estimated using GraphPad Prism 3.02 software (San Diego, CA, USA).

Chemical inhibition studies

The effect of 1 and 3 μM quinidine (Kobayashi *et al.* 1989) on 4-HO-PP formation was assessed in incubations containing 1.0 $\text{mg pHLM protein ml}^{-1}$ and 200 μM MeOPP. Controls contained no quinidine, but the same amount of methanol to control for any solvent effects ($n=3$ each). Significance of inhibition was tested by a one-tailed unpaired *t*-test.

Comparative studies between pHLM and PM HLM

Incubations were carried out at 200 μM MeOPP for 30 min using either 1.0 mg pHLM or $\text{PM HLM protein ml}^{-1}$. The significance of differences in metabolite formation was tested by a one-tailed unpaired *t*-test.

Liquid chromatography-mass spectrometry (LC-MS) conditions and metabolite quantification

MeOPP, 4-HO-PP and 2-HO-PP were separated and quantified using an Agilent Technologies AT 1100 series atmospheric pressure chemical ionization electrospray LC-MSD, SL version and a LC-MSD ChemStation using the A.08.03 software.

LC conditions

Gradient elution was achieved on a Supelco® Discovery HS F5 column (15 $\text{cm} \times 2.1 \text{ mm}$, 3 μm). 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 flow rate were: 0–3.5 min 20% B (flow 0.4 ml min^{-1}), 3.5–7.5 min 90% B (flow 0.55 ml min^{-1}), 7.5–9 min 20% B (flow 0.9 ml min^{-1}), and 9–10 min 20% B (flow 0.4 ml min^{-1}). The injection volume was 10 μl .

Electrospray conditions

The following atmospheric pressure chemical ionization electrospray 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, 5.0 μA ; positive selected-ion monitoring mode; fragmentor voltage, 100 V.

MS conditions

For quantification, the following target ions (m/z) were used in the selected-ion monitoring mode: 179 for 4-HO-PP and 2-HO-PP (IS), 193 for MeOPP.

Metabolite quantification

Quantification was carried out by comparison of the peak area ratios of 4-HO-PP versus IS with the calibration curve in which the peak area ratios of spiked calibration standards (0.5, 1, 2, 3, 4, 5, 7.5, 10 μM) versus IS had been plotted against their concentrations using a simple linear regression model.

Results

Identification of metabolites

The urinary metabolites of MeOPP were separated by GC and identified by EI and PICI mass spectrometry after enzymatic hydrolysis, extraction and acetylation. Additionally to acetylation, trifluoroacetylation was conducted to clarify whether *N*-acetyl compounds were formed by derivatization or by metabolic conjugation. The EI and PICI mass spectra, the structures and predominant

fragmentation patterns of acetylated MeOPP and its acetylated metabolites, are shown in figure 1 (mass spectra 1–6). The data for trifluoroacetylated *N*-acetyl-4-hydroxyaniline (mass spectra 7 in figure 1) is also shown. To check for acidic metabolites, the urine samples were also extracted after cleavage of conjugates at acidic pH (4–5) and the corresponding extracts analysed after methylation followed by acetylation (Kraemer *et al.* 2000). However, no acidic metabolites could be found.

Besides MeOPP (mass spectra number 1 in figure 1), the following metabolites could be identified in rat urine: 4-HO-PP (mass spectra number 2), *N*-(4-methoxyphenyl)ethylenediamine (number 3), 4-methoxyaniline (number 4), 4-hydroxyaniline (after acetylation, numbers 5 and 6) and *N*-acetyl-4-hydroxyaniline (after acetylation: numbers 5 and 6; after trifluoroacetylation: number 7). The numbers of the spectra correspond to those in figures 2 and 7. Figure 2 shows typical reconstructed mass chromatograms with the ions 109, 148, 151, 162, 234 and 262 of an acetylated extract of a rat urine sample collected over 24 h after administration of 1 mg kg⁻¹ BM MeOPP. The ions were selected from the mass spectra recorded during this study. They indicate the presence of MeOPP, 4-HO-PP and 4-hydroxyaniline, metabolites which were detectable after application of a common drug abuser's dose. The peak numbers correspond to those used in figures 1 and 7.

LC-MS procedure

The applied LC-MS conditions provided baseline separation of 4-HO-PP, 2-HO-PP (IS) and MeOPP (figure 3). The calibration curves were linear over the tested calibration range ($r^2 > 0.997$) and were used to determine the metabolite concentration in the incubation samples.

Initial screening studies

Figure 4 illustrates that among the nine CYPs tested, only CYP2D6 supported *O*-demethylation of the methoxy moiety of MeOPP. 4-HO-PP was detectable neither in incubations with other cDNA-expressed CYPs nor in incubations with insect cell control microsomes.

Kinetic studies

All incubations were carried out at initial rate conditions, a prerequisite for Michaelis–Menten kinetics. CYP2D6 showed a typical hyperbolic metabolite formation profile (figure 5). The apparent K_m for CYP2D6 was $48.34 \pm 14.48 \mu\text{M}$ and $V_{\max} = 5.44 \pm 0.47 \text{ pmol min}^{-1} \text{ pmol}^{-1} \text{ CYP}$ (mean $\pm$ standard error). The kinetic parameters for incubations using pHLM were determined to be: apparent $K_m = 204.80 \pm 51.81 \mu\text{M}$ and $V_{\max} = 127.50 \pm 13.25 \text{ pmol min}^{-1} \text{ mg}^{-1}$ of protein.

Chemical inhibition studies

In order to underline the importance of CYP2D6 in MeOPP *O*-demethylation, the CYP2D6-specific inhibitor quinidine (1 and 3 μM) was added to incubation mixtures and the rate of metabolite formation was compared with those of incubations without the inhibitor. The inhibition experiments were performed with 200 μM MeOPP. 4-HO-PP formation was significantly inhibited by 1 μM

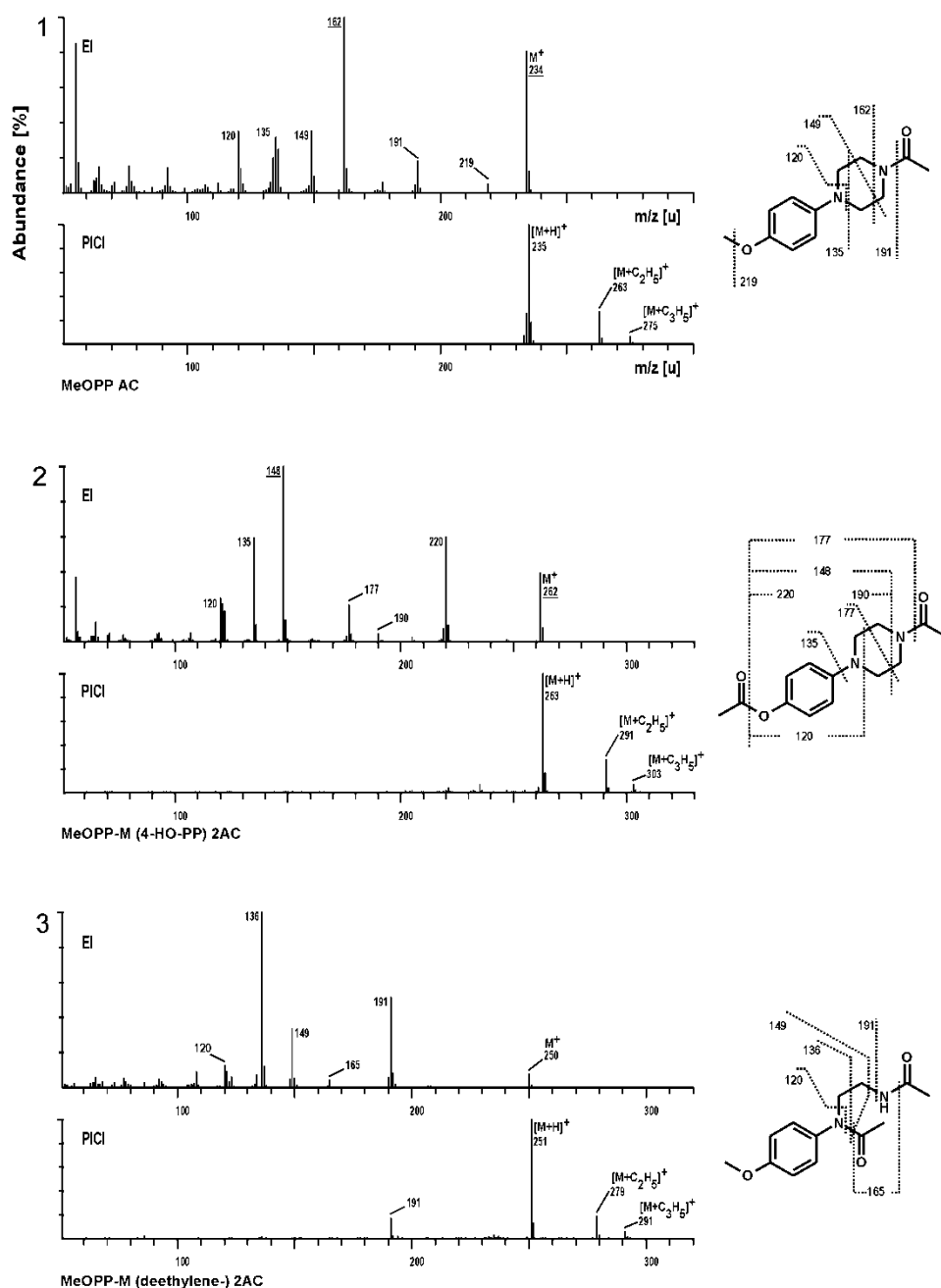


Figure 1. EI and PICI mass spectra, structures and predominant fragmentation patterns of MeOPP and its metabolites after acetylation (mass spectra numbers 1–6) and of the *N*-acetyl conjugate of 4-hydroxyaniline after trifluoroacetylation (spectrum number 7). The numbers of the spectra correspond to those in figures 2 and 7. Ions selected for detection are underlined. (The axes are only labelled for spectra number 1.)

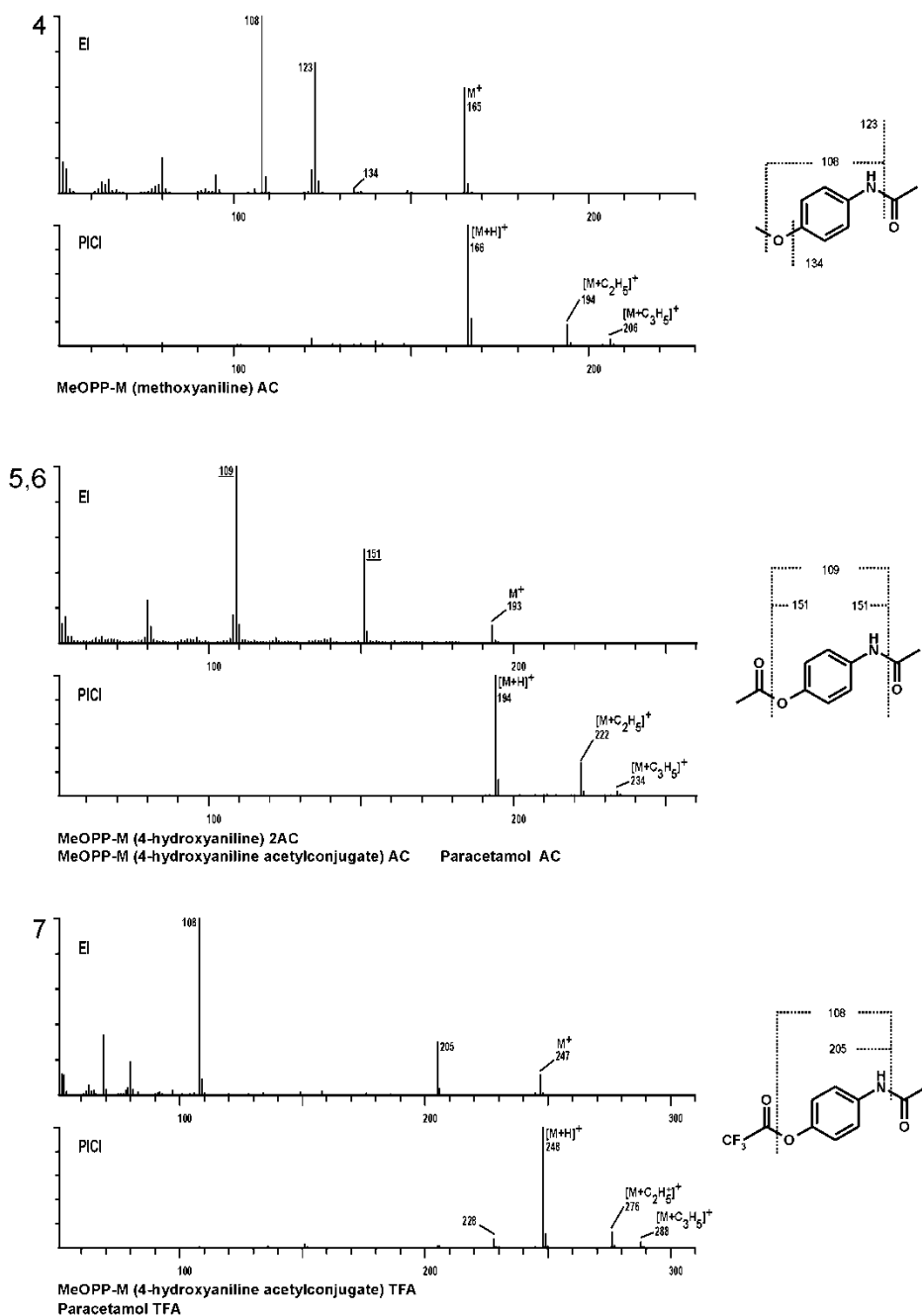


Figure 1. Continued.

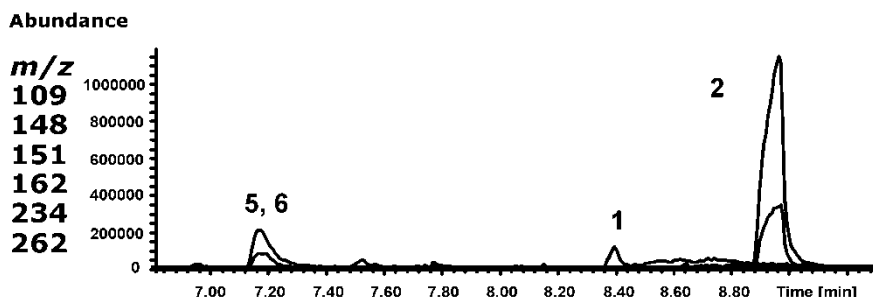


Figure 2. Typical reconstructed mass chromatograms with the given ions of an acetylated extract of a rat urine sample collected over 24 h after administration of a common abuser's dose of 1 mg kg^{-1} BM of MeOPP. The chromatograms indicate the presence of MeOPP, 4-HO-PP and 4-hydroxyaniline. The peak numbers correspond to those used in figures 1 and 7.

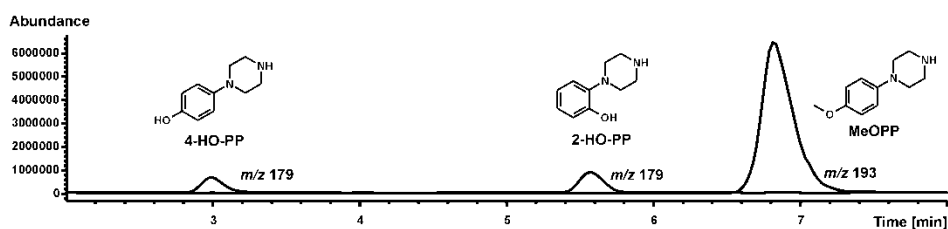


Figure 3. Typical mass fragmentograms of a supernatant of an incubation mixture of $200 \mu\text{M}$ MeOPP with cDNA-expressed CYP2D6 with the following ions: m/z 179 for 4-HO-PP, and 2-HO-PP and 193 for MeOPP.

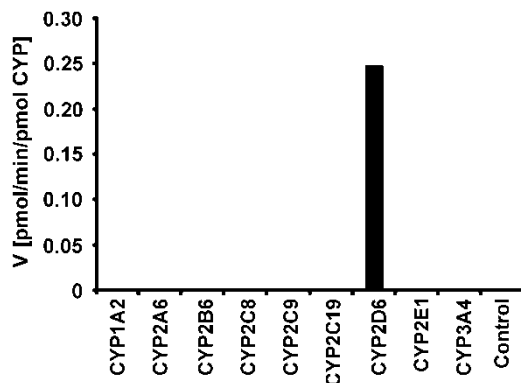


Figure 4. Formation rates (V) for MeOPP *O*-demethylation with $200 \mu\text{M}$ MeOPP with $50 \text{ pmoles ml}^{-1}$ of the given individual CYPs and with insect cell control microsomes. Data are the average of two determinations.

quinidine ($71.9 \pm 4.8\%$; $p = 0.0044$) and by $3 \mu\text{M}$ quinidine ($98.5 \pm 0.5\%$; $p = 0.0009$), respectively (mean $\pm$ standard error of the mean).

Comparative studies between pHLM and PM HLM

To underline further the importance of CYP2D6 in MeOPP *O*-demethylation and to demonstrate differences in CYP2D6 poor (PM) and extensive (EM) metabolizers, the metabolite formation rate of pHLM was compared with that

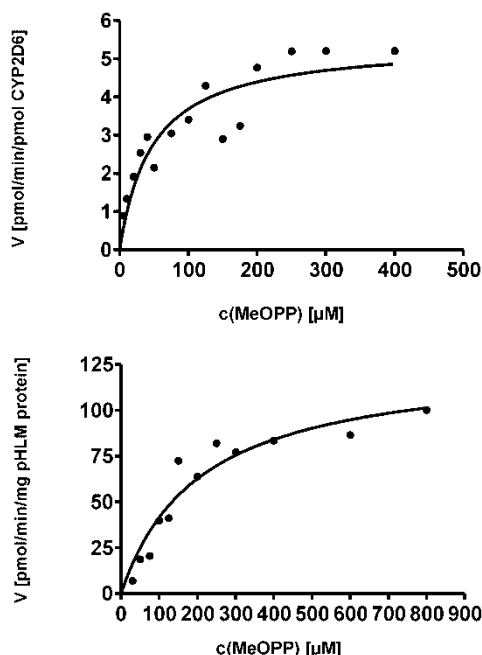


Figure 5. Michaelis–Menten plots for MeOPP *O*-demethylation catalysed by CYP2D6 (upper) and HLM (lower). Values are the mean of duplicate incubations. Solid curves were calculated by non-linear regression according to equation (1) (one site binding model).

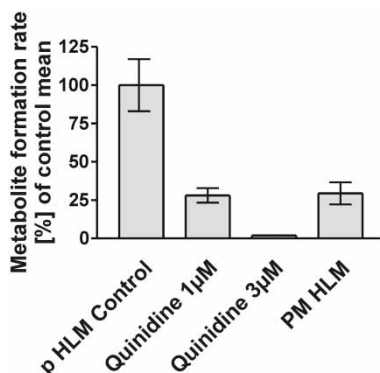


Figure 6. Metabolite formation in PM HLM (right bar) and effect of 1 and 3 μM quinidine on metabolite formation in pHLM (second and third from left) in incubation mixtures containing 200 μM MeOPP. Controls with pHLM without inhibitor (left bar) were set to 100%. Each bar is the mean of six incubations $\pm$ standard error of the mean.

of PM HLM. The metabolite formation rate of PM HLM was significantly lower by $70.6 \pm 7.2\%$ ($p = 0.0136$) than that of pHLM (figure 6).

Discussion

Metabolism studies of new designer drugs are commonly conducted using rat models due to ethical reservation about human studies (Kanamori *et al.* 2002,

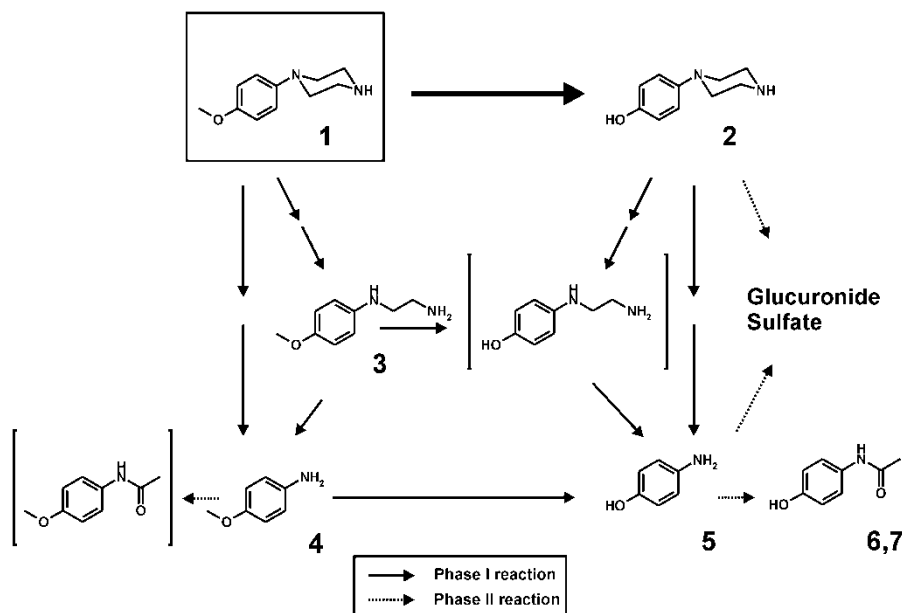


Figure 7. Proposed scheme for the metabolism of MeOPP in rats (metabolites in brackets not detected). Metabolites 2 and 5 were also excreted as glucuronic and/or sulfuric acid conjugates in urine. The numbers of the metabolites correspond to those in figures 1 and 2.

Springer *et al.* 2002, 2003a, b, 2003d, Staack *et al.* 2002, 2003a, b, Paul and Maurer 2003, Staack and Maurer 2003a). The chosen rat model has already proved to be a suitable model for identification of human metabolites (Kraemer *et al.* 2000, Staack *et al.* 2002). The first aim of the present study was to identify the metabolites of MeOPP formed in this rat model. They were identified in urine with and without cleavage of conjugates after extraction and derivatization (acetylation, trifluoroacetylation or methylation plus acetylation) by full-scan EI and PICI GC-MS. Extraction at slightly acidic pH and at pH 8–9 allowed the isolation of acidic, basic and amphoteric compounds. The extraction solvent used has proved to be very efficient in extracting compounds with different chemical properties from biomatrices. It is also routinely used for comprehensive screening of drugs, poisons and their metabolites with very different physicochemical properties, the so-called systematic toxicological analysis (Maurer 2000b).

In order to identify the rat metabolites of MeOPP, the compound was administered at two different doses, a dose that corresponded to a common drug abusers' dose and a high dose also to identify the minor metabolites of MeOPP. Information on these metabolites is necessary as they might be found in urinalysis of human poisoning cases. In such cases, the minor metabolites must be clearly differentiated from additionally taken poisons. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode, which were interpreted in correlation to those of the parent compound according to the rules described by, for example, McLafferty and Turecek (1993) and Smith and Busch (1999). In order to verify the molecular mass of the postulated metabolites, PICI mass spectra were recorded because they contain abundant peaks of the protonated molecules $[M + H]^+$ with adduct ions typical for PICI using methane as reagent

gas. The identity of 4-HO-PP, 4-methoxyaniline and 4-hydroxyaniline could be further confirmed by comparing their mass spectra and the gas chromatographic retention times with those of the corresponding reference substances. The structure could be further confirmed by spiking urine of MeOPP treated rats either with 4-HO-PP, with 4-methoxyaniline or with 4-hydroxyaniline before extraction and acetylation. The corresponding metabolites and the reference substances each resulted in a single peak. This fact together with the identical mass spectrum is a clear confirmation of the structure.

As the peaks of the hydroxy metabolites were more abundant after combined glucuronidase/sulfatase hydrolysis, it can be concluded that they are partly excreted as the corresponding glucuronides and/or sulfates. *N*-acetyl-4-hydroxyaniline was detected in trifluoroacetylated extracts as *O*-trifluoroacetylated derivative (mass spectra number 7 in figure 1). These mass spectra correspond to those of trifluoroacetylated paracetamol. Again, the identity of the structure could be confirmed by spiking urine of MeOPP-treated rats with paracetamol before extraction. After trifluoroacetylation, this MeOPP metabolite and paracetamol resulted in a single peak. This fact together with the identical mass spectrum is a clear confirmation of the structure. Therefore, it could be concluded that this aniline derivative was metabolically acetylated, probably by *N*-acetyltransferase, which is known to be responsible for phase II metabolism of aniline derivatives (Ioannides 2001). 4-Methoxyaniline could not be detected as the acetyl conjugate in trifluoroacetylated rat urine extracts, probably due to its low concentration. A metabolic acetylation of this aniline derivative could only be assumed. *N*-(4-hydroxyphenyl)ethylenediamine, the corresponding *O*-demethyl metabolite of *N*-(4-methoxyphenyl)ethylenediamine, could not be detected. Therefore, its formation could also only be assumed.

Based on the identified metabolites, the following metabolic pathways (figure 7) could be postulated: *O*-demethylation of the methoxy moiety followed by partial glucuronidation and/or sulfation; degradation of the piperazine heterocycle by double *N*-dealkylation of MeOPP to *N*-(4-methoxyphenyl)ethylenediamine (3) or to 4-methoxyaniline (4), and of 4-HO-PP to 4-hydroxyaniline (5), followed by partial *N*-acetylation. 4-Hydroxyaniline was also partially glucuronidated and/or sulfated. These metabolic pathways are in accordance with those described for other phenylpiperazine derivatives (Staack and Maurer 2003a, Staack *et al.* 2003b). Numbers of the metabolites correspond to those in figures 1 and 2.

The two metabolites *N*-(4-methoxyphenyl)ethylenediamine and 4-methoxyaniline were only minor metabolites that could only be detected in rat urine after administration of the high dose. The most abundant metabolite found in the rat urine samples after application of a common abuser's dose of 1 mg kg⁻¹ BM was 4-HO-PP (2) (figure 2). Unfortunately, no authentic human samples were available to confirm these data, but incubation of MeOPP with pHLM confirmed that 4-HO-PP formation was the major CYP-catalysed reaction in humans.

A further aim of the present study was to investigate the human hepatic CYP enzymes catalysing this major metabolic step. The initial screening studies with the nine most abundant human hepatic CYPs were performed to identify their possible role in MeOPP *O*-demethylation. According to the supplier's advice, the incubation conditions chosen were adequate to make a statement about a general involvement of a particular CYP enzyme. The data revealed that of the tested CYPs, only the polymorphically expressed CYP2D6 was capable of demethylating

MeOPP. For this reason, the kinetic profiles of only CYP2D6 as a single CYP enzyme source and that of pHLM were further investigated. The kinetic assays were performed under initial rate conditions, a prerequisite for Michaelis–Menten kinetics (Clarke 1998). As expected, classical hyperbolic Michaelis–Menten plots (figure 5) were found using cDNA-expressed CYPs. The apparent K_m and V_{max} values of the investigated CYPs were calculated by non-linear regression fit according to the Michaelis–Menten equation. Apparent K_m values for CYP2D6 and for pHLM differed somewhat from each other. This may be due to the fact that non-specific protein binding was greater in pHLM (1.0 mg ml⁻¹) compared with CYP2D6 microsomes (0.3 mg ml⁻¹). Such non-specific binding to phospholipid membranes has been described for lipophilic and cationic compounds at pH 7.4, which should be the case for MeOPP (Margolis and Obach 2003).

To confirm the role of CYP2D6 in MeOPP *O*-demethylation, inhibition studies with the CYP2D6-specific chemical inhibitor quinidine were performed. The concentration of the inhibitor (1 and 3 μ M) was based on average literature data (Newton *et al.* 1995, Bourrie *et al.* 1996, Clarke 1998). The substrate concentration examined was 200 μ M, a concentration corresponding to the calculated apparent K_m in pHLM. The results (figure 6) showed that at both inhibitor concentrations, the overall turnover was inhibited markedly. In order to study whether significant differences between PM and EM subjects could be detected, comparative studies using pHLM and PM HLM were conducted. The metabolite formation rate of PM HLM was significantly lower than that of pHLM. This remarkable difference in metabolite formation between PM and EM is further proof of the involvement of CYP2D6 in the major metabolic step of MeOPP and it may give hints for pharmacokinetic differences depending on the CYP2D6 genotype.

The results of the current study are in accordance with the results of studies on the involvement of CYP2D6 in the *O*-demethylation of other methoxy-substituted compounds such as amphetamines, β -carboline alkaloids or tryptophane (Wu *et al.* 1997, Bach *et al.* 1999, Yu *et al.* 2003a, b). The present studies showed that CYP2D6 is the major CYP which supports MeOPP *O*-demethylation. Significant differences could be shown *in vitro* between EM and PM, which account for about 7% of the Caucasian population (Bertilsson 1995, Smith *et al.* 1998). MeOPP might also be susceptible to drug–drug interactions with potent CYP2D6 inhibitory drugs leading to a decreased clearance of MeOPP and elevated plasma concentrations. Whether this genetic polymorphism and/or drug interactions are of clinical relevance for MeOPP toxicokinetics cannot be unequivocally concluded.

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Pharmacokinetics and metabolism of ^{14}C -1,3-dichloropropene in the Fischer 344 rat and the $\text{B}_6\text{C}_3\text{F}_1$ mouse

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1. ^{14}C -1,3-dichloropropene (^{14}C -DCP) is rapidly absorbed and eliminated in both the male F344 rat and $\text{B}_6\text{C}_3\text{F}_1$ mouse following oral administration of 1 or 50 mg kg^{-1} (rat) or 1 or 100 mg kg^{-1} (mouse).

2. It is extensively metabolized in both species. Urinary excretion was the major route of elimination, accounting for 50.9–61.3 and 62.5–78.6% of the administered dose in rat and mouse, respectively.

3. Urinary elimination half-lives ranged from 5 to 6 h (rat) and from 7 to 10 h (mouse). Elimination via faeces or as $^{14}\text{CO}_2$ accounted for 14.5–20.5 and 13.7–17.6% of the administered dose, respectively.

4. Metabolites arising from glutathione conjugation account for 36–55 and 48–50% of the administered dose in excreted from rats and mice, respectively. Hydrolysis of the 3-chloro moiety of DCP accounted for 24–37 and 29% of the dose administered to rats and mice, respectively. Two novel dimercapturic acid conjugates were also identified at low levels that might arise via initial hydrolysis of DCP or of epoxidation of DCP-glutathione conjugate or of DCP itself. Structural confirmation of these dimercapturates was obtained via analysis of deuterium retention from D_4 -DCP in the male F344 rat.

5. Only quantitative differences are seen between the overall metabolic profile of DCP in these two species.

Introduction

1,3-Dichloropropene (DCP) is the active ingredient in Telone[®] II soil fumigant, which is used in the control of nematodes in a variety of food crops (Budavari 1996). Early studies with this compound reported that the material was well absorbed by the rat following oral administration, with 51–54% of the dose excreted in the urine within 96 h (Hutson *et al.* 1971). The major route of metabolism for this compound has been investigated in the rat and has been shown to be glutathione conjugation (Climie *et al.* 1979, Onkenhout *et al.* 1986, Fisher and Kilgore 1988). Mercapturic acid conjugates of DCP, arising following metabolism with glutathione, have also been observed as urinary metabolites in human volunteers exposed to DCP via inhalation (Osterloh *et al.* 1984, van Welie *et al.* 1991, Kežić *et al.* 1996). The only other significant metabolite found has been radiolabelled carbon dioxide (CO_2), following oral administration of ^{14}C -DCP to

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the rat (Hutson *et al.* 1971). CO₂ was found to represent up to 24% of the administered dose of DCP. Epoxidation of DCP, affording 1,3-dichloro-1-propene oxide (DCPO), has been observed in the Swiss-Webster or B₆C₃F₁ mouse (Schneider *et al.* 1998, Bartels *et al.* 2000), but only following intraperitoneal (i.p.) administration of the test material at dosages near to or exceeding the reported LD₅₀ for this compound (215–640 mg kg⁻¹ bw)(WHO 1993).

Whereas there are several studies that report on selected aspects of the metabolism of DCP in the rat, a comprehensive evaluation of the metabolic fate of this compound in any species is not currently available. The current study, therefore, was performed to evaluate the pharmacokinetics and metabolism of ¹⁴C-DCP in the Fischer 344 rat and B₆C₃F₁ mouse following oral administration. Additional experiments were performed to elucidate the mechanism of formation of radiolabelled CO₂ and two dimercapturic acid metabolites of DCP.

Materials and methods

Reagents

DCP, a 52:48 mixture of *cis/trans*-isomers, was obtained from the Dow Chemical Co. (Midland, MI, USA). The purity of this test material was determined to be 98.2%. ¹⁴C-DCP, a 58:42 mixture of *cis/trans*-isomers (19.3 mCi mmol⁻¹), was obtained from Dow AgroSciences (Indianapolis, IN, USA). The purity of this test material was determined to be 98.1%. D₄-DCP, a 67:33 mixture of *cis/trans*-isomers, was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The purity of this test material was reported to be 98%. ¹⁴C-3-chloroallyl alcohol (¹⁴C-CAL), a 59:41 mixture of *cis/trans*-isomers (1.91 mCi mmol⁻¹), and ¹⁴C-3-chloroacrylic acid (¹⁴C-CAA), a 36:64 mixture of *cis/trans*-isomers (1.93 mCi mmol⁻¹) were obtained from Dow AgroSciences. The purities of these test materials were determined to be both 96.5%. Unlabelled CAL and CAA, 45:55 and 51:49 mixtures of *cis/trans*-isomers, respectively, were also obtained from Dow AgroSciences. The purities of these test materials were determined to be 98.6 and 99.5%, respectively. The compound 3,3-*bis*-S-(N-acetylcysteinyl)-1-propanol (3,3-dimercapturate) was a gift from Dr Ali Banijamali (Uniroyal Chemical Co., Middlebury, CT, USA) (Banijamali *et al.* 1999).

Cis- and *trans*-isomers of *S*-(3-chloro-2-propenyl)-acetic acid (DCP-acetate), *S*-(3-chloro-2-propenyl)-pyruvic acid (DCP-pyruvate), and *S*-(3-chloro-2-propenyl)-N-acetyl-L-cysteine (DCP-mercapturate) were prepared from *cis*- or *trans*-DCP and 2-thioacetic acid, 3-thiopyruvic acid or N-acetyl-L-cysteine, respectively. The general preparation method for these compounds was based on the method of Kaye *et al.* (1972) and Climie *et al.* (1979), and involved addition of 10 mM thioalkylacid to a stirred solution of 10 mM DCP in 18 ml aq. NaOH (1.4 M) and 27 ml ethanol (degassed with N₂ stream for 25 min). Following reaction at room temperature for 16 h, the reaction mixture was acidified with HCl and extracted with ethyl acetate (2 × 10 ml). The combined extracts were taken to dryness (N₂ stream) and the crude products characterized via LC/MS/MS. The *cis/trans*-isomer mixture of *S*-(3-chloro-2-propenyl)-lactic acid (DCP-lactate) was prepared in a similar fashion from 3-chloro-propenyl mercaptan (*cis/trans*) and 3-chlorolactic acid. 2,3-*bis*-S-(N-acetylcysteinyl)-1-propanol (2,3-dimercapturate) was also prepared in a similar fashion from 2,3-dibromo-1-propanol and N-acetyl-L-cysteine. The *cis*- and *trans*-isomers of *S*-(3-chloro-2-propenyl)-N-acetyl-L-cysteine sulfoxide (DCP-sulfoxide) were prepared from H₂O₂-mediated peroxidation of *cis*- and *trans*-DCP-Merc, according to the method of Park *et al.* (1992). All other compounds and solvents were reagent grade or better.

Experimental groups

The pharmacokinetics and metabolism of ¹⁴C-DCP was examined as follows. Radiolabelled DCP was administered via a feeding needle to groups of male F344 rats (1 and 50 mg kg⁻¹ bw) or male B₆C₃F₁ mice (1 and 100 mg kg⁻¹ bw) (6 animals/species/dose level). Urine, faeces, volatiles in expired air and CO₂ were collected from three animals/species/dose level at 6, 12, 24, 36 and 48 h. Urine and faecal samples only were collected from the remaining three animals/species/dose level at 12, 24 and 48 h for metabolite identification. Volatiles in expired air were collected via charcoal traps. CO₂ was collected after the charcoal traps by trapping in a 2-methoxyethanol:ethanolamine (7:3) solution (Timchalk *et al.* 1994). Distribution of radioactivity in selected tissues and remaining carcass was determined in three animals/species/dose level at 48 h. Pooled urine samples and selected faecal samples were analysed via high-pressure liquid chromatography (HPLC) and HPLC-mass spectrometry (LC/MS/MS) to identify and quantify metabolites arising from the test material.

The mechanism of DCP-dimercapturate formation was examined as follows. Two male F344 rats were administered D_4 -DCP via a feeding needle at a dose level of 50 mg kg^{-1} bw. Urine samples were collected for 9 h. The two urine samples were pooled and analysed via LC/MS/MS for structural characterization of the two DCP-dimercapturic acid metabolites arising from DCP.

The pharmacokinetics and metabolism of ^{14}C -CAL and ^{14}C -CAA were examined as follows. Radiolabelled CAL or CAA was administered via a feeding needle to groups of male F344 rats (5 and $65 \text{ mg } ^{14}\text{C-CAL kg}^{-1}$ bw or 5 and $75 \text{ mg } ^{14}\text{C-CAA kg}^{-1}$ bw; four animals/test material/dose level). Urine samples were collected at 6, 12, 24, 48, 72, 96, 120, 144 and 168 h. Faecal samples were collected daily from all animals up to 168 h. Volatiles in expired air were collected for 24 h and CO_2 were collected at 6, 12, 32, 48 and 72 h, as described above. Distribution of radioactivity in selected tissues and remaining carcass was determined in all animals at 168 h. Pooled urine samples and selected faecal samples were analysed via HPLC and LC/MS/MS to identify and quantify metabolites arising from the test material.

Test animals

Male F344 rats (161–246 g) and male $\text{B}_6\text{C}_3\text{F}_1$ mice (26–30 g) were purchased from Charles River Breeding Laboratories (Raleigh, NC, USA). Upon receipt, the animals were examined by a veterinarian and found to be in good health. Before use, the animals were acclimated to the laboratory environment for at least 1 week and Roth-type metabolism cages for 2 days. The rooms in which the animals were housed had a 12-h photocycle and were designed to maintain adequate environmental temperature, relative humidity and airflow for the mouse and rat. Municipal drinking water and Purina Certified Rodent Chow #5002 (Purina Mills, Inc., St Louis, MO, USA) were provided *ad libitum* to animals administered ^{14}C -DCP or D_4 -DCP. Food was withdrawn from mice and rats given ^{14}C -CAL or ^{14}C -CAA at approximately 15 h before the administration of the test material, respectively. Food was returned to these animals approximately 4 h post-dosing. Food was available *ad libitum* for the animals administered labelled DCP.

The protocol and conduct of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The Dow Chemical Co.

Dose preparation

The oral dose solutions of ^{14}C -DCP were prepared in corn oil (Acros Organics, Pittsburgh, PA, USA) from ^{14}C -DCP and unlabelled DCP at concentrations of 0.2 mg g^{-1} corn oil (1 mg kg^{-1} bw), 10 mg g^{-1} corn oil (50 mg kg^{-1} bw) and 20 mg g^{-1} corn oil (100 mg kg^{-1} bw). The radioactivity levels were $3.0 \mu\text{Ci g}^{-1}$ corn oil (1 mg kg^{-1} bw) and $80 \mu\text{Ci g}^{-1}$ corn oil (50 and 100 mg kg^{-1} bw). The oral dose solution of D_4 -DCP was prepared in corn oil from D_4 -DCP at a concentration of 10 mg g^{-1} corn oil (50 mg kg^{-1} bw).

The oral dose solutions of ^{14}C -CAL were prepared in corn oil from ^{14}C -CAL and unlabelled CAL at concentrations of 1 mg g^{-1} corn oil (5 mg kg^{-1} bw) and 10 mg g^{-1} corn oil (65 mg kg^{-1} bw). The radioactivity levels were $20 \mu\text{Ci g}^{-1}$ corn oil for both dose levels. The oral dose solutions of ^{14}C -CAA were prepared in corn oil from ^{14}C -CAA and unlabelled CAA at concentrations of 1 mg g^{-1} corn oil (5 mg kg^{-1} bw) and 10 mg g^{-1} corn oil (75 mg kg^{-1} bw). The radioactivity levels were $20 \mu\text{Ci g}^{-1}$ corn oil for both dose levels.

Dose administration

The test animals were weighed before dose administration. Based on body weight, a measured volume of the dose solution was administered, by gavage, using a disposable glass syringe and stainless steel feeding needle. Actual dose amounts administered were determined by weighing the syringe before and following dosing. Dose amounts, for all test materials, was $5 \text{ g dose solution kg}^{-1}$ bw. Animals were returned to the metabolism cages immediately after dosing.

Sample collection

Following the administration of test materials, urine and faecal samples were collected on dry ice. Urine separators were rinsed after each time interval and the radioactivity in these samples added to the urine values. Each urine and rinse sample was weighed individually and separately weighed aliquots were analysed for radioactivity. Pooled urine samples (by equal volume) were stored at -80°C before being analysed via HPLC. Each faecal specimen was weighed and an aqueous homogenate (about 25% w/w) of faeces was prepared. Weighed aliquots of faecal homogenates were placed in scintillation vials, solubilized and analysed for radioactivity. Pooled faeces (by equal volume) were stored at -80°C before being extracted and analysed via HPLC. Once the study was terminated (168 h post-dosing), the cages were washed with water, the weight of the final cage wash (FCW) obtained and a weighed aliquot analysed for radioactivity with a liquid scintillation spectrometer (LSS). The charcoal trap samples were desorbed with a weighed amount of toluene and a weighed aliquot was then analysed for radioactivity. Weighed aliquots of the CO_2 trapping solution were analysed directly for radioactivity.

At the termination of experiments, animals were euthanized with CO₂ and exsanguinated via cardiac puncture. Tissue samples were collected and either homogenized with a weighed aliquot of water and solubilized, or analysed directly for radioactivity.

¹⁴C-analysis

Radioactivity was quantified via LSS (Beckman LS3801, LS1801 or LS6000). Counts per minute (cpm) were corrected for quench and background and converted to disintegrations per minute (dpm). At least one sealed ¹⁴C-standard was counted with each group of samples to monitor the performance of the LSS. Samples with dpms less than twice the concurrently run background (blanks) were considered to contain insufficient radioactivity to reliably quantify.

Chemical analysis

Urine samples were analysed via HPLC with radiochemical detection (HPLC/RAM) or HPLC with electrospray mass spectral detection (ESI-LC/MS) following centrifugation, filtration and/or dilution with deionized water, to remove or dissolve suspended solids. Faecal homogenate aliquots were extracted with acetonitrile/water (10/90 + 1% acetic acid), concentrated and centrifuged before HPLC analysis. Quantitation of 2,3- and 3,3-dimercapturate isomers in rat and mouse urine was performed via ESI-LC/MS in the following manner. Aliquots of urine samples (50 µl) were diluted with 100 µl deionized water. Matrix standards were prepared by fortifying control urine from male F344 rats with 2.6–131 and 4.0–202 µg ml⁻¹ of 2,3- and 3,3-DCP dimercapturate, respectively.

Separation of DCP, CAL or CAA metabolites in excreta samples was achieved with a YMC ODS-AQ guard column (4 × 23 mm × 5 cm) and a YMC-ODS-AQ analytical column (4.6 mm × 25 cm × 5 µm) (Kyoto, Japan). A Hitachi L6200A ternary gradient pump (Tokyo, Japan) was used to deliver the eluents A (deionized water + 1% acetic acid) and B (acetonitrile + 1% acetic acid) at a flow rate of 1 ml min⁻¹ with the following gradient programme. Initial hold for 2 min at 100% A, 2-min linear ramp to 99% A, 2-min hold at 99% A, 3-min linear ramp to 92% A, 1 min linear ramp to 83% A, 30-min linear ramp to 81% A, 15-min linear ramp to 95% B, 5-min hold at 95% B. Radiolabelled metabolites were detected via an in-line radiochemical detector (Berthold Model LB509, Bad Wildbad, Germany) or via collection of 1-min eluent fractions (Isco Foxy 200 collector, Lincoln, NE, USA) followed by LSC analysis. Dpms from the LSC analysis of the eluent fractions were converted into chromatographic data files compatible with the laboratory chromatography data system (PENelson TurboChrom). Metabolite profiles were then calculated by integration of the reconstructed chromatograms for these samples.

Mass spectral identification of DCP metabolites was achieved with either a Hewlett Packard 1100 LC/MSD or a Finnigan MAT LCQ-Duo mass spectrometer, in the negative-ion electrospray ionization mode. Representative LC/MS conditions for the Hewlett Packard system were: nitrogen drying gas at 8.0 l min⁻¹ and 300°C; nebulizer pressure 40 psig; capillary voltage at 3 kV; fragmenter setting at 60; 60–450 amu at 1.9 s/scan. HPLC conditions and column were the same as above. Representative LC/MS/MS conditions for the Finnigan system were: nitrogen sheath gas at 90 units; electrospray voltage 5.5 kV; capillary heater 225°C; ion-trap collision energy 22%. Quantitation of the 2,3- and 3,3-dimercapturate isomers in urine was performed by monitoring the *m/z* 252 and 162 product ions arising from the *m/z* 381 precursor ion, respectively. HPLC conditions and column were the same as above.

Data analysis

Descriptive statistics were conducted, i.e. mean ± standard deviation (SD). Calculations were performed using Microsoft Excel® (v.7.0) in full precision mode. Pharmacokinetic parameters were estimated using a commercially available Excel-based program (PK Solutions v.2.0, Summit Research Services, Ashland, OH, USA).

Results and discussion

The primary routes of excretion for total radioactivity in rats and mice, following a single oral dose of ¹⁴C-DCP, were via the urine, faeces and as expired ¹⁴CO₂. The majority of recovered radioactivity was found in the urine of all four dose groups, accounting for 50.9–78.6% of the administered dose (table 1). Faecal elimination accounted for an additional 14.5–20.5% of the administered dose, with an additional 13.7–17.6% collected as radiolabelled CO₂. The distribution of radioactivity remaining in the carcass at 48 h post-dosing was slightly higher in the rat (5.6–6.0%) versus the mouse (1.8–2.0%). Overall, the routes of ¹⁴C-elimination were fairly comparable, both between dose levels and species.

Table 1. Per cent of administered ^{14}C -activity recovered in the excreta and carcasses of male rats and mice during 48 h after a single oral dose of ^{14}C DCP.^a

Sample	Administered ^{14}C activity recovered(%)	
	1 mg kg ⁻¹	50 mg kg ⁻¹
Rats		
Urine	50.9 ± 4.6	61.3 ± 4.3
Faeces	20.5 ± 0.3	17.1 ± 1.6
CO ₂	17.6 ± 0.4	15.1 ± 0.9
Tissues and remaining carcass ^b	6.0 ± 1.0	5.6 ± 1.0
Cage wash	1.0 ± 0.3	1.0 ± 0.5
Charcoal Trap	0.1 ± 0.04	0.6 ± 0.8
Total recovery	96.0 ± 5.5	100.5 ± 4.6
	1 mg kg ⁻¹	100 mg kg ⁻¹
Mice		
Urine	78.6 ± 6.7	62.5 ± 3.6
Faeces	15.7 ± 2.7	14.5 ± 3.0
CO ₂	14.4 ± 0.4	13.7 ± 2.0
Tissues and remaining carcass ^b	2.0 ± 0.2	1.8 ± 0.2
Cage wash	0.9 ± 0.5	4.0 ± 2.8
Charcoal trap	0.1 ± 0.1	0.2 ± 0.1
Total recovery	111.7 ± 7.3	96.7 ± 7.8

^aEach value is the mean ± SD for three animals.^bSum of all tissues analysed; individual tissue concentrations are shown in table 4.

Similar elimination profiles were reported by Hutson *et al.* (1971), who reported 51.1 and 6.8% of the dose excreted in the 0–96-h urine and faeces, respectively, following oral administration of ^{14}C -DCP to the male rat. Eder and Dornbusch (1988) evaluated the metabolic fate of the related compound, 2,3-dichloro-1-propene (2,3-DCP) and reported 56.7, 1.6 and 0.3% of an oral dose (175 mg kg⁻¹) eliminated within 20 h via urine, faeces and as $^{14}\text{CO}_2$, respectively. Similar data were reported by Bond *et al.* (1985) following inhalation exposure of F344 rats to 2,3-DCP via inhalation (7.5 ppm in air). Elimination via urine, faeces and as $^{14}\text{CO}_2$ accounted for 54.6, 16.8 and 3.2% of total recovered radioactivity within 65 h after exposure, respectively. These results indicate that DCP is well absorbed and eliminated primarily in the urine, as is the related dichloropropene, 2,3-DCP.

To identify metabolites that arise via potential hydrolysis of the 3-chloro moiety of DCP, the metabolic fates of ^{14}C -CAA and ^{14}C -CAA, both environmental hydrolysis products of DCP (WHO 1993), were examined in the male F344 rat. In contrast to DCP, the majority of orally administered ^{14}C -CAL or ^{14}C -CAA was eliminated as radiolabelled CO₂ in the male rat (table 2). A total of 50.2–51.8% of an oral dose of ^{14}C -CAL was eliminated as $^{14}\text{CO}_2$ in 168 h. An even greater percentage of the corresponding ^{14}C -CAA was found as $^{14}\text{CO}_2$ in the male rat (60.4–68.2%). Urinary elimination accounted for 21.0–21.3 and 15.9–16.7% of the administered dose of ^{14}C -CAL and ^{14}C -CAA, respectively. Faecal elimination was a minor route of elimination for both compounds, accounting for 13% (^{14}C -CAL) and 3.3–3.9% (^{14}C -CAA) of the dose. These data indicate that both compounds are well absorbed and metabolized primarily to $^{14}\text{CO}_2$ in the rat.

The rate of elimination for absorbed ^{14}C -DCP was quite rapid in both the rat and mouse. The urinary elimination half-lives ranged from 5.43–5.91 h in the rat

Table 2. Per cent of administered ^{14}C -activity recovered in the excreta and carcasses of male rats during 168 h after a single oral dose of ^{14}C -CAA or ^{14}C -CAA.^a

Sample	Administered ^{14}C -activity recovered	
	5 mg kg ⁻¹	65 mg kg ⁻¹
^{14}C -CLA		
Urine	21.3 ± 3.5	21.0 ± 1.6
Faeces	13.0 ± 2.2	13.0 ± 2.4
CO ₂	51.8 ± 0.8	50.2 ± 3.8
Tissues and remaining carcass	5.5 ± 0.3	5.3 ± 0.4
Cage wash	0.3 ± 0.2	0.3 ± 0.1
Charcoal trap	0.0 ± 0.0	0.0 ± 0.0
Total Recovery	91.9 ± 1.1	89.8 ± 2.7
	5 mg kg ⁻¹	75 mg kg ⁻¹
^{14}C -CAA		
Urine	16.7 ± 0.9	15.9 ± 3.6
Faeces	3.9 ± 0.6	3.3 ± 3.0
CO ₂	68.2 ± 1.5	60.4 ± 2.0
Tissues and remaining carcass	7.0 ± 1.1	6.8 ± 0.2
Cage wash	0.3 ± 0.4	0.3 ± 2.8
Charcoal trap	0.0 ± 0.0	0.0 ± 0.1
Total recovery	96.1 ± 6.1	86.7 ± 4.4

^aEach value is the mean ± SD for three to four animals.Table 3. Elimination half-lives for radioactivity following oral administration of ^{14}C -DCP to rat and mouse or ^{14}C -CAL or ^{14}C -CAA to rat.

Test material/species	Elimination half-lives*		
	Urine	CO ₂	Faeces
^{14}C -DCP (rat)			
1 mg kg ⁻¹	5.91 ± 0.40	4.44 ± 0.23	6.91 ± 0.85
50 mg kg ⁻¹	5.43 ± 0.64	4.34 ± 0.53	10.43 ± 4.70
^{14}C -DCP (mouse)			
1 mg kg ⁻¹	9.62 ± 3.30	8.45 ± 0.97	10.24 ± 2.96
100 mg kg ⁻¹	6.60 ± 0.83	6.52 ± 0.41	11.15 ± 6.54
^{14}C -CLA (rat)			
5 mg kg ⁻¹	7.65 ± 2.01	9.32 ± 0.47	9.47 ± 1.57
65 mg kg ⁻¹	11.07 ± 0.78	13.96 ± 2.46	6.84 ± 0.69
^{14}C -CAA (rat)			
5 mg kg ⁻¹	7.87 ± 2.26	8.49 ± 0.30	11.44 ± 1.34
75 mg kg ⁻¹	9.72 ± 0.64	15.77 ± 2.77	10.78 ± 1.21

*Values are the mean ± SD for three to four animals/test material/dose level.

and 6.60–9.62 h in the mouse (table 3). Comparable half-lives were observed for ^{14}C -CAL (7.65–11.07 h) and ^{14}C -CAA (7.87–9.72 h). Faecal elimination rates for ^{14}C -DCP were similar to urine, with half-lives of 6.91–10.43 and 10.24–11.15 h for rats and mice, respectively. The elimination of radiolabelled CO₂ was quite consistent with the corresponding urinary elimination rates for all three test materials, with the $^{14}\text{CO}_2$ half-lives generally within 20% of the urine values.

Low levels of radioactivity were found in a number of tissues in the rat and mouse at 48 h following oral administration of ^{14}C -DCP (table 4). The highest amount of radioactivity was found in the stomach of the F344 rat, accounting for

Table 4. Tissue concentrations of ^{14}C -activity in male rats and mice 48 h after a single oral dose of ^{14}C -DCP.^a

Tissue	Microgram equivalents of ^{14}C -DCP g ⁻¹ tissue	
	1 mg kg ⁻¹	50 mg kg ⁻¹
Rats		
Non-glandular stomach	0.29 ± 0.07	12.07 ± 1.13
Glandular stomach	0.23 ± 0.03	10.23 ± 1.37
Liver	0.24 ± 0.02	9.92 ± 0.38
Kidney	0.27 ± 0.02	10.91 ± 1.13
Bladder	0.31 ± 0.07	7.22 ± 1.45
Fat	0.08 ± 0.01	2.62 ± 1.45
Skin	0.09 ± 0.04	3.78 ± 0.97
Blood	0.05 ± NA ^b	1.88 ± 0.82
Remaining carcass	0.04 ± 0.01	1.80 ± 0.14
Mice		
Non-glandular stomach	0.23 ± 0.08	15.25 ± 1.70
Glandular stomach	0.04 ± 0.01	3.15 ± 0.29
Liver	0.10 ± 0.02	5.79 ± 0.91
Kidney	0.09 ± 0.01	7.28 ± 0.85
Bladder	0.13 ± 0.10	3.27 ± 0.65
Fat	0.04 ± 0.03	4.65 ± 1.29
Skin	0.02 ± 0.02	1.64 ± 0.83
Blood	n.d. ^c	0.79 ± 0.21
Remaining carcass	0.01 ± 0.002	1.31 ± 0.20

^aEach value is the mean ± SD for three animals unless otherwise noted.^bDetectable in only one of three rats.^cn.d., Not detectable.

0.52 and 22.30 µg equivalents g⁻¹ tissue in the 1 and 50 mg kg⁻¹ dose groups, respectively. Similar amounts were also seen in the corresponding dose groups of the mouse. Comparable levels of radioactivity were recovered in the liver of both species. These results most probably represent binding of the test material or incorporation of radiolabelled carbon into portal-of-entry tissues. The other significant location of retained radioactivity was in the kidney and bladder of both species. This radioactivity may represent contribution by urinary metabolites, since this was the major route of elimination in both species.

The metabolic profile of radiolabelled metabolites of DCP in the urine of rats and mice administered ^{14}C -DCP was determined via HPLC, with radiochemical detection (HPLC/RAM). Pooled urine samples were analysed from all four dose groups. Extracts of faecal samples from the high-dose groups only were analysed for metabolite composition due to the low level of radioactivity present in the 1 mg kg⁻¹ dose samples.

Twenty-one metabolites of ^{14}C -DCP were characterized via reverse-phase HPLC at levels of at least 1% of the administered dose (table 5 and figure 1). Five of six major metabolites > 5% of the administered dose were identified via ESI-LC/MS analysis (L, S, W, Z, BB). The most polar major metabolite, peak D, was not identified but characterized as arising via hydrolysis of DCP based on similar a HPLC profile to comparable metabolites of CAL and CAA. All six major metabolites were observed in the mouse. Only four of the major metabolites, peaks D, L, W and Z, were quantifiable in the rat via HPLC/RAM detection. However, all six metabolites were observed at trace levels, via ESI-LC/MS analysis, in the pooled urine of rats administered 50 mg kg⁻¹ ^{14}C -DCP (figure 2).

Table 5. Distribution of ^{14}C -DCP metabolites in the excreta of male F344 rats and male B6C3F1 mice.

Approximate retention time (min)	Peak name	F344 rat				B6C3F1 mice				Metabolite structure
		1 mg kg ⁻¹ Urine (%)	50 mg kg ⁻¹			1 mg kg ⁻¹ Urine (%)	100 mg kg ⁻¹			
			Urine (%)	Faeces (%)	Totals (U+F) (%)		Urine (%)	Faeces (%)	Totals (U+F) (%)	
3.5	A	2.51	0.95	ND	0.95	0.84	1.03	2.96	3.99	*
3.9	B	0.49	0.13	1.02	1.15	2.16	0.98	ND	0.98	*
5.3	C	0.93	0.38	ND	0.38	4.90	1.17	ND	1.17	*
6.5	D	5.14	1.66	ND	1.66	0.79	2.50	ND	2.50	*
14.3	I	0.30	0.02	ND	0.02	0.39	0.44	1.51	1.95	*
15.3	J	0.36	0.48	ND	0.48	1.07	2.71	ND	2.71	*
15.6	K	0.70	0.46	ND	0.46	2.91	0.49	0.50	0.99	*
16.0	L	18.53	10.13	1.69	11.82	7.38	3.82	1.00	4.82	<i>cis/trans</i> -DCP-sulfoxide and dimercapturate isomers
17.4	M	ND	0.05	0.39	0.44	1.09	0.35	ND	0.35	**
21.5	R	ND	ND	ND	ND	ND	2.52	ND	2.52	<i>cis</i> -DCP-N-acetyl-glutathione ***
23.5	S	ND	ND	ND	ND	14.87	9.65	ND	9.65	<i>cis</i> -DCP-pyruvate
25.1	T	ND	1.28	ND	1.28	ND	1.07	ND	1.07	<i>trans</i> -DCP-N-Acetyl-glutathione **
26.0	U	ND	ND	1.20	1.20	ND	0.12	ND	0.12	**
26.2	V	ND	ND	ND	ND	1.47	2.41	ND	2.41	<i>cis</i> -DCP-lactate
27.5	W	21.99	30.35	ND	30.35	5.39	11.25	2.49	13.74	<i>cis</i> -DCP-mercapturate
28.8	X	ND	ND	ND	ND	2.59	1.39	1.17	2.55	<i>trans</i> -DCP-pyruvate
30.5	Y	ND	ND	ND	ND	0.80	1.02	1.02	2.04	<i>trans</i> -DCP-lactate
32.0	Z	5.57	13.91	ND	13.91	0.43	3.42	ND	3.42	<i>trans</i> -DCP-mercapturate
34.8	BB	ND	ND	ND	ND	8.87	4.34	ND	4.34	<i>cis</i> -DCP-acetate
42.0	CC	ND	ND	ND	ND	2.99	1.75	ND	1.75	<i>trans</i> -DCP-acetate
44.9	EE	ND	ND	ND	ND	1.25	0.35	ND	0.35	**

ND, not detected at < 0.01% of administered dose.

* Metabolite structure unknown, but also seen in urine of rats administered 3-chloroallyl alcohol or 3-chloroacrylic acid.

** Metabolite structure unknown.

*** Metabolite identification tentative, based on LC/MS spectra.

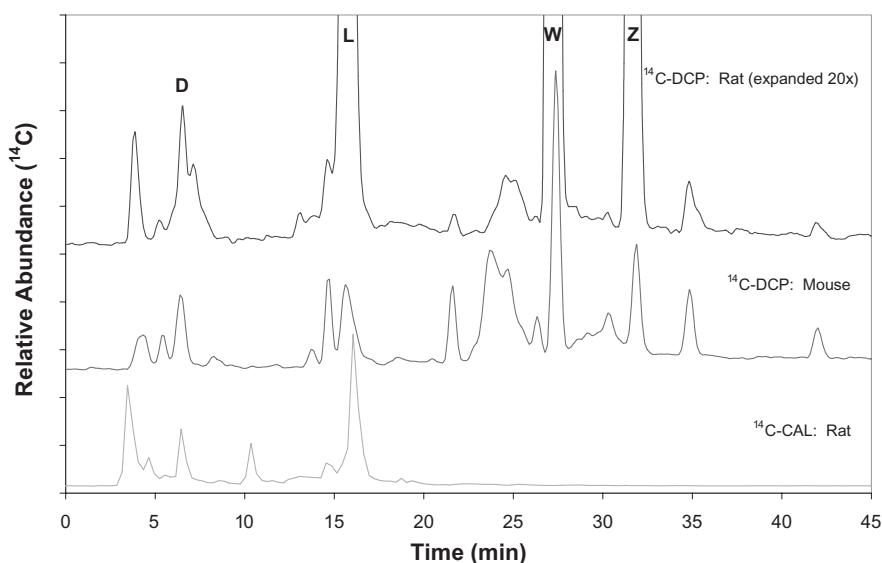


Figure 1. HPLC separation of radiolabelled metabolites in (top) 0–12-h urine of Fischer 344 rats administered 50 mg kg^{-1} ^{14}C -DCP, (middle) 0–12-h urine of $\text{B}_6\text{C}_3\text{F}_1$ mice administered 100 mg kg^{-1} ^{14}C -DCP and (bottom) 6–12-h urine of Fischer 344 rats administered 65 mg kg^{-1} ^{14}C -CAA.

Several early eluting metabolites were observed in rat and mouse excreta samples (figure 1 and table 5). No identification of these metabolites was achieved via ESI-LS/MS analysis of the samples. However, comparable peaks were observed for most of these metabolites in urine samples from F344 rats administered ^{14}C -CAL, a probable hydrolysis product of DCP in mammals. One metabolite unique to ^{14}C -CAL, at a retention time of 10.5 min, may be a result of conjugation of this alcohol when administered directly to the rat. However, no structural data were obtained for this compound.

Peak L in urine samples from both species was found to contain four metabolites: 2,3-dimercapturate, 3,3-dimercapturate, *cis*- and *trans*-DCP-sulfoxide. These metabolites were not resolved via HPLC separation and eluent fraction collection (figure 1). However, ESI-LC/MS analysis of these compounds afforded identification of four metabolites (figures 2 and 3). The isomers of DCP-sulfoxide have been reported previously (Kaye *et al.* 1972). The early eluting dimercapturate metabolite had a comparable retention time and product ion mass spectrum with the 2,3-dimercapturate standard (figure 4A, B). This metabolite, obtained from the D_4 -DCP test material, was found to contain three deuterium atoms (figure 4C), consistent with glutathione addition to the 2-carbon of an epoxide of DCP or DCP-glutathione, followed by rearrangement of the epoxide to an acyl chloride, with concomitant loss of the deuterium atom on the 3-carbon (figure 9A). The later eluting dimercapturate metabolite was identified as 3,3-dimercapturate based on retention time and product ion mass spectral matches (figure 5A, B). This latter metabolite was also observed as a urinary metabolite from F344 rats administered ^{14}C -CAL (figure 5C) and ^{14}C -CAA (data not shown). This metabolite, obtained from the D_4 -DCP test material, was found to contain two deuterium atoms (figure 5D), consistent with loss of two deuterium atoms on formation of 3-chloroacrylic acid, a proposed intermediate in this metabolic pathway (figure 9B).

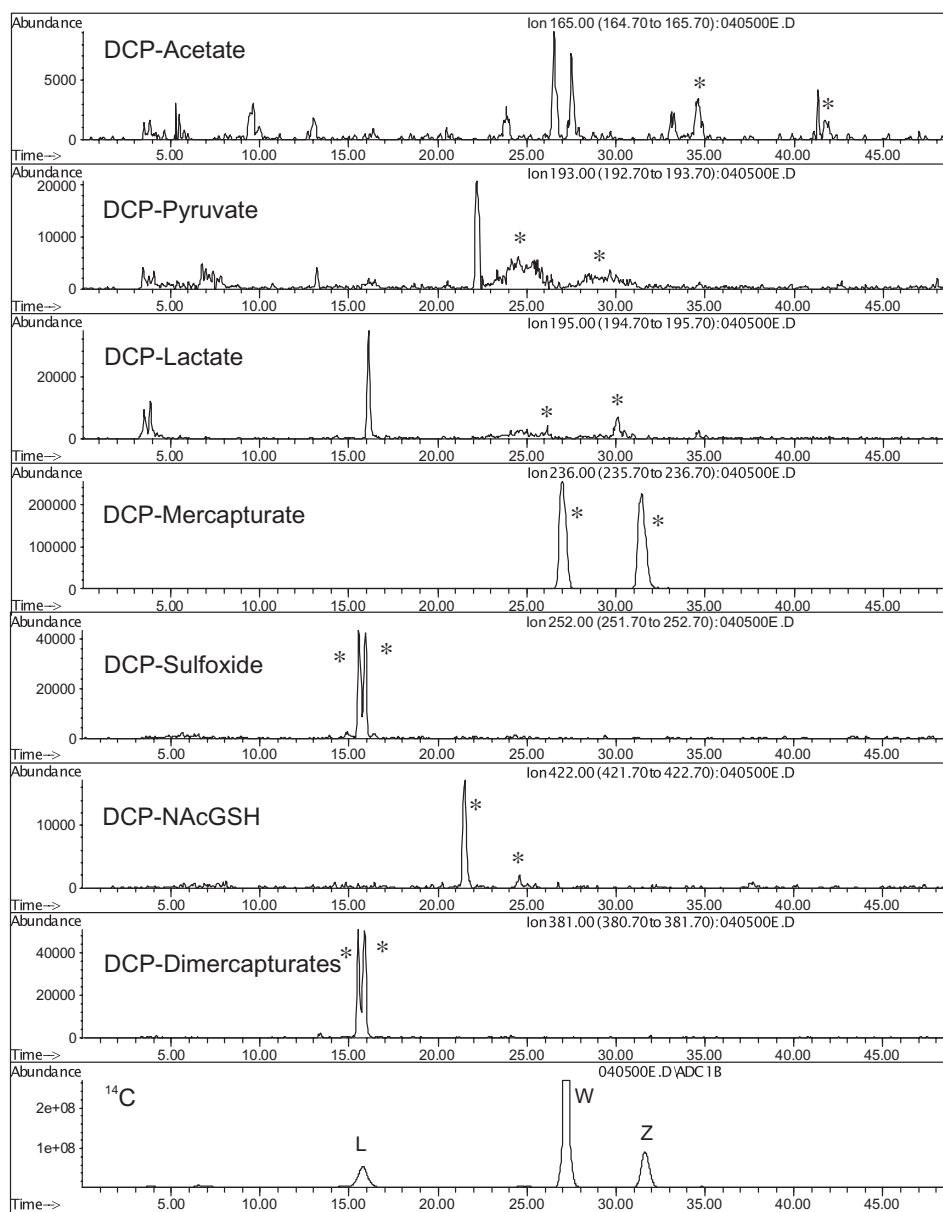


Figure 2. Selected-ion monitoring chromatograms of the major radiolabelled metabolites in the 0–12-h urine of F344 rats administered 50 mg kg⁻¹ ¹⁴C-DCP.

Peaks R and T comprised ND (0.01)–2.5% of the administered dose in mice and rats. These metabolites were tentatively assigned the structure of *cis*- and *trans*-*S*-(3-chloro-2-propenyl)-N-acetyl-glutathione (*cis*-DCP-NACGSH), respectively. As shown in figure 6A, the MS spectrum for peak T (*trans*-DCP-NACGSH) indicates a molecular weight of 423, with retention of one chlorine atom. MS/MS analysis of this metabolite afforded product ions consistent with the proposed structure (figure 6B). The tentative assignment of *cis*- and *trans*-configurations to the early and late eluting isomers of this metabolite was based on the consistent

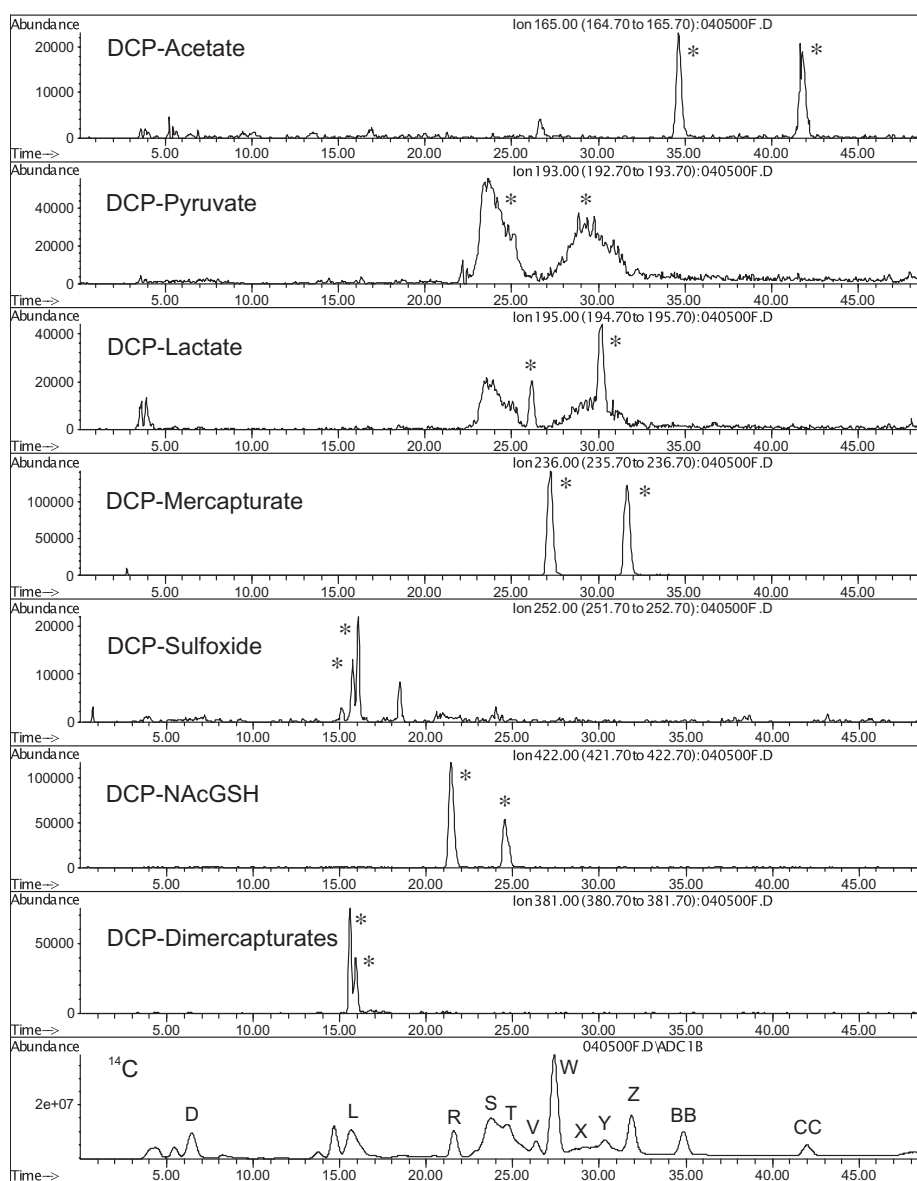


Figure 3. Selected-ion monitoring chromatograms of the major radiolabelled metabolites in the 0–12-h urine of $\text{B}_6\text{C}_3\text{F}_1$ mice administered 100 mg kg^{-1} ^{14}C -DCP.

elution order of *cis* < *trans* for other pairs of DCP metabolite standards (DCP-acetate, DCP-pyruvate, DCP-mercapturate, DCP-sulfoxide; data not shown). This metabolite has not been reported in the literature. However, Fisher and Kilgore (1988) did identify the intact glutathione conjugate of DCP as a circulating metabolite in the blood of male Sprague–Dawley rats exposed to DCP vapour via inhalation. In addition, intact glutathione conjugates have been identified as urinary metabolites of efavirenz and spinosad (Domoradzki *et al.* 1996, Mutlib *et al.* 1999).

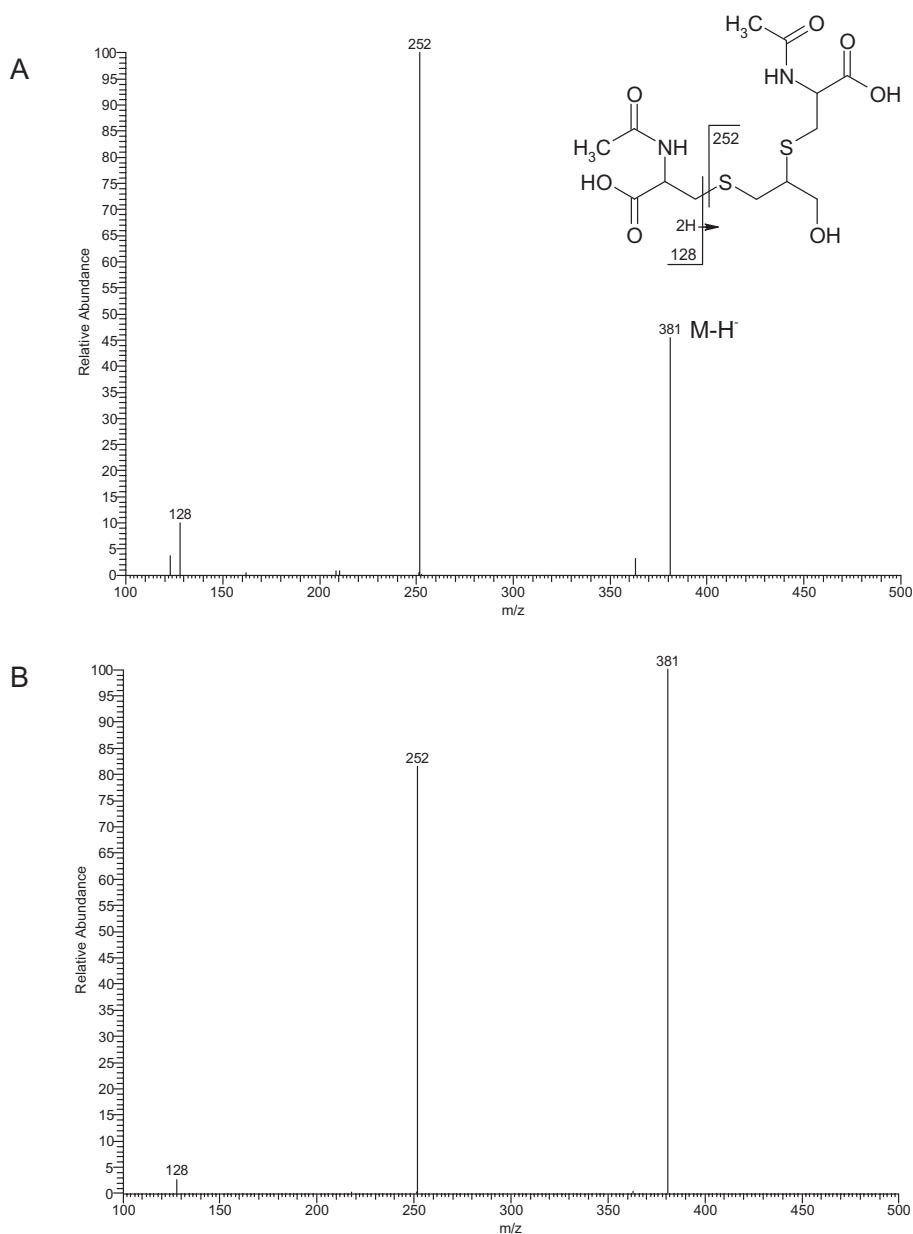


Figure 4. Negative-ion ESI-product ion mass spectra: (A) synthetic standard of 2,3-dimercapturate; (B) metabolic 2,3-dimercapturate in peak L of 0–12-h urine of F344 rats administered 50 mg ^{14}C -DCP mg^{-1} body weight. Negative-ion ESI-product ion mass spectra: (C) metabolic 2,3-dimercapturate in peak L of 0–9-h urine of F344 rats administered 50 mg D_4 -DCP mg^{-1} body weight.

Peak S was quantifiable, via radiochemical detection, as a DCP metabolite in mice, but not in rats. However, as stated above, trace levels of this metabolite were observed as a urinary metabolite of DCP in the rat, via ESI-LC/MS analysis (figure 3). The MS spectrum for this compound affords a molecular weight of 194 for this metabolite, with retention of one chlorine atom (figure 7A).

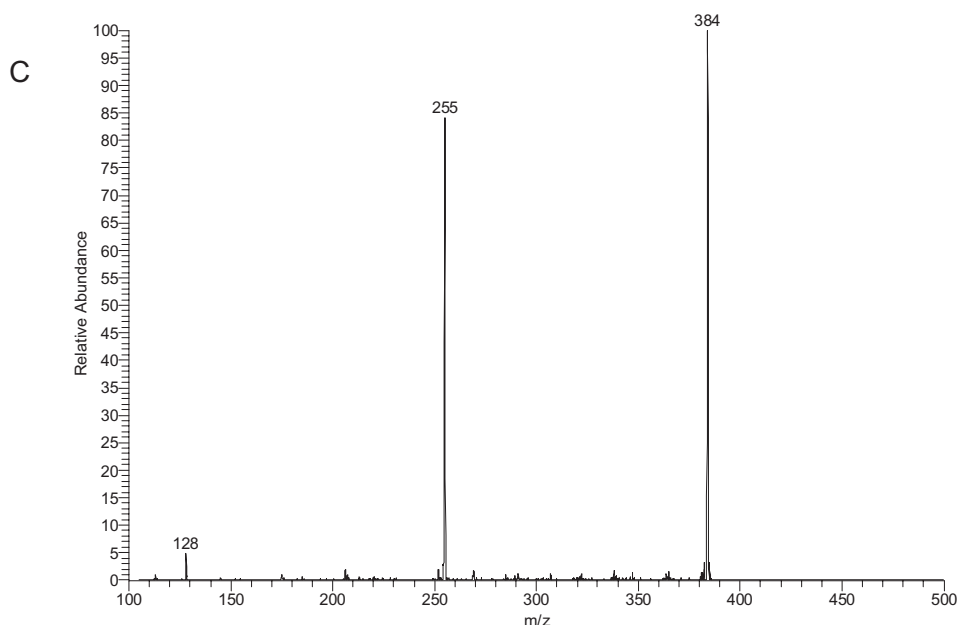


Figure 4. Continued.

MS/MS analysis of this metabolite afforded product ions consistent with the proposed structure for DCP-pyruvate (figure 7B). Preliminary ESI-LC/MS analysis of the synthesized *cis*-isomer of this of this compound afforded a retention time and mass spectral match with peak S (data not shown). However, definitive MS/MS spectra were not obtained due to degradation of this standard during storage. The *trans*-isomer of this metabolite was identified in the same manner as peak X (data not shown).

Peaks V, Y, BB and CC were quantitated only in the mouse (figure 3). These metabolites were identified as the *cis*- and *trans*-isomer pairs of DCP-lactate and DCP-acetate, respectively, based on retention time and spectral match with synthesized standards (data not shown). These metabolites were also seen at trace levels in the rat, via ESI-LC/MS analysis (figure 2). The tentative assignment of *cis*- and *trans*-configurations to the early and late eluting isomers of DCP-lactate was based on the consistent elution order of *cis* before *trans* for other pairs of DCP metabolite standards, as described previously (see peaks R and T).

These metabolite identification data show that the major pathway for DCP metabolism in the F344 rat and B₆C₃F₁ mouse involves glutathione conjugation, as has been reported (Climie *et al.* 1979). In addition to the N-acetylcysteine and N-acetylcysteine sulfoxide metabolites identified previously, numerous additional novel metabolites of the glutathione conjugation pathway were identified in the rat and mouse. As shown in figure 8, these additional metabolites represent subsequent metabolism of an intermediate *S*-(3-chloro-2-propenyl)-L-cysteine metabolite to the corresponding thiopyruvate, thiolactate and thioacetate conjugates.

Hydrolysis of the 3-chloro moiety of DCP, is proposed as the mechanism for production of CO₂ from this compound (figure 8). A total of 13.7–17.6% of radiolabelled DCP was converted to $^{14}\text{CO}_2$ in the rat or mouse following oral

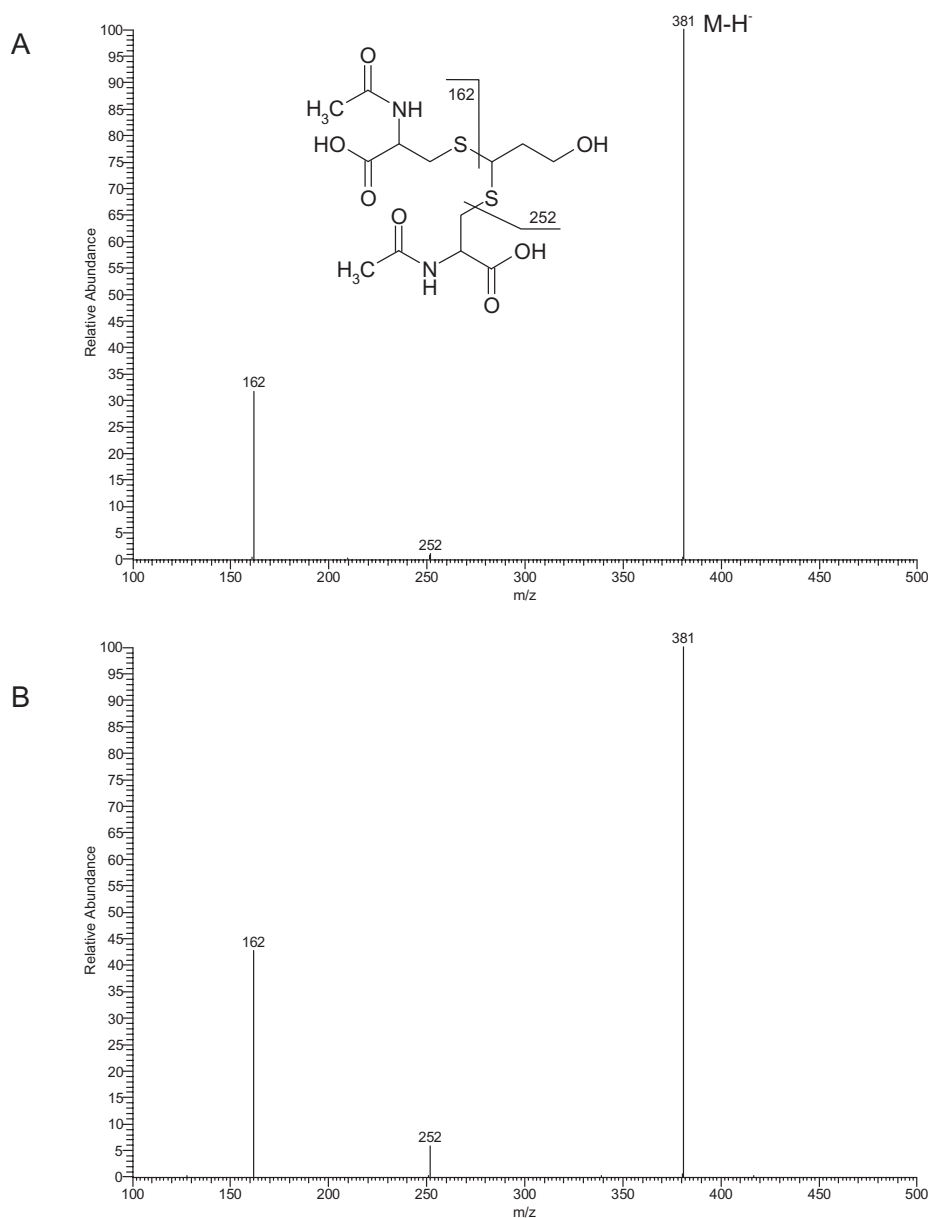


Figure 5. Negative-ion ESI-product ion mass spectra: (A) synthetic standard of 3,3-dimercapturate; (B) metabolic 3,3-dimercapturate in peak L of 0–12-h urine of male F344 rats administered 50 mg ¹⁴C-DCP mg⁻¹ body weight. Negative-ion ESI-product ion mass spectra: (C) metabolic 3,3-dimercapturate in 12–24-h urine of male F344 rats administered 65 mg kg⁻¹ ¹⁴C-CAL; (D) metabolic 3,3-dimercapturate in peak L of 0–9-h urine of male F344 rats administered 50 mg D₄-DCP mg⁻¹ body weight.

administration (table 1). The results of the current study also show that administration of ¹⁴C-labelled CAL or CAA to male F344 rats affords even higher amounts of the dose as radiolabelled CO₂ (50–52 and 60–68%, respectively). These data, showing metabolic CO₂ production increasing from DCP to CAL, with the highest levels arising from CAA acid, support the premise of CO₂ arising from

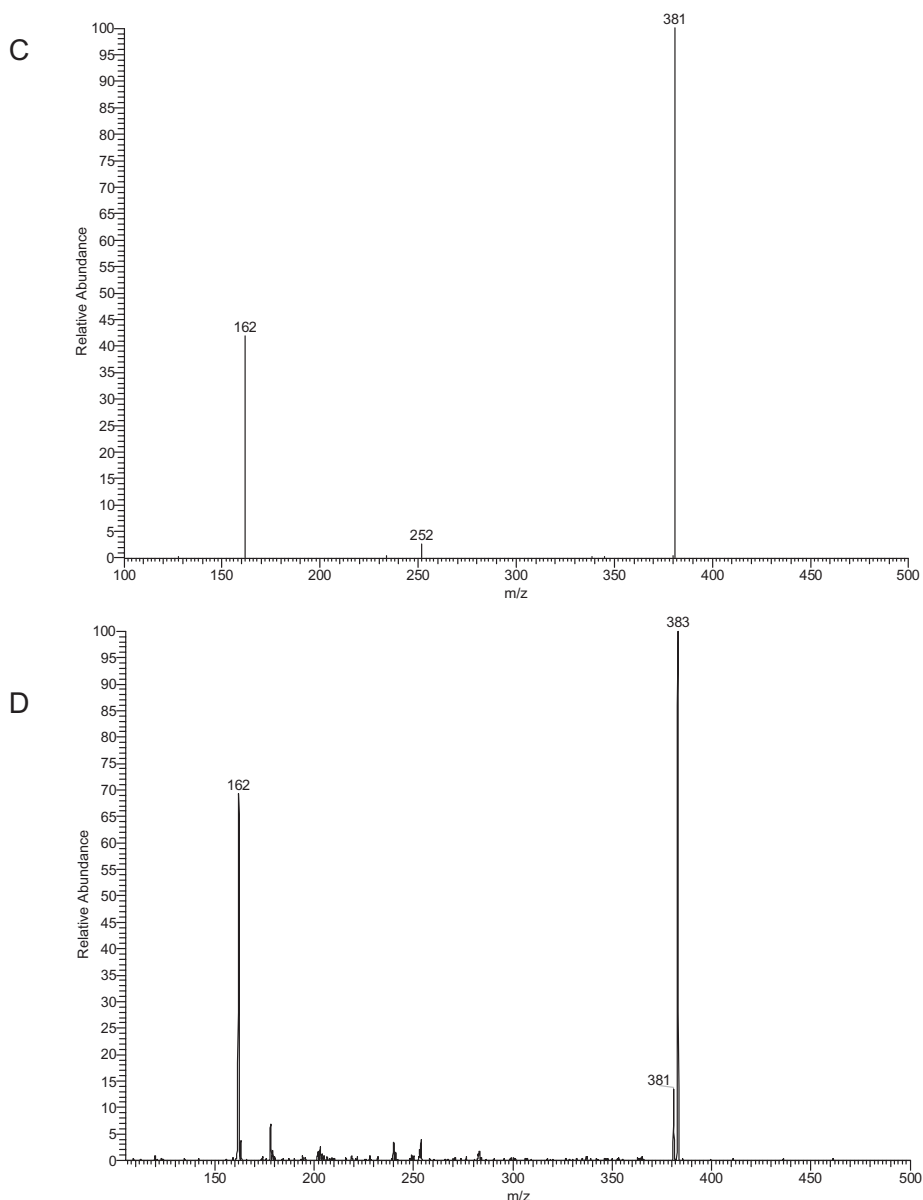


Figure 5. Continued.

DCP via initial hydrolysis of this test material. This is consistent with the proposed route of metabolism of the related compound, ^{14}C -epichlorohydrin to $^{14}\text{CO}_2$, via the intermediate 3-chloro-1,2-propanediol (Gingell *et al.* 1985). The latter authors report 38% of this test material converted to radiolabelled CO_2 .

Since CAL was not identified as a metabolite of DCP in the present study, one cannot rule out oxidative dechlorination as a route of DCP conversion to CAA, and subsequent metabolites. However, several pieces of data support an initial hydrolysis of DCP to CAL. Numerous polar metabolites of DCP were seen in the urine of both species administered this test material. Most of these HPLC peaks were

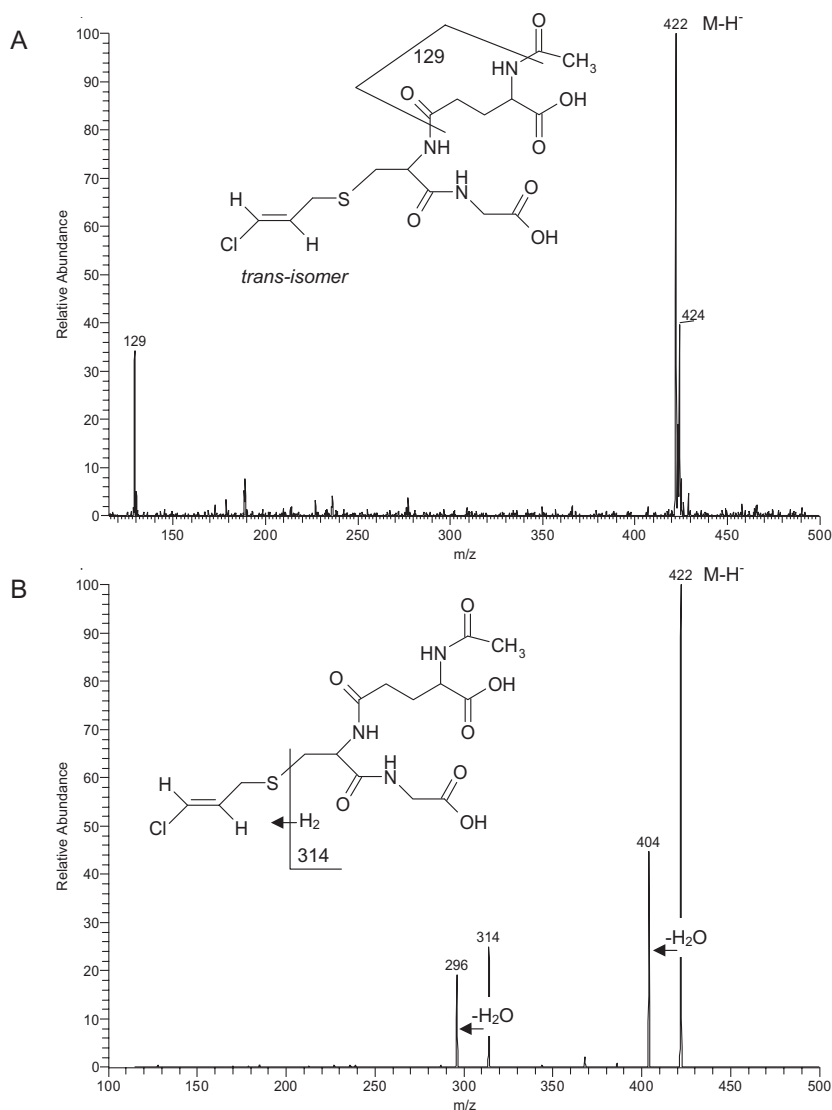


Figure 6. Peak T (*trans*-DCP-N-acetyl-glutathione conjugate; tentative structure) in 0–12-h urine of male B₆C₃F₁ mice administered 100 mg ¹⁴C-DCP mg⁻¹ body weight: (A) negative-ion ESI-mass spectrum; (B) negative-ion ESI-product ion mass spectrum.

also observed as metabolites of ¹⁴C-CAL or ¹⁴C-CAA in the male rat. Whereas these polar metabolites were not fully identified, the similarity in the profile of these early eluting HPLC peaks with those arising from CAL or CAA suggest that these metabolites represent products of initial hydrolysis of the 3-chloro moiety of DCP. DCP has been shown to be hydrolysed to CAL in aqueous conditions (WHO 1993), suggesting that some test material could be hydrolysed in the gastrointestinal tract before systemic absorption. Eder and Dornbusch (1988) also studied the metabolic fate of the related compound 2,3-dichloro-1-propene. They found both the 2-chloroallyl alcohol and 2-chloroacrylic acid metabolites in rat and suggested that they arise via hydrolysis of the test material. The novel

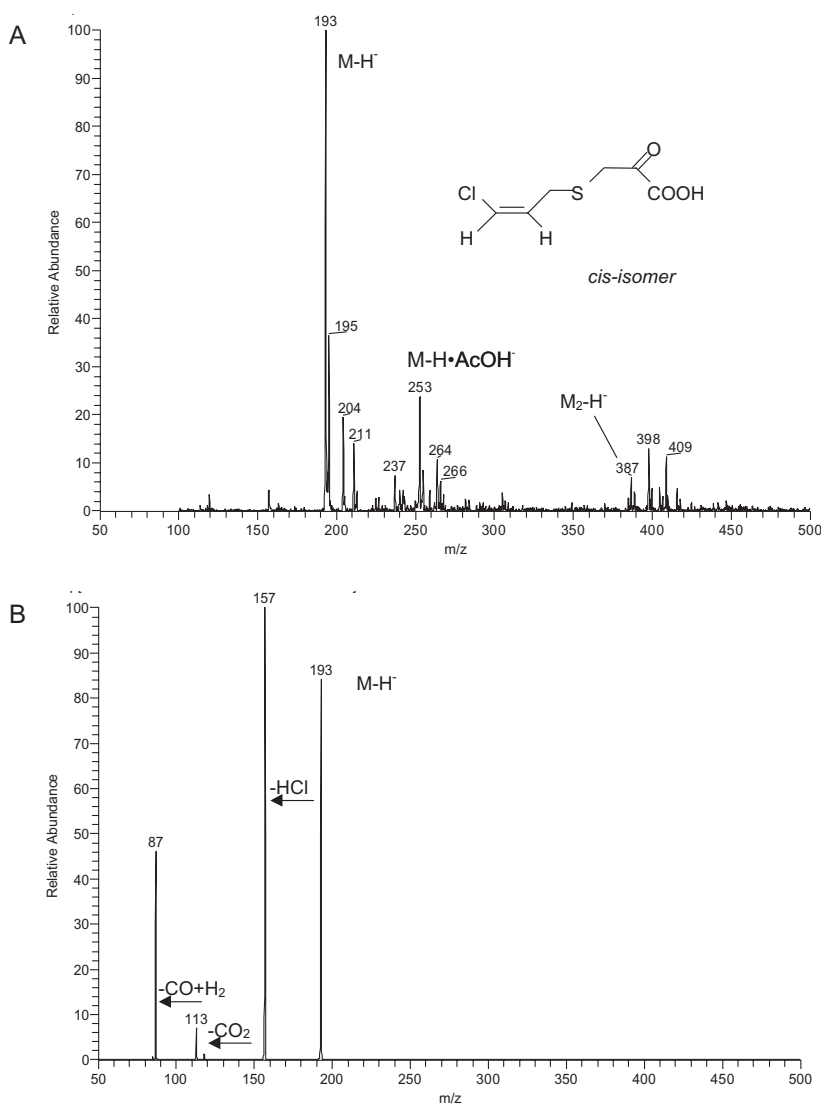


Figure 7. Peak S (*cis*-DCP-pyruvate; tentative structure) in 0–12-h urine of male $\text{B}_6\text{C}_3\text{F}_1$ mice administered $100\text{ mg }^{14}\text{C}\text{-DCP mg}^{-1}$ body weight: (A) negative-ion ESI-mass spectrum; (B) negative-ion ESI-product ion mass spectrum.

3,3-dimercapturate metabolite was also identified as a metabolite of DCP in the rat and mouse. This compound was also identified as a major urinary metabolite of CAL and CAA in the present study and has been found as a metabolite of propargyl alcohol (Banijamali *et al.* 1999). This compound is thought to arise via $\text{S}_\text{N}2$ - and/or Michael-type additions of glutathione to the terminal carbon atom of these compounds (figure 9). Formation of 3,3-dimercapturate from DCP is therefore most probably via an initial hydrolysis to 3-chloroallyl alcohol, oxidation to 3-chloroacrylic acid, followed by glutathione conjugation via the previously reported mechanism. Reduction to the final propanol moiety may occur via dehydrogenases or acid reductases (Natarajan *et al.* 1984). Quantitative analysis

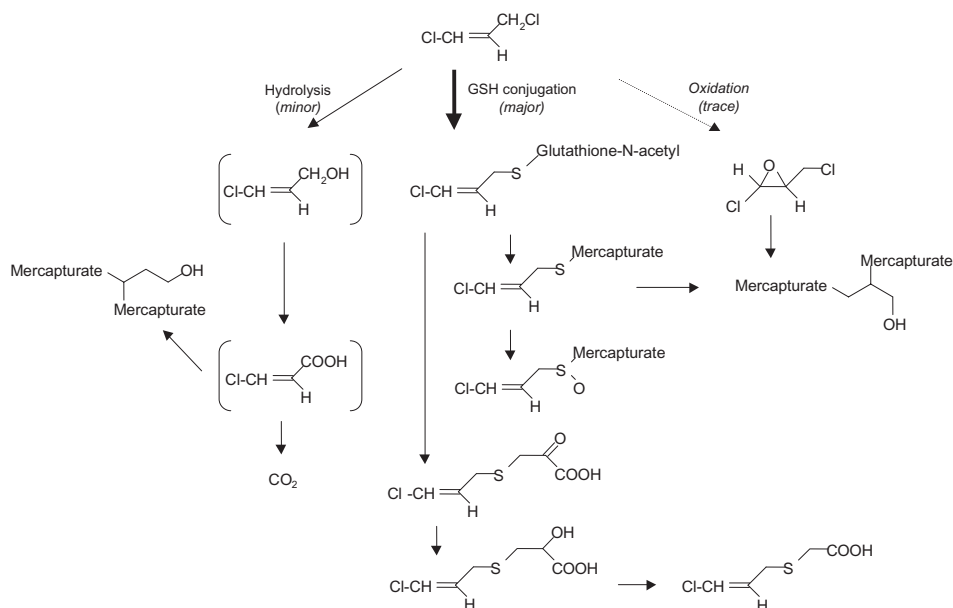


Figure 8. Metabolic fate of ^{14}C -DCP in the F344 rat and the $\text{B}_6\text{C}_3\text{F}_1$ mouse.

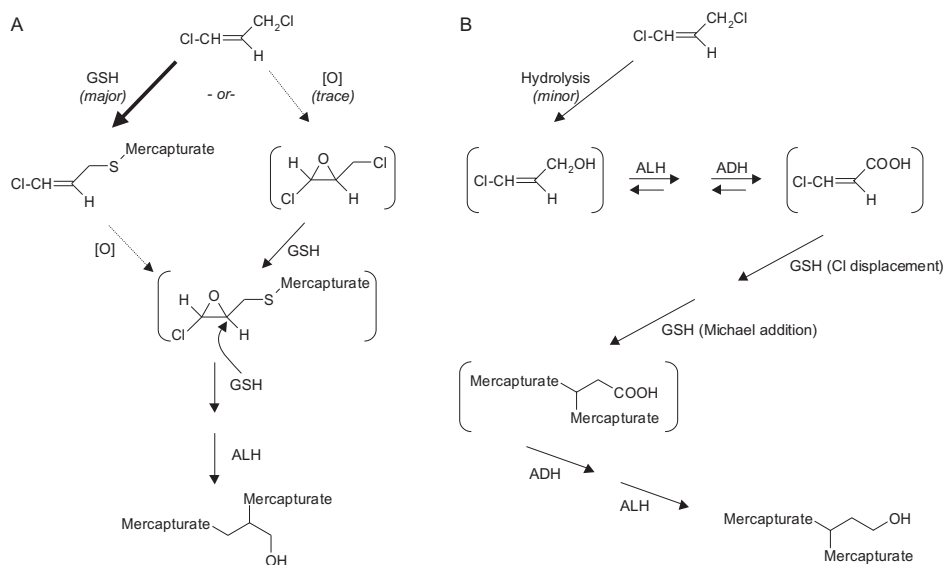


Figure 9. Proposed mechanism for the formation of dimercapturic acid metabolites of DCP in the F344 rat and the $\text{B}_6\text{C}_3\text{F}_1$ mouse: (A) 2,3-dimercapturate; (B) 3,3-dimercapturate.

via ESI-LC/MS identified 4.2–8.8 and ND–0.7% of the administered dose as this metabolite in rats and mice, respectively (table 6).

Another novel dimercapturic acid metabolite was identified in the rats and mice administered ^{14}C -DCP. The structure of this metabolite was determined to be the 2,3-configuration of DCP-dimercapturate. This metabolite was present at trace

Table 6. Distribution of ^{14}C -DCP metabolites in excreta via glutathione conjugation, hydrolysis and epoxidation pathways.

	F344 rat		B6C3F1 mouse	
	1 mg kg ⁻¹ Urine (%)	50 mg kg ⁻¹ Urine+Faeces (%)	1 mg kg ⁻¹ Urine (%)	100 mg kg ⁻¹ Urine+Faeces (%)
HPLC peaks A-K	10.43	5.16	14.20	14.76
3,3-Dimercapturate in peak L	8.83	4.17	ND(0.2)	0.72
$^{14}\text{CO}_2$ total	17.60	15.10	14.40	13.70
Hydrolysis pathway total	36.86	24.44	28.60	29.18
2,3-Dimercapturate in peak L	1.62	0.62	2.06	0.54
Epoxidation metabolites total	1.62	0.62	2.06	0.54
DCP-Sulfoxides in peak L + peaks M-FF	8.08	7.03	5.32	3.56
Glutathione metabolites total	35.64	54.71	47.70	50.17
Total per cent of Dose in analyzed excreta + CO_2	74.12	79.77	78.36	79.89

levels only, accounting for 0.6–1.6 and 0.5–2.1% of the administered dose in rats and mice, respectively (table 6). As shown in figures 8 and 9, this metabolite might arise from epoxidation of the glutathione adduct of DCP. Alternately, epoxidation of DCP, followed by glutathione conjugation could give rise to this minor metabolite. The data obtained in the present study do not provide sufficient information for elucidation of the mechanism of formation for this metabolite in the rodent.

The overall quantitative comparison for the various major metabolic pathways for ^{14}C -DCP is shown in table 6. This distribution of radioactivity present in the analysed excreta samples and via $^{14}\text{CO}_2$ elimination does not account for radioactivity present in faecal samples from the 1 mg kg⁻¹ dose groups (9–15%), tissues plus carcass (1.8–6.0%), and cage wash plus charcoal trap (1.0–4.2%). However, these totals do provide information on the relative metabolic fate of ^{14}C -DCP via these three metabolic pathways. Metabolites assumed to arise via initial glutathione conjugation (DCP-sulfoxides plus peaks M-EE) account for approximately 36–55 and 48–50% of the administered dose excreted from rats and mice, respectively. Initial hydrolysis of the 3-chloro moiety of DCP, assumed to result in formation of the polar metabolites of DCP (peaks A-K plus 3,3-dimercapturate), accounted for approximately 9–19 and 14–15% of the administered dose in rats and mice, respectively. Inclusion of $^{14}\text{CO}_2$ formation data affords a total of 24–37 and 29% of the administered dose being metabolized via initial hydrolysis of DCP. Formation of 2,3-dimercapturate, which may represent epoxidation of DCP or a DCP-glutathione conjugate, was a minor metabolic pathway, comprising 0.6–1.6 and 0.5–2.1% of the administered dose in rats and mice, respectively. These low levels of metabolites arising via epoxidation of the test material or metabolite(s) is consistent with previous work in the authors' laboratory in which it was shown that epoxidation of DCP to DCPO is a minor route of metabolism at dose levels that cause no acute hepatic injury or death (Bartels *et al.* 2000). These overall metabolic fate data are also consistent with the metabolism of a related compound,

2,3-dichloropropene (2,3-DCP). Eder and Dornbusch (1988) reported that 2,3-DCP is metabolized via glutathione conjugation, hydrolysis and epoxidation in the female Wistar rat, accounting for 67.1, 29.2 and 3.6% of the urinary radioactivity, respectively.

In summary, ^{14}C -DCP is rapidly absorbed and eliminated in both the male F344 rat and $\text{B}_6\text{C}_3\text{F}_1$ mouse. The absorbed test material is extensively metabolized in both species. Metabolites arising from initial glutathione conjugation account for approximately 36–55 and 48–50% of the administered dose excreted from rats and mice, respectively. Hydrolysis of the 3-chloro moiety of DCP, assumed to result in formation of the polar metabolites of DCP, accounted for approximately 9.3–19.3 and 14–16% of the administered dose in rats and mice, respectively. Inclusion of $^{14}\text{CO}_2$ formation affords 24–37 and 29% of the administered dose being metabolized via initial hydrolysis of DCP. Numerous metabolites were identified that arise from both these metabolic pathways. Two novel dimercapturic acid conjugates were also identified at low levels that are proposed to arise via initial hydrolysis of DCP or epoxidation of DCP-glutathione conjugate or DCP itself. Formation of 2,3-dimercapturate, which may represent epoxidation of a DCP-glutathione conjugate or DCP, was a minor metabolic pathway, comprising 0.6–1.6 and 0.5–2.1% of the administered dose in rats and mice, respectively. Quantitative differences, only, are seen between the overall metabolic profile of DCP in these two species.

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Prediction of *in vivo* drug interactions with eplerenone in man from *in vitro* metabolic inhibition data

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1. The inhibition kinetics of eplerenone (EP) 6 β -hydroxylation by 10 drugs were determined *in vitro* using human liver microsomes. Inhibition factors were calculated from *in vitro* inhibition constant (K_i) and three different inhibitor $C_{\max}$ values (liver $C_{\max}$ of total and unbound inhibitor, and maximum influx concentration of inhibitor into the liver). Subsequently, the inhibition factors were compared with available pharmacokinetic data derived from clinical interaction trials conducted by Pfizer involving EP and these drugs. EP was also evaluated for its effect on the metabolism of 10 drugs *in vitro*, and again the *in vitro* data were compared with results from the clinical trials.

2. The K_i values for the inhibition of EP 6 β -hydroxylation by cisapride, cyclosporine, digoxin, erythromycin, fluconazole, ketoconazole, midazolam, saquinavir, simvastatin and verapamil were 2.90, 1.24, > 75.0, 9.50, 59.0, 0.160, 8.10, 0.546, 6.23 and 13.3 μ M, respectively. Among the three methods, inhibition factors (R_b) calculated using the K_i and estimated liver $C_{\max}$ values of the unbound drug were best correlated with the *in vivo* area under the curve-fold increases of EP in humans. The R_b values for the drugs listed above were 1.04, 1.69, 1.00, 2.17, 2.24, 4.90, 1.00, 1.82, 1.01 and 1.04, respectively, and the *in vivo* area under the curve-fold increases of EP by these drugs were 1.04, 1.16, 0.930, 2.87, 2.24, 5.39, 1.00, 2.07, 1.03 and 1.98, respectively.

3. EP did not have any significant effects on the drugs tested *in vitro* or in the clinic.

4. Using *in vitro* metabolic interaction data, human *in vivo* pharmacokinetic interactions involving EP could be predicted nearly quantitatively. The lack of effects of EP on the pharmacokinetics of other drugs in man was also suggested in the *in vitro* data.

Introduction

Eplerenone (EP, pregn-4-ene-7,21-dicarboxylic acid, 9,11 α -epoxy-17 α -hydroxy-3-oxo- γ -lactone, methyl ester) (figure 1) is a highly selective aldosterone blocker (McMahon 2001). Blockade of aldosterone is known to be beneficial in the treatment of cardiovascular and renovascular diseases (Pitt *et al.* 1999). Although angiotensin-converting enzyme inhibitors and angiotensin-type 1 receptor antagonists act to suppress the renin–angiotensin–aldosterone system, these agents do not control plasma aldosterone levels adequately, which is known as ‘aldosterone synthesis escape’ (MacFayden *et al.* 1997, Pitt *et al.* 1999). Spironolactone, a non-selective aldosterone receptor antagonist, is effective in suppressing the actions of aldosterone. However, it is associated with progestational and anti-androgenic side-effects due to its promiscuous binding to other steroid receptors (Sun *et al.* 1997, Pitt *et al.* 1999). To avoid these side-effects, a new highly selective aldosterone blocker, EP, has been developed and approved in the

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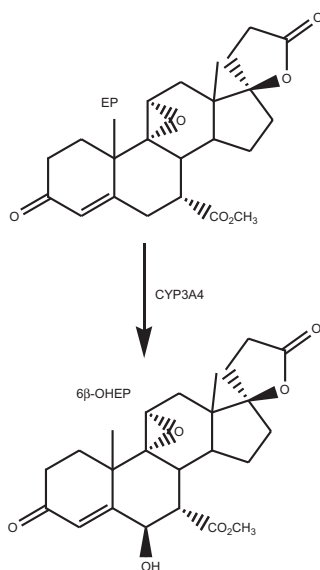


Figure 1. Chemical structure of EP and its metabolism to 6β-OHEP.

USA for the treatment of hypertension and heart failure. The presence of a 9,11-epoxide group in the EP molecule dramatically reduced progestational and anti-androgenic side-effects compared with spironolactone (McMahon 2001).

The 6β-hydroxylation was the primary route of EP metabolism in humans, which is mediated by CYP3A4 (Cook *et al.* 2002, 2003). Therefore, to evaluate their potential to bring about a pharmacokinetic interaction, 10 test drugs that are CYP3A inhibitors/substrate or known to be frequently involved in drug interactions were examined for their potency in inhibiting EP metabolism in human liver microsomes. Using *in vitro* K_i values for each of the 10 drugs, inhibition factors (R) were calculated, representing the potential for the test drugs to influence EP metabolism. EP was also evaluated for its effect on the metabolism of 10 drugs *in vitro*. The *in vitro* results were compared with corresponding clinical pharmacokinetic interaction data available internally through Pfizer Global Research in order to determine whether *in vitro* data could be used to predict the *in vivo* situation.

Materials and methods

In vitro metabolic interaction experiments

EP (lot no. RCT 10056, purity > 99%) and 6β-hydroxyeplerenone (6β-OHEP) were from Pfizer Corp. (St. Louis, MO, USA). Cisapride, cyclosporine, digoxin, erythromycin, 17α-ethinylestradiol, ketoconazole and (*R,S*)-verapamil were from Sigma-Aldrich (St Louis, MO, USA). Saquinavir was from Roche (Neuilly, France). Midazolam was from Ultrafine (Manchester Science Park, Manchester, UK). Fluconazole was from Ranbaxy Laboratories Ltd (New Delhi, India). Simvastatin was provided by Cedra Corp. (Austin, TX, USA). Human liver microsomes were from Cedra Corp. or XenoTech, L.L.C. (Kansas City, KS, USA). Buffer salts and other reagents were from Sigma-Aldrich, Burdick & Jackson (Muskegon, MI, USA) or Mallinckrodt (Hazelwood, MO, USA) and were of the highest quality available.

Inhibition constants (K_i) for the *in vitro* metabolic interactions between EP and potential inhibitors of EP 6β-hydroxylation were determined from incubations containing 0.1 mg ml⁻¹ human liver microsomes, 1 mM EDTA, 1 mM NADPH and 100 mM potassium phosphate buffer, pH 7.4, in a

500- μ l incubation mixture. EP dissolved in acetonitrile (final organic concentration < 1% v/v) was added to the mixture at concentrations of 25, 50, 100, 200 or 400 μ M. The concentrations of potential inhibitors used (also dissolved in acetonitrile) depended on the individual inhibitor and spanned a range from approximately 25–500% of the expected K_i . After equilibrating the incubation mixture, substrates and inhibitors for 3 min at 37°C, reactions were initiated by the addition of the NADPH. Samples were incubated for 15 min and terminated by the addition of the extraction solvent ethyl acetate. Preliminary experiments indicated that under these incubation conditions, rates of EP 6 β -hydroxylation were within the linear range with respect to both enzyme concentration and incubation time. Samples were incubated in duplicate. Following centrifugation of the samples, the supernatants were transferred to new test tubes, dried under a stream of nitrogen, reconstituted in liquid chromatography mobile phase and analysed for 6 β -OHEP on a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system validated as described below.

After formation rates for 6 β -OHEP were calculated, Lineweaver–Burke double-reciprocal plots were created and a mechanistic model of inhibition was chosen for subsequent data analysis. The K_i values were estimated by non-linear regression of the untransformed data using Systat® 6.0.1 (SPSS, Inc., Chicago, IL, USA) programmed with the model of inhibition that provided the best fit of the data. Inhibition factors (R) were calculated using three different methods:

$$\text{Method A: } R_a = 1 + C_{\max, I, L} / K_i \quad (1)$$

$$\text{Method B: } R_b = 1 + f_u * C_{\max, I, L} / K_i \quad (2)$$

$$\text{Method C: } R_c = 1 + f_u^* C_{\max, I, IN} / K_i, \quad (3)$$

where $C_{\max, I, L}$ is the liver $C_{\max}$ of the corresponding inhibitor after oral administration and f_u is the fraction unbound in plasma. Plasma $C_{\max}$ values for the inhibitors in humans after an oral dose ($C_{\max, I, P}$) were determined in their respective clinical trial as described below. $C_{\max, I, L}$ was estimated through a review of the literature and subsequent calculations. That is, for each inhibitor, a liver–plasma concentration ratio or partition coefficient (P_L) was found in a rat tissue distribution study. Multiplying this value by $C_{\max, I, P}$ provided an estimate for $C_{\max, I, L}$. When P_L is concentration dependent, a P_L corresponding to inhibitor concentrations at or near $C_{\max, I, P}$ after therapeutic doses was chosen for subsequent calculations. In equation (3) (Ito *et al.* 1998):

$$C_{\max, I, IN} = C_{\max, I, P} + (F_a \cdot K_a \cdot D) / Q_h,$$

where $C_{\max, I, IN}$ is the maximum influx concentration of the inhibitor into the liver, F_a is the fraction of the dose absorbed for each inhibitor, K_a is the first-order rate constant for inhibitor gastrointestinal absorption determined in the respective clinical trial as described below, D is the inhibitor dose and Q_h is hepatic blood flow in humans (86.8 l h⁻¹).

For examination of the potential inhibitory effects of EP on the metabolism of other drugs, IC₅₀ values were determined from the disappearance of the following drugs in the presence of EP at concentrations of 0, 1, 5, 25, 50 and 100 μ M: cisapride, cyclosporine, digoxin, erythromycin, midazolam, norethindrone, saquinavir, simvastatin and *R,S*-verapamil. The IC₅₀ value for 17 α -ethinylestradiol was also determined at final EP concentrations of 0, 25, 50, 100, 200 and 400 μ M. Interacting drugs were included at two concentrations, one approximating the $C_{\max, I, P}$, the other at five times $C_{\max, I, P}$. The incubations involving midazolam and saquinavir were performed using 0.1 and 0.05 mg ml⁻¹ protein, respectively. Incubations for the other drugs used microsomal protein at 0.5 mg ml⁻¹. Reactions were performed using a single time-point assay; however, the incubation time used ranged from 10 to 60 min and depended on the specific rate of metabolism of the test drug without inhibitor such that the rate was within the linear range with respect to time.

LC-MS/MS assay for 6 β -OHEP in human liver microsomes

An LC-MS/MS analytical method was developed and validated for the determination of 6 β -OHEP extracted from incubation buffer containing human liver microsomes. Briefly, 6 β -OHEP was dissolved in acetonitrile and infused directly into a Micromass Quattro II LC-MS/MS to determine the parent and product ions, and the instrument was tuned to maximize the signal. As a validation exercise, a standard curve (6 β -OHEP concentrations of 10.0, 20.0, 50.0, 100, 200, 1000 and 2000 ng ml⁻¹) was extracted in triplicate from suspensions of human liver microsomes using ethyl acetate, transferred and evaporated to dryness under a stream of nitrogen. Residues were reconstituted in mobile phase (40% aqueous acetonitrile) and injected onto the LC-MS/MS system equipped with 5- μ m reverse-phase Zorbax XDB-C8 (2.1 $\times$ 50 mm) analytical column. The m/z 431 $\rightarrow$ 211 product ions of 6 β -OHEP were monitored. In addition, six quality control (QC) samples were extracted and analysed at each of three concentrations (high, medium, low) prepared from a separate weighing of 6 β -OHEP. Relative extract stability was established later to 65 h by re-injecting a standard curve and associated QC samples and assessing the calculated concentrations. The specificity of the assay with regard to EP or to each potential inhibitor was evaluated by analysing triplicate medium 6 β -OHEP QC samples fortified with

EP or with high concentrations of potential inhibitors. On the day of validation, the coefficient of determination (R^2) for the standard curve was 1.000 and the coefficient of variation ($n=3$) varied from 1.10 to 5.72% over the range 20.0–2000 ng ml⁻¹. The coefficients of variation for high, medium and low QC samples ($n=6$) were 5.90, 8.53 and 6.91%, respectively. The lower limit of quantification for the assay was 10.0 ng ml⁻¹. In the evaluation of specificity, there was general agreement between the mean concentration of 6 β -OHEP in the four medium QC samples and the mean of QC samples with added inhibitor.

Source of the clinical interaction data

The *in vitro* data were compared with clinical interaction data available internally through Pfizer Global Research, most of which were published as abstracts by Tolbert *et al* (2000, 2002a–d) and Reid *et al* (2003a–d). The clinical interaction data were the results of 11 clinical trials designed to assess the potential for interaction between EP and several drugs (each administered orally) known to be potent CYP3A inhibitors and/or are possible candidates for involvement in pharmacokinetic interactions. The *in vivo* pharmacokinetic interaction data were considered to be reliable since the clinical interaction trials were performed using accurate methodology and according to appropriate protocols as briefly summarized below.

In each trial, eligible subjects were determined by clinical laboratory tests (haematology, biochemistry and urinalysis including microscopic examination), medical history, physical examination (including vital signs, height and weight), 12-lead electrocardiogram, drug and alcohol toxicity screen, and serum pregnancy test (when applicable), and monitored for adverse events throughout the trial period. All oral doses of EP consisted of one 100-mg once-daily (QD) film-coated tablet. All trials were approved by the Ethics Committee, Institutional Review Board at each clinical study site and conducted in accordance with the Declaration of Helsinki, and Food and Drug Administration Good Clinical Practices.

Six of the trials involving orally administered cisapride (Tolbert *et al*. 2002a), digoxin (Tolbert *et al*. 2002b), erythromycin (Tolbert *et al*. 2002c), saquinavir (Reid *et al*. 2003a), simvastatin (Reid *et al*. 2003b) or verapamil (Reid *et al*. 2003c) were designed to determine the effect of the test drug on the steady-state pharmacokinetics of EP, as well as on the effect of EP on the steady-state pharmacokinetics of the test drug, each in 24 (18 in the case of cisapride and simvastatin) healthy adults. Each of these interactions was assessed in a randomized, multiple-dose, six-sequence, three-period, three-treatment crossover study (table 1). Doses of each test drug, administered alone or with EP, were clinically relevant, and the number of days in each treatment period differed depending on which test drug was administered as described below. Cisapride (Propulsid[®], lot no. 99P0309) was administered at doses of 20 mg twice daily (BID) for the first 5 days of each 6-day treatment and once on the sixth day. Digoxin (Lanoxicap[®], lot no. SF089303) was administered at doses of 200 μ g QD on all 10 days of each treatment period. Erythromycin (Ery-Tab[®], lot no. 59427AF21) was administered at doses of 500 mg BID for the first 6 days of each 7-day treatment period and once on the seventh day. Saquinavir (Fortovase[®], lot no. 0F897K) was administered at doses of 1200 mg three times daily (TID) for the first 5 days of each 6-day treatment period and once on the sixth day. Simvastatin (Zocor[®], lot no. J0998) was administered at doses of 40 mg QD on all 7 days of each treatment period. Lastly, verapamil (Calan[®] SR, lot no. 0F897K) was administered at doses of 240 mg QD on all 7 days of each treatment period. In each trial, EP was also administered over the entire treatment period, alone or concomitantly with the first daily dose of each test drug. Regardless of the test drug involved, patients were allowed a

Table 1. Study design for the clinical interaction trials involving cisapride, cyclosporine, digoxin, erythromycin, saquinavir, simvastatin and verapamil. Each treatment group (A, B or C) includes three subjects for cisapride and simvastatin, and four subjects for the other test drugs.

Treatment sequence	Period		
	1	2	3
I	A	B	C
II	B	C	A
III	C	A	B
IV	A	C	B
V	B	A	C
VI	C	B	A

A = 100 mg EP, QD; B = test drug; C = 100 mg EP, QD + test drug.

2-day rest between treatment periods. Following doses on the last day of each treatment period, plasma samples were collected at predetermined intervals for determination of concentrations of EP and the appropriate test drug.

Two trials involving ketoconazole and fluconazole were designed to determine only the effect of the test drug administered orally on the pharmacokinetics of EP. In these studies, the effect of steady-state ketoconazole or fluconazole on the single-dose pharmacokinetic profile of EP, each in 18 healthy adults, was determined in a randomized, multiple-dose, two-sequence, two-period crossover study (Tolbert *et al.* 2000). For both trials, each subject received a single initial oral dose of EP 3 days before the first treatment period. On days 1–6 of the first ketoconazole treatment period, nine subjects received oral ketoconazole (Nizoral[®], lot no. 98P0579E, 200 mg tablet BID) while nine subjects received a placebo (lot no. RCT10903, 200 mg tablet BID). In the second treatment period, the nine subjects who received ketoconazole in the first treatment period received placebo in the second, while the nine subjects who received a placebo in the first treatment period received ketoconazole in the second. On day 6 of each treatment period, all subjects received a concomitant dose of EP. The fluconazole trial was performed in the same fashion as the ketoconazole trial in a different group of 18 subjects. However, the subjects received fluconazole (Diflucan[®], lot no. 84P005E, 200 mg QD) in place of ketoconazole. Plasma samples for determination of EP concentrations were collected at predetermined intervals after the initial dose of EP and on the sixth day of each treatment period. In addition, blood samples for determination of either ketoconazole or fluconazole concentrations were collected from subjects receiving active doses of either drug on days 1–6 of each treatment period.

Two trials involving cyclosporine (Reid *et al.* 2003d) and midazolam (Tolbert *et al.* 2002d) were designed only to determine the effect of EP on the steady-state pharmacokinetics of the test drugs, each in 20 healthy adults. Pharmacokinetic effects of EP administration were assessed in a randomized, multiple-dose, two-sequence, two-period crossover study. In the cyclosporine interaction study, each subject received a single initial oral dose of cyclosporine (400 mg QD, Neoral[®], lot no. 305515) 3 days before the first treatment period. During each 5-day treatment period, subjects received cyclosporine plus placebo or cyclosporine plus EP. In the midazolam interaction study, each subject received a single initial oral dose of midazolam (10 mg, Versed[®], lot no. 0004) 3 days before the first treatment period. During the 6-day treatment periods, subjects received either EP or a placebo QD. On the sixth day of each treatment period, subjects were administered a concomitant 10-mg dose of midazolam. For the cyclosporine and midazolam interaction studies, plasma samples were collected at predetermined intervals following the initial doses of test drug for determination of test drug concentrations. Plasma samples were also collected following doses on the last day of each treatment period for determination of EP and test drug concentrations.

Lastly, an interaction trial involving oral contraceptives (ethinylestradiol and norethindrone) was also designed only to determine the effect of EP on the steady-state pharmacokinetics of the test drug in 24 healthy adults. The effect was assessed in a multiple-dose, single-sequence, two-period, crossover study. In the first period, subjects received a 28-day treatment of oral contraceptive tablets (Ortho-Novum 1/35[®] 28-day regimen, lot no. 29N215), co-administered with placebo tablets QD on days 1–11. In the second period, subjects received a 28-day treatment of contraceptive tablets, co-administered with EP on days 1–11. Plasma samples were collected at predetermined intervals on day 11 of each treatment period for determination of 17 α -ethinylestradiol and norethindrone concentrations.

Plasma samples were analysed for EP as indicated above using a validated LC-MS/MS system (Zhang *et al.* 2003). Interacting compounds were also analysed using an appropriate LC-MS/MS procedure. Pharmacokinetic parameters (K_a , AUC_{0-lq} , $t_{1/2}$, T_{max} and C_{max} , — where AUC is the area under the curve) were calculated with the aid of WinNonlin Professional v.3.1 (Pharsight Corp., Mountain View, CA, USA). Least-squares means from an analysis of variance (ANOVA) model with effects for subject, treatment and period are presented for AUC_{0-lq} , $t_{1/2}$, T_{max} and C_{max} . p values for the difference in these parameters were obtained from the pairwise comparison within the ANOVA model.

The cyclosporine and midazolam interaction trials were designed primarily to determine the effects of EP on the pharmacokinetics of the test drugs, and baseline EP pharmacokinetic parameters were not measured. However, since EP concentrations were measured in the presence of the interacting drug on the last day of each treatment period, a baseline EP AUC, obtained by averaging the results from the other eight interaction studies, was used only for gross estimation of the potential changes in EP pharmacokinetics in the presence of cyclosporine and midazolam and not for statistical comparison.

Results

In vitro metabolic interactions

The types of inhibition of *in vitro* EP metabolism by 10 drugs were evaluated using the Lineweaver–Burke double reciprocal plots (figure 2, digoxin

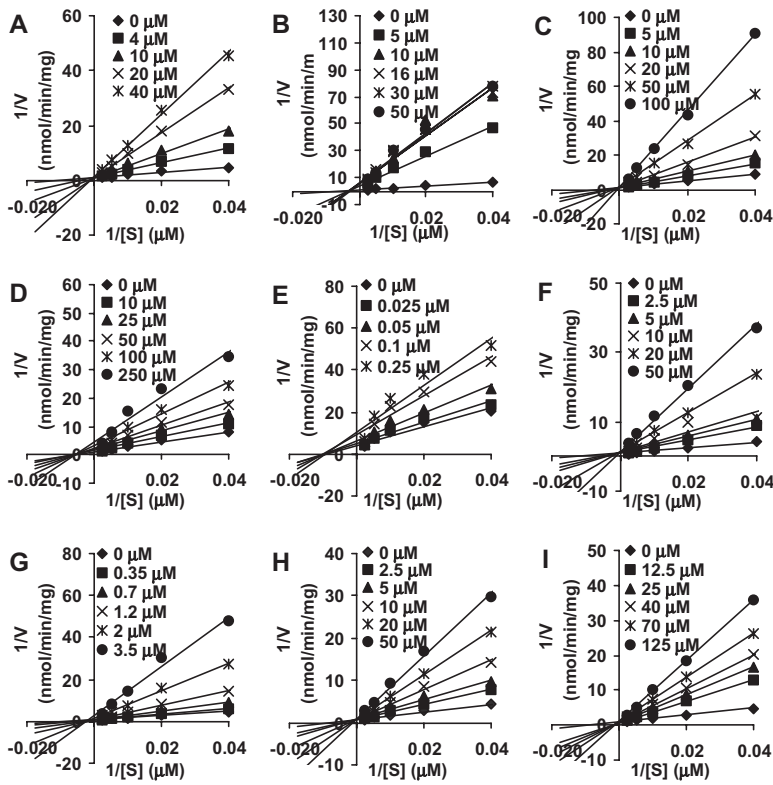


Figure 2. Lineweaver–Burke plots for the 6 β -hydroxylation of EP in the absence and presence of cisapride (A), cyclosporine (B), erythromycin (C), fluconazole (D), ketoconazole (E), midazolam (F), saquinavir (G), simvastatin (H) or verapamil (I) in human liver microsomes. V , rate of production of 6 β -OHEP in microsomal incubates (nmol min⁻¹ mg⁻¹ protein); S , concentration of EP (μ M). Each symbol represents different concentrations of the inhibitors and is derived from duplicate determinations.

not shown), and K_i values were determined directly from the non-transformed data according to the type of inhibition (table 2). Inhibition of EP metabolism by cisapride, erythromycin, simvastatin and verapamil was competitive. Inhibition by cyclosporine, fluconazole, ketoconazole and midazolam was non-competitive. Inhibition of EP metabolism by saquinavir was mixed. Digoxin did not inhibit EP metabolism sufficiently at the concentrations used to allow the definitive determination of K_i values for this drug. The drugs with a $K_i < 10 \mu\text{M}$ were cisapride, cyclosporine, erythromycin, ketoconazole, midazolam, saquinavir and simvastatin. The drugs with K_i values between 10 and 75 μM were fluconazole and verapamil. The drug with a $K_i > 75 \mu\text{M}$ was digoxin.

Using K_i and total or unbound $C_{\text{max},\text{I,L}}$ or $C_{\text{max},\text{I,IN}}$, inhibition factors for these drugs were calculated according to Methods A–C, as described and shown in table 3. With Method A, cyclosporine, erythromycin, ketoconazole and saquinavir yielded an $R_a > 10$ for each. R_a values of cisapride and fluconazole were between 2 and 3. With Method B, the drugs with $R_b \geq 1.5$ were cyclosporine, erythromycin, fluconazole, ketoconazole and saquinavir. With Method C, R_c values for only fluconazole and ketoconazole were slightly greater than 1.5.

Table 2. Inhibition type and K_i values for inhibitors of EP 6 β -hydroxylation *in vitro*.

Drugs	Inhibition type	K_i (μ M)
Cisapride	competitive	2.90
Cyclosporine	non-competitive	1.24
Digoxin	ND	> 75.0
Erythromycin	competitive	9.50
Fluconazole	non-competitive	59.0
Ketoconazole	non-competitive	0.160
Midazolam	non-competitive	8.10
Saquinavir	linear-mixed	0.546
Simvastatin	competitive	6.23
Verapamil	competitive	13.3

ND, not determined.

Data are derived from duplicate determinations.

When inhibition of cisapride, cyclosporine, digoxin, ethinylestradiol, erythromycin, midazolam, norethindrone, saquinavir, simvastatin and verapamil metabolism by EP were examined in human liver microsomes, < 50% inhibition was observed for ethinylestradiol at EP concentrations of up to 400 μ M and for the other test drugs at EP concentrations of up to 100 μ M. Thus, IC_{50} values for these interactions could not be calculated.

Summary of clinical interaction data

Effects of cisapride, digoxin, erythromycin, fluconazole, ketoconazole, saquinavir, simvastatin and verapamil on EP pharmacokinetics were determined (table 4). Administration of EP with one of the most potent CYP3A inhibitors, ketoconazole resulted in significant ($p < 0.001$) prolongation of mean EP T_{max} by about 1 h and an approximately 1.7-fold increase in C_{max} . Apparent $t_{1/2}$ was 2.5 times longer with concomitant administration of ketoconazole and the mean EP AUC was approximately 5.4-fold higher. Co-administration of erythromycin with EP resulted in a significant ($p < 0.001$) increase in mean C_{max} (1.6-fold), apparent $t_{1/2}$ (1.6-fold) and AUC (2.9-fold), although there was no significant change in T_{max} . Co-administration of fluconazole also resulted in significant increases in mean C_{max} (1.4-fold), apparent $t_{1/2}$ (1.7-fold) and AUC (2.2-fold). Saquinavir and verapamil significantly increased C_{max} and apparent $t_{1/2}$, and AUCs of EP in the presence of these drugs were approximately twofold higher. However, cisapride, digoxin and simvastatin did not appear to alter the pharmacokinetics of EP.

Effects of EP treatment on pharmacokinetics of the following drugs were determined: cisapride, cyclosporine, digoxin, ethinylestradiol, erythromycin, midazolam, norethindrone, saquinavir, simvastatin and verapamil (table 5). Changes of AUCs of these drugs with concomitant administration of EP ranged from approximately 79% (saquinavir) to 117% (norethindrone) of the values obtained without EP treatment. Although the differences in AUCs for several drugs (digoxin, erythromycin and norethindrone) were statistically significant, these results indicate that EP did not substantially change the pharmacokinetics of these drugs, and that co-administration of EP did not have clinically significant effects on exposure to these drugs.

Table 3. Inhibition factors for EP metabolism calculated from *in vitro* inhibition constant (K_i) and $C_{\max,I,L}$ without f_u (R_a) and with f_u (R_b), and $C_{\max,I,IN}$ (R_c).

Test drug	Dose ^a (mg)	F_a^b (%)	K_a^a (h ⁻¹)	f_u^b	P_L^b	$C_{\max,I,P}^a$ (μ M)	$C_{\max,I,L}^c$ (μ M)	$C_{\max,I,IN}^c$ (μ M)	Inhibition factor ^c		
									R_a	R_b	R_c
Cisapride	20	100 ^d	0.679	0.02 ^f	31.1 ^h	0.173	5.38	0.509	2.86	1.04	1.00
Cyclosporine	172	35 ^d	0.605	0.07 ^f	11.0 ⁱ	1.11	12.2	1.46	10.8	1.69	1.08
Digoxin	0.2	70 ^d	2.03	0.75 ^f	8.04 ^j	0.00251	0.0202	0.00657	1.00	1.00	1.00
Erythromycin	500	35 ^d	0.266	0.16 ^f	30.4 ^k	4.24	129	4.97	14.6	2.17	1.08
Fluconazole	200	100 ^d	2.36	0.89 ^f	2.50 ^l	33.0	82.5	50.8	2.40	2.24	1.77
Ketoconazole	200	59 ^e	0.838	0.01 ^f	6.00 ^l	10.4	62.4	12.5	390	4.90	1.78
Midazolam	10	44 ^f	3.60	0.02 ^f	8.8 ^m	0.177	1.56	0.737	1.19	1.00	1.00
Saquinavir	1200	30 ^g	0.562	0.02 ^f	14.0 ⁿ	1.60	22.4	5.07	41.0	1.82	1.19
Simvastatin	40	5 ^f	0.744	0.06 ^f	44.2 ^o	0.0217	0.959	0.0627	1.15	1.01	1.00
Verapamil	240	100 ^d	0.446	0.10 ^f	10.0 ^p	0.561	5.61	3.27	1.42	1.04	1.02

^aDose, K_a (absorption rate constant) and $C_{\max,I,P}$ (maximum concentration of test drug in the plasma) were determined from clinical interaction studies following administration of test drug alone.

^b F_a (fraction of test drug dose absorbed), f_u (fraction of test drug unbound in plasma) and P_L (liver partition coefficient) were compiled from various sources.

^c $C_{\max,I,L}$ (maximum concentration of test drug in the liver), $C_{\max,I,IN}$ (maximum influx concentration of test drug into the liver) and inhibition factor were calculated using the appropriate parameters.

^dZhao *et al.* (2001); ^eIto *et al.* (1998); ^fThummel and Shen (2001); ^gParrott and Lave (2002); ^hMichiels *et al.* (1987); ⁱKawai *et al.* (1998); ^jWeinhouse *et al.* (1985); ^kYamano *et al.* (2000); ^lErvine and Houston (1994); ^mBjorkman *et al.* (1996); ⁿDenissen *et al.* (1997), Shetty *et al.* (1996); ^oGermershausen *et al.* (1989); ^pLevy and Boddy (1991).

Table 4. Pharmacokinetic parameters for EP following EP administration alone or with test drugs.

Test drug	EP alone				EP with test drug				
	$C_{\max}$ (ng ml ⁻¹)	$T_{\max}$ (h)	$t_{1/2}$ (h)	$AUC_{(0-lqc)}$ (h*ng ml ⁻¹)	$C_{\max}$ (ng ml ⁻¹)	$T_{\max}$ (h)	$t_{1/2}$ (h)	$AUC_{(0-lqc)}$ (h*ng ml ⁻¹)	AUC-fold increase
Cisapride	2170	1.67	4.82	12 800	2210	1.42	4.64	13 300	1.04
Cyclosporine ^a	NA	NA	NA	NA	1850	2.18	3.30	13 400	1.16
Digoxin	2040	1.75	4.01	12 200	1870*	1.88	3.83	11 300	0.930
Erythromycin	1480	1.96	4.40	8330	2380***	1.61	6.63	23 900***	2.87
Fluconazole	1550	1.76	3.47	9550	2190***	1.77	5.99	21 400***	2.24
Ketoconazole	1550	1.72	3.63	9560	2610***	2.53***	9.21	51 500***	5.39
Midazolam ^a	NA	NA	NA	NA	1700	2.70	3.46	11 100	1.00
Saquinavir	2140	2.60	4.87	16 200	3000***	3.10**	6.49	33 700***	2.07
Simvastatin	1790	2.11	4.19	12 200	1890	1.78	5.05	12 600	1.03
Verapamil	1890	1.69	4.50	11 600	2560***	1.77	7.84	22 800***	1.98

^aCyclosporine and midazolam interaction trials were designed to determine the effect of EP on the pharmacokinetics of the test drug, and baseline EP concentrations were not measured. Therefore, AUC-fold increases were calculated based on the mean baseline EP AUC from the eight other interaction studies (mean of 11 600 h*ng ml⁻¹) and a statistical analysis was not performed.

NA, not available; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 5. Pharmacokinetic parameters for test drugs following administration of test drug alone or with EP.

Interacting drug	Test drug alone			Test drug with EP			
	$C_{\max}$ (ng ml ⁻¹)	$T_{\max}$ (h)	$AUC_{(0-lqc)}$ (h*ng ml ⁻¹)	$C_{\max}$ (ng ml ⁻¹)	$T_{\max}$ (h)	$AUC_{(0-lqc)}$ (h*ng ml ⁻¹)	AUC-fold increase
Cisapride	80.5	1.69	540	84.1	1.58	579	1.07
Cyclosporine	1350	1.38	8580	1280	1.43	8690	1.01
Digoxin	1.96	0.75	11.1	2.05	0.79	12.9*	1.16
17 α -Ethinylestradiol ^a	0.138	1.54	1.20	0.135	1.66	1.19	0.992
Erythromycin	3110	3.75	20 000	2500**	4.39	17 100*	0.855
Midazolam	49.2	0.61	119	48.5	0.55	114	0.958
Norethindrone ^a	21.1	1.22	139	25.3**	1.41	162***	1.17
Saquinavir	1070	2.33	3310	750*	1.96*	2610	0.789
Simvastatin	9.09	NA	51.0	6.17***	NA	43.9	0.861
R-verapamil ^b	204	4.92	2330	168*	5.38	2130	0.914
S-verapamil ^b	51.1	5.63	546	40.9*	6.46	493	0.903

^aAdministered as Ortho-Novum 1/35[®] 28-day regimen.

^bAdministered as a racemic dose.

NA, not available; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Comparison of *in vitro* data and clinical interaction data

When the R_a , R_b and R_c values (table 3) were compared with the actual fold increases in AUC (table 4) after co-administration of EP with cisapride, cyclosporine, digoxin, erythromycin, fluconazole, ketoconazole, midazolam, saquinavir, simvastatin or verapamil, an excellent correlation was observed with R_b (figure 3). R_a and R_c were less well correlated with the *in vivo* data (data not shown).

Discussion

The present study has demonstrated that the potent inhibitors of EP metabolism *in vitro* ($K_i < 10 \mu\text{M}$) were cisapride, cyclosporine, erythromycin,

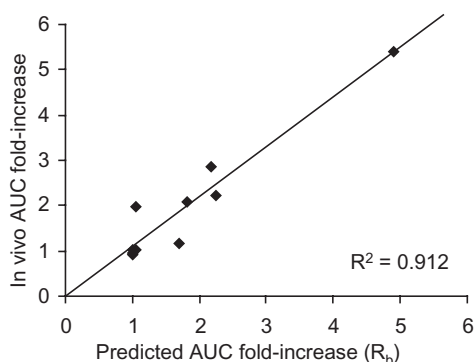


Figure 3. Correlation between predicted AUC-fold increase using Method B (R_b) and AUC-fold increases obtained from the *in vivo* interaction studies of EP with cisapride, cyclosporine, digoxin, erythromycin, fluconazole, ketoconazole, midazolam, saquinavir, simvastatin or verapamil.

ketoconazole, midazolam, saquinavir and simvastatin. However, metabolic interactions *in vivo* are expected to be dependent not only on the inhibition constants K_i , but also on inhibitor concentrations following clinically relevant doses. Since it was not initially clear which inhibitor concentrations are more appropriate for use in translating *in vitro* data into predictions of the *in vivo* condition, three different approaches were evaluated, using liver $C_{\max}$ of total inhibitor (R_a), liver $C_{\max}$ of unbound inhibitor (R_b) and maximum influx concentration of inhibitor into the liver (R_c). The R_b values of erythromycin, fluconazole, ketoconazole and saquinavir were substantially higher than 1.5 (predicting a 50% or greater increase in EP AUC) and the fold increases in AUC for these drugs were predicted nearly quantitatively using Method B. These results suggest that drugs with a potential for interacting significantly with EP in the clinic could be identified using *in vitro* metabolic inhibition results. However, prediction of a verapamil and cyclosporine effect using R_b deviated slightly from the actual fold increase in AUCs. The R_b for verapamil was 1.04, whereas the fold increase in AUCs of EP after co-administration of verapamil was 1.98. The exact reason for this discrepancy (approximately 46% underestimation using the *in vitro* data) is not known. However, the following two possibilities may be suggested. First, verapamil is a chiral compound that shows enantioselective metabolism, plasma protein binding and pharmacological activity (Nelson and Olsen 1988, Mehvar *et al.* 2002). Therefore, the K_i of one enantiomer may be appreciably lower than that of the racemate (13.3 μM) and R_b values calculated from the K_i of racemic verapamil may have been underestimated. Second, verapamil is a good substrate for P-glycoprotein and more extensively metabolized in the gastrointestinal tract than other drugs that are rapidly absorbed, metabolized by CYP3A4, but not a P-glycoprotein substrate (Johnson *et al.* 2003). In this case, *in vivo* EP metabolism may have been inhibited not only by verapamil, but also by circulating metabolites of verapamil to a greater extent in the liver and gastrointestinal tract. If the production of these metabolites during the *in vitro* experiment is substantially lower than that *in vivo* (e.g. due to gastrointestinal metabolism of verapamil), an underestimation of the fold increase in AUCs will occur. In the case of cyclosporine, the $R_b=1.69$ and slightly higher than the actual fold increase (1.16) in AUC (approximately 45% deviation). However, in the cyclosporine clinical trial,

the EP AUC was not determined in the absence of cyclosporine treatment. An average EP AUC was obtained from the other eight trials and used in the calculation of R_b . This may, at least in part, account for the deviation observed with the prediction for cyclosporine.

Method A was also useful in predicting an interaction. However, the predicted AUC-fold increases were greatly exaggerated relative to R_b , as in the cases of cisapride, cyclosporine, erythromycin, ketoconazole and saquinavir. Without a function for f_u , these exaggerated results appeared to be due to the overestimation of the amount of inhibitor available to bind to the metabolizing enzymes in the liver, in this case CYP3A4.

Using $C_{\max, I, IN}$ (Ito *et al.* 1998), Method C predicted interactions for ketoconazole and fluconazole. However, these and other predicted AUC-fold increases were greatly underestimated relative to R_a and R_b although relatively accurate parameters were used in this calculation. $C_{\max, I, IN}$, D and K_a were determined from the actual interaction trials presented above. F_a values were found in the literature and not expected to change much from trial to trial. The Q_h used is a well-accepted value for human hepatic blood flow. It should be noted that this method assumes effective concentrations of an inhibitor for its interaction with a substrate are the same as maximum free concentrations in plasma and fails to consider active, carrier-mediated or any other partitioning of the inhibitor against concentration gradient into the hepatocytes. This may underestimate the amount of inhibitor available to bind to CYP3A4 in the liver, causing an underestimation of the *in vivo* inhibition effect.

EP is metabolized to 6 β -OHEP by CYP3A4 in humans (Cook *et al.* 2002). Since approximately half of all marketed drugs are metabolized by CYP3A (Benet *et al.* 1996), it is important to find a simple *in vitro* method for predicting pharmacokinetic interactions with EP. EP is extensively metabolized in humans and total plasma CL is primarily due to metabolic CL (Cook *et al.* 2003). Thus, inhibition of metabolism will have more pronounced effects on total clearance of EP compared with other drugs that are metabolically cleared only in part. This makes EP a good candidate for investigating the predictability of *in vivo* pharmacokinetic interactions using *in vitro* metabolism methods. Good correlation with unbound concentrations of inhibitor in the liver can be justified by the fact that EP is primarily metabolized in the liver; hence, this is where metabolic interactions will take place. Further, only unbound drug is expected to be available to affect metabolism. In this calculation, it was assumed that the fraction of the inhibitors available for metabolic interaction in hepatocytes might be similar to the unbound fraction in plasma. This assumption is supported by findings that when *in vivo* CLs are predicted from the *in vitro* CLs, cancellation of plasma and microsomal protein binding gave good predictions for many drugs (Obach 1999). It is noteworthy, however, that Method B (as well as Method C) is highly sensitive to protein binding for compounds where f_u is low (i.e. <0.05). An example can be found in the case of the *in vitro* metabolic interaction between EP and ketoconazole. The f_u for ketoconazole reported in the literature (Thummel and Shen 2001) is 0.01, yielding an $R_b = 4.9$. If the measured f_u differed by 0.005 or less in either direction, R_b could range from 2.95 to 6.85, presumably depending on the error in the binding assay. Furthermore, ketoconazole is very promiscuous in its interactions with other drugs (e.g. potent inhibitor of efflux transporters) and causes a range of fold changes in AUCs and other parameters depending on the

drug that is co-administered (Venkatakrisnan *et al.* 2000). In these cases, the predictions may also need to consider relative contributions of routes of elimination other than liver metabolism as well as other possible sites of interactions such as P-glycoprotein in the gut and liver. Therefore, while in the present paper a metabolic interaction between EP and ketoconazole in the liver is quantitatively predicted using liver microsomes, care must be taken in assuring the accuracy of all parameters, as well as considering these other possibilities if accurate predictions are desired for other drugs using Method B. Additionally, although good prediction was made for a drug interaction between EP and erythromycin, inhibition of CYP3A4 metabolism by erythromycin is generally known to be mechanism based (Jones and Hall 2002) and Method B may not be adequate for other drug interactions involving erythromycin. Good prediction of the interaction between erythromycin and EP may be because the primary *in vivo* mechanism of inhibition of EP metabolism is competitive, as in the *in vitro* study.

As a part of the clinical trials, EP was also evaluated for its effect on the pharmacokinetics of the test drugs including cisapride, cyclosporine, digoxin, ethinylestradiol, erythromycin, midazolam, norethindrone, saquinavir, simvastatin and verapamil. There were no clinically significant changes in AUCs of these drugs (table 5). This finding was in agreement with the *in vitro* IC₅₀ experiments performed in the present study, which indicated that IC₅₀ values for all these drugs were greater than the highest EP concentration tested, >100 µM (>400 µM in the case of ethinylestradiol). Cisapride, erythromycin, midazolam, saquinavir, simvastatin and verapamil are metabolized primarily by CYP3A in man (Parkinson 2001). However, IC₅₀ values for EP inhibition of CYP3A4 activity, as well as activities of CYP1A2, CYP2C9, CYP2C19 and CYP2D6, were >300 µM, much higher than the expected liver C_{max} of EP (25 µM) at the therapeutic dose (Cook *et al.* 2002). Therefore, EP is not expected to inhibit metabolism of any drugs that are primarily metabolized by these enzymes. Digoxin is not metabolized by CYP450 enzymes but is a good substrate of P-glycoprotein (Zhang and Morris 2003). Because EP is neither a substrate nor a good inhibitor of P-glycoprotein (C. S. Cook and L. M. Berry, unpublished data, 2000), clinically significant pharmacokinetic interactions with digoxin were not expected. Therefore, the lack of effect of EP on digoxin pharmacokinetics could be predicted from the *in vitro* results.

In summary, potent CYP3A inhibitors such as ketoconazole, fluconazole, erythromycin and saquinavir increase systemic exposure to EP when administered to subjects receiving EP. However, EP did not change the pharmacokinetics of the drugs that are CYP450 or P-glycoprotein substrates. The pharmacokinetic interactions with EP in humans can be nearly quantitatively predicted by data from *in vitro* metabolism experiments using human liver microsomes.

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Prediction of whole-body metabolic clearance of drugs through the combined use of slices from rat liver, lung, kidney, small intestine and colon

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1. The aim was to investigate whether precision-cut rat tissue slices could be used to predict metabolic drug clearance *in vivo*. To obtain a complete picture, slices not only from liver, but also from lung, kidney, small intestine and colon were included.

2. The metabolic clearances of 7-ethoxycoumarin, 7-hydroxycoumarin, testosterone, methyltestosterone and warfarin were determined by measuring the disappearance of these compounds during incubation with slices prepared from liver, lung, kidney, small intestine and colon.

3. The total *in vitro* metabolic clearance was determined by adding the individual *in vitro* organ clearances from the slices. Prediction based on the *in vitro* clearance was within an order of magnitude to the corresponding *in vivo* values. Interestingly, the relative contribution of extrahepatic metabolic clearance of the studied compounds to total clearance was remarkably high, ranging from 35 to 72% of the total metabolic clearance.

4. It is concluded that the model of multi-organ precision-cut slices is a useful *in vitro* tool for prediction of *in vivo* metabolic clearance. In addition, it provides information about the relative contribution of the liver, lung, kidney, small intestine and colon to the total metabolic clearance.

Introduction

The removal of drugs from the body is usually expressed in the parameter clearance. Several *in vitro* approaches have proven useful in the prediction of *in vivo* clearance (Ito *et al.* 1998). The classical *in vitro* method for this involves the incubation of liver-derived microsomes with a range of parent drug concentrations to quantify the rate of metabolite formation. To calculate the overall drug conversion, a so-called 'metabolic fraction factor' is used that represents the percentage of the particular metabolite measured to the total amount of metabolite formation (Houston 1981). From the relationship between the particular rate of parent drug conversion and the (unbound) concentration, the $V_{\max}$ and K_m values of the particular process can be determined. Dividing $V_{\max}$ by K_m yields the intrinsic clearance. These values are then multiplied by a scaling factor that takes into account the fraction of the total liver that was used in the experiment. After

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taking into account plasma protein binding and liver blood flow, the hepatic *in vivo* metabolic clearance can be calculated.

There are three important disadvantages to this approach. First, for quantification, authentic metabolites as standards are necessary. Second, knowledge about the relative importance of the particular metabolic pathway under study to the overall metabolic clearance of the compound is required. For many drugs, in particular new candidate drugs within the pharmaceutical industry, such data are not available. In addition, the metabolic fraction factor may vary in different organs and between species, reflecting the organ- and species-dependent metabolic profile of the compound under study. Finally, poor solubility often does not allow one to create an appropriate Michaelis–Menten plot.

A much simpler approach that circumvents these disadvantages is based on one incubation at a single, relatively low initial concentration and monitoring the depletion of the compound of interest (Houston 1994). This does not require *a priori* knowledge of the metabolic fate of the drug. This drug depletion approach has become very popular, due to the availability of sensitive analytical techniques, such as LC-MS/MS (Obach *et al.* 1997, Obach 1999, Shibata *et al.* 2000, Clarke and Jeffrey 2001, Naritomi *et al.* 2001). However, only liver preparations have been used to date. The present authors have reported that the lung, kidney and intestine have a considerable capacity for metabolism, at least *in vitro* (De Kanter *et al.* 2002a). Therefore, the present authors consider it important to incorporate not only liver, but also lung, kidney and intestinal tissue to predict the overall metabolic clearance of drugs.

There are several *in vitro* models to study metabolism of xenobiotics in these organs, all with their own distinct advantages and disadvantages. Ideally, an *in vitro* model should be simple, applicable in a similar way for each organ and also should include all metabolic pathways that are defined *in vivo*. The disadvantage of the use of microsomes lies in the lack of cytosolic enzymes and in the non-physiological co-factor supply. When using isolated cells, only one cell type is considered in most cases, whereas in most organs, several cell types may contribute to the metabolic clearance, or can influence each other in this regard (Neyrinck *et al.* 1999). In contrast, in precision-cut organ slices, all relevant cell types, metabolic enzymes, transporters and co-factors are present. Slices are applicable to liver, kidney, lung as well as to the intestines (De Kanter *et al.* 2002b). Further, up-scaling to whole organs is straightforward using slices. However, in several quantitative studies, it was shown that rat liver slices exhibit lower drug-metabolizing activity when compared with isolated hepatocytes, and also if compared with total *in vivo* metabolism (Worboys *et al.* 1995, 1996a, b, 1997, Carlile *et al.* 1999). Therefore, it has been concluded from these data that liver slices are not very attractive for quantitative studies (Houston and Carlile 1997). A recent report provided evidence that the underprediction of metabolic clearance by liver slices may be caused by a low ratio between the characteristic rates for metabolism and diffusion within the slices (Haenen *et al.* 2002). Consequently, liver slices in principle can be useful in the determination of the clearance when the diffusion into the slice is faster than metabolism of the compound involved (Haenen *et al.* 2002). Maybe for this reason, comparable drug metabolism activity using dog or human liver slices was found when compared with isolated hepatocytes from the same liver (Powis *et al.* 1989, Olinga *et al.* 1993, Olinga *et al.* 1998).

The present study used precision-cut slices from rat liver, kidney, lung, small intestine and colon to incorporate those organs that reasonably can be expected to contribute to the total metabolic clearance. The aim of the current study was to determine if organ slices can be used to predict adequately the *in vivo* metabolic clearance using the drug depletion method. Apart from the liver, the relative contribution of the lung, kidney and intestines to metabolism was assessed. 7-Ethoxycoumarin, 7-hydroxycoumarin, testosterone, methyltestosterone and warfarin were selected on the basis of knowledge with respect to their unbound fractions (f_u) and clearances in the rat aiming at a broad range of these values within this study.

Materials and methods

Materials

The following compounds were obtained from the sources indicated: 7-ethoxycoumarin from Fluka (Buchs, Germany); testosterone, 7-hydroxycoumarin, warfarin, cremophor EL and low melting agarose (type VII-A) from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands); Viaspan organ preservation solution (UW) from Barr Laboratories (Pomona, NY, USA); and amphotericin B (Fungizone), penicillin-streptomycin solution and Williams' medium E (with Glutamax) from Gibco (Breda, the Netherlands). Methyltestosterone was kindly provided by Organon N. V. (Oss, the Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

In vivo data

In vivo clearance and plasma protein binding data were obtained from literature data for 7-ethoxycoumarin (Carlile *et al.* 1998), 7-hydroxycoumarin (Ritschel *et al.* 1992) and warfarin (Shibata *et al.* 2000) from Sprague-Dawley rats, and the data for testosterone and methyltestosterone were generated by Organon N. V. as described below.

Animal handling was in accordance with the national laws on animal experimentation for the protection of vertebrate animals used for experimental and other scientific purposes.

The *in vivo* total clearance of testosterone was determined in castrated, overnight fasted, male Wistar (HsdCpb:WU) rats (Harlan, Horst, the Netherlands), 275–350 g, after cannulation of the vena cava under isofurane and N₂O/O₂ anaesthesia. A single bolus of 1 mg kg⁻¹ testosterone or methyltestosterone was administered via the vena cava. Testosterone and methyltestosterone were dissolved in 100% ethanol, after which the solution was first diluted 1:2 with cremophor EL and then diluted (1:8.5) with a solution containing 5% (w/v) mannitol (final concentration 1 mg ml⁻¹). Nine blood samples within 4 h were taken via the vena cava, blood was collected in ethylenediamine tetra-acetic acid-coated syringes and plasma was prepared by centrifugation and stored at -20°C until analysis by LC-MS/MS.

The pharmacokinetic parameters of testosterone and methyltestosterone were calculated by a non-compartment analysis using mean concentrations of three animals at each time point. The *in vivo* clearance was calculated by dividing the dose by the area under the plasma concentration curve.

In vitro experiments

Male Wistar (HsdCpb:WU) rats, mean body weight 360 g (Harlan), were housed in standard cages and had free access to food (RMH-chow, Hope Farms, Inc., Woerden, the Netherlands) and tap water. Rats were anaesthetized by isofurane and N₂O/O₂, and the liver, lungs, kidneys and intestines were excised and placed in ice-cold Krebs-Henseleit buffer containing 10 mM Hepes and 25 mM glucose, pH 7.4. The intestines were flushed thoroughly with ice-cold Krebs-Henseleit buffer to remove the contents. The lungs were filled with agarose solution as described by De Kanter *et al.* (1999).

Preparation of slices

Tissue cylinders from liver, agarose-filled lung and kidney were prepared with a coring tool, i.d. 8 mm (Alabama R&D, Munford, AL, USA), attached to a drilling machine with a variable rotation speed. The organ cylinders were stored in ice-cold UW until slicing.

To prepare agarose filled slices about 30 cm of the intact duodenum and ileum was cut in about 10 cm parts, and these parts and the whole colon were ligated on one side. These parts were then filled with 3% (w/v) low melting agarose solution in 0.9% (w/v) NaCl at 37°C and allowed to gel in ice-cold Krebs-Henseleit buffer. The agarose-filled intestines were cut in 1 cm parts and embedded in the low melting agarose solution at 37°C using the Tissue Embedding Unit from Alabama R&D so that agarose gel cylinders with a diameter of 16 mm were formed.

Organ cylinders from liver, lung, kidney and intestines were used to prepare precision-cut slices (about 0.25 mm thickness, but 0.5 mm for lung slices), using a Krumdieck tissue slicer (Alabama R&D), precooled and filled with oxygenated, ice-cold Krebs–Henseleit buffer. Kidney slices were derived mainly from cortex tissue. All slices were stored in ice-cold UW until incubation. The time gap between coring and incubation was a maximum of 2 h.

Incubation of slices

Slices were incubated in 3.2 ml Williams' medium E prewarmed at 37°C, gassed with 95% O₂/5% CO₂ and supplemented with glucose (final concentration 25 mM). For liver and lung slices, gentamicin (50 µg ml⁻¹) was added. For kidney slices, penicillin (100 U ml⁻¹) plus streptomycin (100 µg ml⁻¹) was used, while for intestine slices, gentamicin (50 µg ml⁻¹) and amphotericin B (2.5 µg ml⁻¹) was added. These agents are poorly metabolized themselves and therefore assumed not to interfere with biotransformation enzymes. Slices were individually incubated in six-well culture plates, which were placed in a plastic container, continuously gassed with humidified 95% O₂/5% CO₂, and shaken back and forth (90 times min⁻¹) in a cabinet at 37°C. During incubation, the gel surrounding the slices, but not inside the ring of intestinal tissue, separated from the slices.

Metabolic activity of slices

Metabolism of the compounds was studied by the addition of 32 µl of a 100× stock-solution in methanol to 3.2 ml Williams' medium E. In each experiment, three individual slice incubations from each organ were analysed for parent drug concentration as described below after the incubation periods indicated in the legends of figures 3–7.

Incubations with substrates, but without slices, served as controls and showed no depletion of the particular substrates. All samples were stored at –20°C until analysis. Control experiments showed that 7-ethoxycoumarin or 7-hydroxycoumarin were not significantly retained in the slices at the concentration used (data not shown) and therefore only medium samples were used for analysis. For analyses of testosterone, methyltestosterone and warfarin, slices and incubation media (3.2 ml) were harvested together after incubation and homogenized using sonication to extract the compounds from the slice. Medium samples (1 ml) of 7-ethoxycoumarin incubations were acidified by adding 10 µl 2 M HCl after sampling in order to prevent spontaneous degradation of metabolites that occurs if the pH of the incubation exceeds 8.0 (De Kanter *et al.* 1998). After thawing of 7-ethoxycoumarin and 7-hydroxycoumarin samples, sodium azide was added to inhibit bacterial contamination (final concentration 0.1 mg ml⁻¹) and 50 µl of the centrifuged sample were analysed using HPLC as described (Walsh *et al.* 1995). For testosterone, methyltestosterone and warfarin, an aliquot of the slice-homogenates in medium was 1:1 diluted with acetonitrile and after precipitation of the proteins by centrifugation, 10 µl of the supernatant were injected by a Pal HTS autosampler (CTC, Zwingen, Switzerland) for a short (1.5 min) HPLC separation over a Zorbax C₈ precolumn (12.5 × 4.6, 5 µm) (Agilent, Palo Alto, CA, USA) using a 10 mM ammonium formate (pH 4) acetonitrile gradient and analysed by a MS/MS system using a PE Sciex API 2000 with Turbo ionspray (Applied Biosystems, Foster City, CA, USA).

Protein content

The protein content of preweighed pieces of small intestine tissue and five slices of each of the other organs was determined after sonication and storage at –20°C. After thawing, the homogenates were diluted with 0.1 M NaOH and the protein content was determined using the Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) against a bovine serum albumin standard curve. All metabolic rates were expressed per µg protein.

Calculation

From the difference between the initial amount of substrate added and the amount of parent compound left after incubation, the intrinsic clearance (CL_{int}) of each organ was calculated as follows. CL_{int} is defined as:

$$CL_{int} = \text{vol.} * (dC/dt)/C, \quad (1)$$

where vol. * dC/dt is equal to the amount of parent drug disappearing from the incubation per unit time in the volume of the incubation medium (vol.) used, which was 3.2 ml.

On integration of equation (1) from time zero to time *t*, one obtains:

$$CL_{int} \int dt = \text{vol.} * \int 1/C dC$$

$$CL_{int} * t = \text{vol.} * (\ln(C) + \text{constant})$$

for *t* = 0: *C* = *C*_{initial}, thus ln(*C*_{initial}) + constant = 0.

Filling in:

$$\begin{aligned} \text{CL}_{\text{int}} * t &= \text{vol.} * [\ln(C) - \ln(C_{\text{initial}})] \\ \text{CL}_{\text{int}} &= \text{vol.} * \ln(C_{\text{initial}}/C)/t, \end{aligned} \quad (2)$$

where C_{initial} is the substrate concentration at time zero and C is the substrate concentration at time t .

The CL_{int} by the slices was expressed per μg slice protein, thus:

$$\text{CL}_{\text{int slice}} = \text{CL}_{\text{int measured}}/\text{protein content of the slices } (\mu\text{g}),$$

and then multiplied with a scaling factor:

$$\text{CL}_{\text{int organ}} = \text{CL}_{\text{int slice}} * \text{scaling factor.}$$

The scaling factors (table 1) were calculated as follows. The organ weight was determined for each liver, lung (after filling with agarose), kidney, and small intestine and colon (after removing the contents but before filling with agarose) from each animal used in the *in vitro* studies. Organ protein content as a percentage of the wet weight was determined by dividing the mean protein content of five slices (or in case of the small intestine and colon, a piece of organ without agarose) by the wet weight of the samples * 100%. The scaling factors were then derived by multiplying the organ weight with the protein percentage and essentially represent the total organ protein content of the organ.

Using the venous equilibration (well-stirred) model (equation 3), the *in vivo* metabolic clearance for each organ was calculated from intrinsic clearance, blood flow (Q) data from figure 2, and unbound fraction (f_u) (table 2), assuming blood-to-plasma ratios to be unity:

$$\text{CL}_{\text{organ}} = \frac{Q * f_u * \text{CL}_{\text{int}}}{Q + f_u * \text{CL}_{\text{int}}} \quad (3)$$

The total metabolic clearance (CL_{calc}) can be estimated from the calculated clearances of the contributing organs (figure 2). Total metabolic clearance is defined here as the total metabolic conversion rate divided by the central venous blood concentration. The contribution of parallel clearance processes can be added up. For serial processes, the extraction by the preceding organs must be taken into account. Since the extraction ratio in the lung was small in all cases, the extraction by the lung was not taken into account for the calculation of the clearance of the other organs, and the clearance by the lung was added to the contribution of the other organs.

The liver is supplied by the portal vein that collects blood from the stomach, spleen, small intestine and colon, organs where already drug elimination takes place (Richter *et al.* 2000). Thus, the liver is exposed to a lower drug concentration, due to prehepatic extraction, than the concentration in the central venous compartment of the body. Therefore, the calculated liver clearance for clearance by intestine and colon was corrected.

The concentration in portal blood as a fraction of the central venous blood concentration, F , is equal to $1 - E$, where E is the extraction ratio in small intestine and colon. Since small intestine and colon are located parallel, their contribution to clearance can be added up and their extraction ratio follows from:

$$E = (\text{CL}_{\text{si}} + \text{CL}_{\text{colon}})/(Q_{\text{si}} + Q_{\text{colon}}), \quad (4)$$

where Q_{si} and Q_{colon} are the blood flows through the small intestine and colon, respectively, and CL_{si} and CL_{colon} are the clearances for small intestine and colon, respectively.

Since only a fraction F of drug is present in the portal blood, the effective clearance of the liver, i.e. the clearance related to the central venous concentration, is diminished by a factor F . Assuming an arterial and portal blood supply to the liver of 20 and 80%, respectively, the total metabolic clearance can be calculated from:

$$\text{CL}_{\text{calc}} = (0.20 + 0.80 * F) * \text{CL}_{\text{liver}} + \text{CL}_{\text{lung}} + \text{CL}_{\text{kidney}} + \text{CL}_{\text{si}} + \text{CL}_{\text{colon}}. \quad (5)$$

For comparison, CL_{calc} was expressed per kg body weight.

Results

Prediction of metabolic clearance by slice incubations

As shown in figure 1, five model compounds were used to evaluate the possibility of using rat organ slices prepared from liver, lung, kidney, small intestine and colon to predict total clearance *in vivo*. The concentrations of the incubated substrates remaining after a certain incubation period were determined and the intrinsic clearances of the slices were calculated using equation (2).

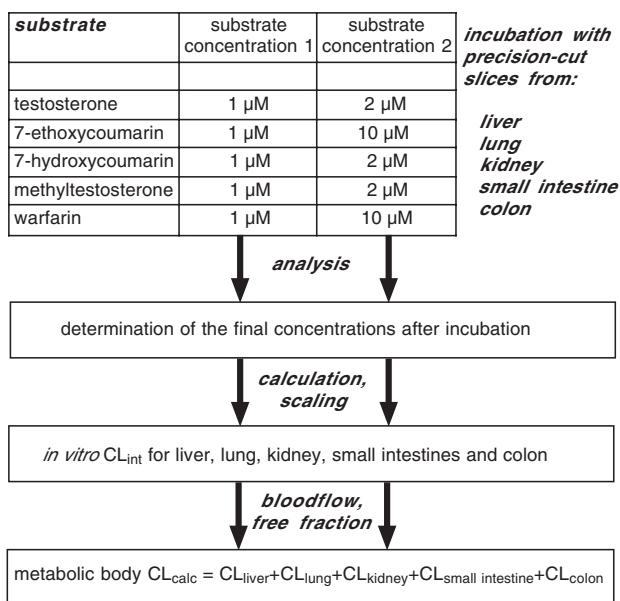


Figure 1. Four-stage strategy for the determination of clearance. Substrate concentration 1 is used as a drug-depletion concentration, while substrate concentration 2 is used as a control concentration (see the Discussion).

Table 1. Organ weights, slice protein percentage and scaling factors.

	Organ weight (g)	Slice protein percentage ^a	Scaling factor ($\times 1000$) ^b	Slice protein content (μ g)
Liver	15.3	10.8	1655	1565
Lung (agarose filled)	7.2	1.0	75	231
Kidney	2.9	7.5	217	1227
Small intestine	6.9	12.1	836	434
Colon	1.0	12.1	126	458

Values are means of at least eight experiments.

The slice protein content of individual experiments was used in the calculations.

^aDetermined through wet weight and protein content of the slices.

^bThese factors were calculated by multiplying the organ weight with protein content (μ g protein/organ).

To convert the results of the individual slices into data for the whole organ, scaling factors were used that take into account the fraction of the organ used in the experiments. The scaling factors for rat liver, lung, kidney, small intestine and colon are shown in table 1.

The contribution of the organs to the total metabolic clearance was calculated using the blood flows shown in figure 2 and the unbound fractions as shown in table 2.

Metabolic clearances are shown in figures 3–7 for the compounds studied. Of note, the amount of compound taken up into, or which was secreted unmetabolized from the slices, was not included in the measured clearances. This because the slices were disrupted in their own incubation medium and the total (= slice + medium) amount of parent compounds for analysis was extracted.

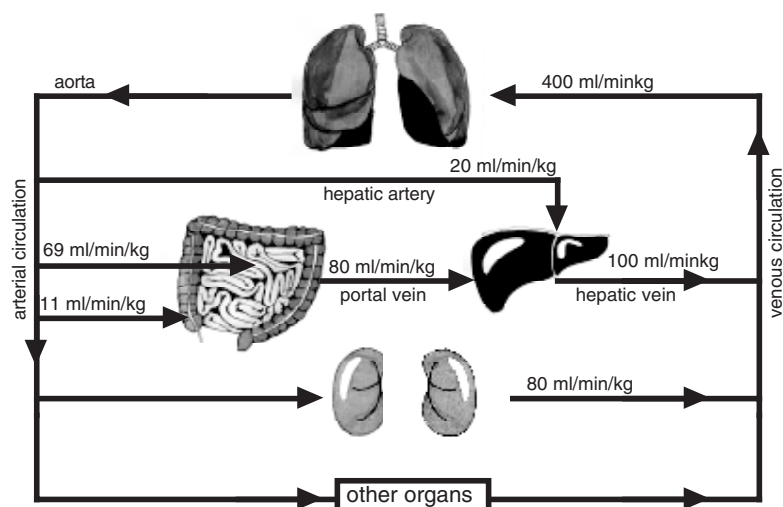


Figure 2. Scheme of blood flow through the body as used in the study for physiology-based pharmacokinetic modelling. Data for rat blood flow through the liver are from (Carlile *et al.* 1998), while liver and kidneys receive 25 and 20%, respectively, of the total blood flow. A total of 80% of the blood flow to the liver is supplied by the portal vein and 20% by the hepatic artery (Richter *et al.* 2000). The ratio of blood flow through the small intestine and colon corresponds to the ratio of the wet weight of these organs in the study.

Table 2. Unbound fractions, *in vivo* prediction, contribution of the liver and *in vivo* clearances.

	Unbound fraction in blood (fu)	<i>In vivo</i> prediction ^c (%)	Extrahepatic contribution (%)	CL _{<i>in vivo</i>} (ml min ⁻¹ kg ⁻¹)
7-Ethoxycoumarin	0.22 ^a	111	72	40 ^a
7-Hydroxycoumarin	0.10 ^b	25	63	97 ^b
Testosterone	0.40 ^c	99	42	62 ^f
Methyltestosterone	0.01 ^f	124	35	51.5 ^f
Warfarin	0.011 ^d	50	56	0.18 ^d

From ^aCarlile *et al.* (1998), ^bRitschel *et al.* (1992), ^cPardridge (1986) and ^dShibata *et al.* (2000).

^eCL_{calc}/CL_{*in vivo*}.

^fFrom Organon N. V. (Oss, the Netherlands).

Figures 3–7 show that the *in vivo* clearances were closely predicted by the present data, although some underprediction was observed for warfarin (50%) and 7-hydroxycoumarin (25%) (table 2).

Incubation at the two different concentrations used yielded about the same clearance predictions for all compounds studied (figures 3–7). Therefore, it was concluded that the chosen concentrations were well under the total K_m of the metabolic reactions involved (see below).

Discussion

The present study incubated slices from several organs and measured drug depletion to predict *in vivo* clearance. Organ slices resemble the *in vivo* situation more closely than isolated cells or cell fractions, and consequently allow simple scaling procedures. Moreover, slices from different organs are prepared in essentially the same manner making them a convenient tool to study different organs

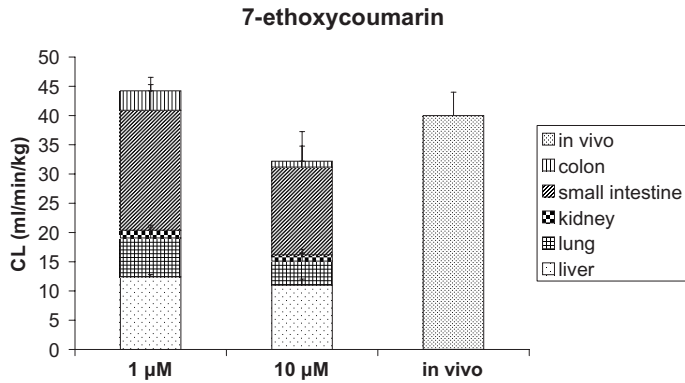


Figure 3. 7-Ethoxycoumarin clearance. The *in vitro* clearance of 7-ethoxycoumarin was determined with slices at 1 and 10 μ M and compared with the *in vivo* value. The incubation period was 3 h. Data are the mean $\pm$ SEM from three animals.

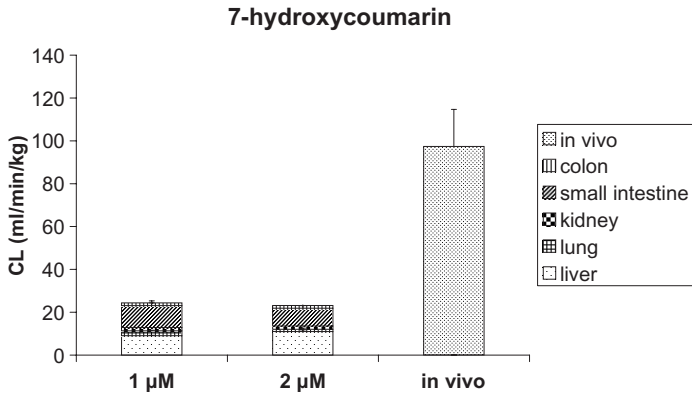


Figure 4. 7-Hydroxycoumarin clearance. The *in vitro* clearance of 7-hydroxycoumarin was determined with slices at 1 and 2 μ M and compared the *in vivo* value. The incubation period was 3 h. Data are the mean $\pm$ SEM from three animals.

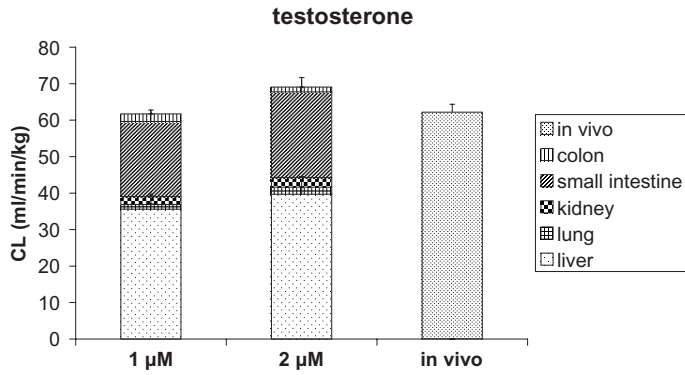


Figure 5. Testosterone clearance. The *in vitro* clearance of testosterone was determined with slices at 1 and 2 μ M and compared with the *in vivo* value. The incubation period for liver slices was 0.5 h. The incubation period for lung, kidney small intestine and colon slices was 3 h. Data are the mean $\pm$ SEM from three animals.

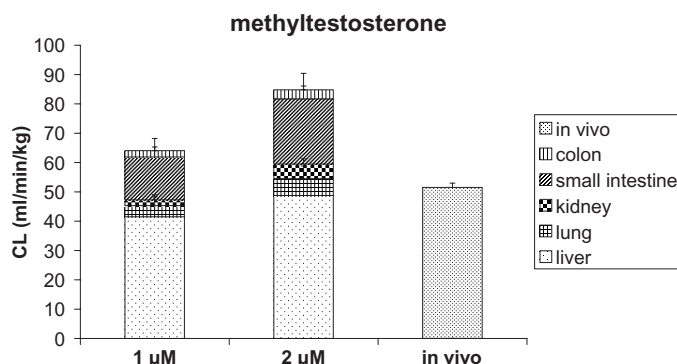


Figure 6. Methyltestosterone clearance. The *in vitro* clearance of methyltestosterone was determined with slices at 1 and 2 μM compared with the *in vivo* value. The incubation period for liver, lung, kidney small intestine and colon slices was 3 h. Data are the mean $\pm$ SEM from four animals.

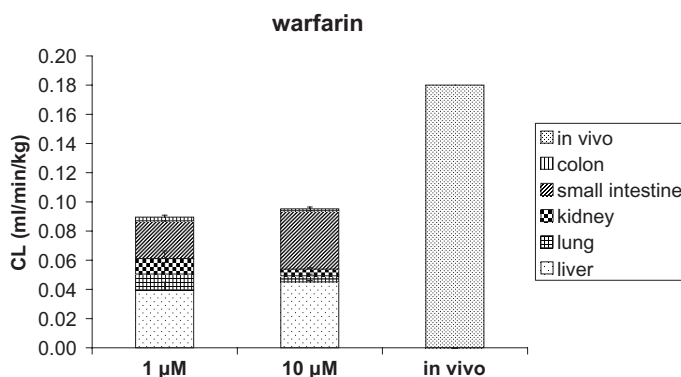


Figure 7. Warfarin clearance. The *in vitro* clearance of warfarin was determined with slices at 1 and 10 μM compared with the *in vivo* value. The incubation period for liver, lung, kidney small intestine and colon slices was 24 h. Data are the mean $\pm$ SEM from four animals.

simultaneously. A limitation of the use of slices, just as is the case with hepatocytes and microsomes, is that only *metabolic* clearance can be estimated and not clearance by excretion of the parent drug. In addition, one should keep in mind that not all cells in the slices may be viable. The cells on the cutting edges might be damaged by the slicing procedure and in the cells in the interior can be hypoxic. Dead cells cannot be removed by special centrifugation techniques as can be used for isolated hepatocytes.

The present method is based on measuring the rate of drug disappearance under linear kinetic conditions. A prerequisite for the drug depletion approach is that the chosen substrate concentration (C_{initial}) should be well under the overall K_m of the enzymes involved in the substrate metabolism (Houston 1994). In the case that the chosen substrate concentrations would not be clearly below the overall K_m , a lower intrinsic clearance would be expected with the higher substrate concentration (2 or 10 μM in the present study), compared with the (lower) depletion substrate concentration (1 μM in the present study). The present study confirmed that the initial substrate concentration was chosen low enough because experiments with higher substrate concentrations did not show significantly lower clearances for all compounds tested (figures 3–7).

To estimate the total metabolic clearance for the comparison with *in vivo* clearance, the individual contributions of the various organs were added. This method implies that potential extrahepatic metabolism is included, in contrast to most other *in vitro* studies where only hepatic metabolism is considered. Especially, the inclusion of extrahepatic metabolism in the gut was considered to be of major importance, even after intravenous dosage (Lin *et al.* 1999).

Further, the fact that 80% of the liver blood flow originates from the gastrointestinal tract was considered, where metabolism can already take place. Extraction in the gut influences hepatic extraction not only after oral, but also after intravenous administration (Lin *et al.* 1997). Therefore, the apparent contribution of the liver to the total metabolic clearance was corrected, for the fraction eliminated in the gastrointestinal tract and consequently is not available for elimination by the liver. As a result, not only blood flow to the liver limits the supply of substrate available for elimination by the liver, but also elimination in the gut may limit the contribution of hepatic clearance. In other words, the liver operates at a lower substrate concentration than indicated by the systemic circulation. It was concluded that the influence of blood flow of the liver is taken into account in most *in vitro* studies but that the influence of prehepatic elimination in the gut has often been overlooked, most likely because liver and intestine are not frequently studied together.

After intravenous administration, drugs will first reach the lungs as a site of potential clearance. Therefore, in theory, it would be more accurate to correct the clearance of the other organs for the extraction ratio of the lung. However, the extraction fraction (CL/Q) in the lung is in general small, as a result of its modest metabolic activity and the fact that the lung blood flow is large (figure 2), although in some cases, drug metabolism in the lungs can be substantial (Gram 1997).

The present study considered the organs studied as homogeneously mixed compartments and consequently used the 'well-stirred' model to calculate their clearance. However, it is known that the distribution of drug-metabolizing enzymes varies along the length of the small intestine and along the sinusoids of the liver. For practical reasons, intestinal slices were prepared only from the initial part from the small intestine. As a consequence, an over prediction of intestinal contribution would occur if there is a significant loss of metabolic activity along the gastrointestinal tract, for example as described to be the case for various human and rat CYP isoforms (Zhang *et al.* 1999, Takemoto *et al.* 2003).

The present study could not make a direct comparison with regard to the contribution of the individual organs *in vivo*. Nevertheless, the *in vitro* prediction of the clearance by the summed contribution of the studied organs was reasonably close to the *in vivo* clearance (table 2). Remarkably, the contribution of the liver to total clearance was only 28–65%, depending on the compound studied (table 2). It will be of interest to investigate further whether this relatively high extrahepatic clearance is applicable for many drugs or is related to the rather arbitrary selection of model compounds used in this study. In the case of the well-studied model compound, 7-ethoxycoumarin, the contribution of the extrahepatic organs are in agreement with the finding that the human CYP1A1, which is expressed extrahepatically, shows the highest catalytic affinity for its dealkylation, when compared with other CYP isoforms (Shimada *et al.* 1999). Also, in a limited number of other *in vitro* studies, a relatively high drug-metabolizing activity was found in

extrahepatic tissue, especially in intestinal preparations, which is in agreement with the current results (Vickers *et al.* 1992, 2001, De Graaf *et al.* 2002).

The present study observed some underprediction of the *in vivo* clearances for two of the five compounds. Underprediction of *in vivo* clearance among others might be the result of decreased viability of the slices and/or to limited diffusion of substrates into the slices, which has been shown to occur at high metabolic turnover rates (Ekins *et al.* 1995). In addition, underprediction can be the result if excretion of unchanged drug is a major clearance route in the body. On the other hand, an overprediction may result if enterohepatic circulation *in vivo* occurs.

Several *in vitro* clearances that were reported until now yielded an underprediction of the *in vivo* value (e.g. Andersson *et al.* 2001), especially when only liver slices were used (Houston and Carlile 1997). If only the contribution of liver slices as single metabolizing system would be used in the present study, a considerable underprediction would also have been found. However, when extrahepatic clearance was included, *in vivo* prediction turned out to be considerably better (table 2). Therefore, it is concluded that slices are a useful preparation for *in vitro* prediction of clearance, when the contribution of the lung, kidney and, especially the intestines, are included. When only the liver is considered for *in vitro* prediction of metabolic clearance, the use of isolated hepatocytes predicts *in vivo* clearance more closely than the use of liver slices (Houston and Carlile 1997). It remains to be established if the use of isolated hepatocytes, where higher clearances were found than in slices (Houston and Carlile 1997), is to be preferred above liver slices, or that isolated hepatocytes actually *overpredict* the liver clearance, and in fact compensate for the contribution of the other organs.

In conclusion, the present has shown the usefulness of a straightforward approach to predict clearance using precision-cut slices from liver, lung, kidney, small intestine and colon. This approach also gives important information about the contribution of the various organs towards total metabolic clearance. Most importantly, this technique can be applied to human tissues, allowing prediction of metabolic clearance in man in an early phase of development. Such an *in vitro* approach might contribute to the reduction of the use of experimental animals in research on drug disposition.

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Phase I and II enzyme characterization of two sources of HepG2 cell lines

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1. The metabolism by HepG2 cell from two sources (M1, M2) of 12 substrates is reported: ethoxyresorufin, ethoxycoumarin, testosterone, tolbutamide, chlorzoxazone, dextromethorphan, phenacetin, midazolam, acetaminophen, hydroxycoumarin, *p*-nitrophenol and 1-chloro-2,4-dinitrobenzene (CDNB), and a pharmaceutical compound, EMD68843.

2. Activities varied markedly. Some were present in M1 (CYP1A, CYP2C9, CYP2E1) but absent in M2. M1 had a more complete set of Phase I enzymes than M2. CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities were present at levels similar to human hepatocytes. Phase II metabolism differed between M1 and M2. M1 conjugated hydroxycoumarin and *p*-nitrophenol to glucuronides only, whereas M2 produced sulfates. Glutathione conjugation of CDNB metabolism was 10-fold higher in M1 than in M2, but was still much lower than in human hepatocytes. CYP2E, CYP2C, CYP2B6 and CYP3A (but not CYP1A, glucuronyl *S*-transferase or *S*-transferase) were inducible in M1. Metabolites of EMD68843, produced by induced (but not uninduced) M1 were the same as those produced in human hepatocytes.

3. In conclusion, HepG2 cells have both Phase I and II enzymes, which activities and at what levels depend on the source and culture conditions. Therefore, HepG2 cells routinely used in *in vitro* assays should be characterized for their drug-metabolizing capabilities before any results can be fully interpreted.

Introduction

HepG2 cell lines derived from a Caucasian human hepatoblastoma were developed with the intention that they would replace fresh human hepatocytes in early toxicology screening. They have an obvious advantage of their ready availability and assurance of a certain reproducibility of experiments. Moreover, they have been shown to display many genotypic and phenotypic characteristics of human hepatocytes (Sassa *et al.* 1987). Early studies showed these cells to have extensive aldehyde dehydrogenase activities and functional glutathione regulation (Dierickx 1987, Marselos *et al.* 1987). More recent studies on HepG2 cells report their extensive glucuronyl *S*-transferase activities (Walle *et al.* 2000, O'Leary, 2003). HepG2 cells have been, and still are, used in a wide range of studies to measure drug metabolism and toxicity (Parker *et al.* 2000, Allen *et al.* 2001, Scheers *et al.* 2001, Yoshitomi *et al.* 2001, Feerman *et al.* 2002). Some have modified these cells and characterized their enzyme activities, for example, Yoshitomi *et al.* (2001), who established transformed HepG2 cells containing

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one specific cytochrome P450 (CYP). They found transformed cell activities to be similar to those in human microsomes. Other investigators have focussed on CYP1A and CYP3A activities and their involvement in substrate metabolism, cytotoxicity or induction potential (Parker *et al.* 2000, Allen *et al.* 2001, Feierman *et al.* 2002). Few have considered the possibility that their HepG2 cells contain appreciable amounts of enzymes other than those they have focussed on and some have not reported enzyme characteristics at all, although their assay may rely upon metabolic activation. Some assume that no metabolism of the test compound will occur, while others have used HepG2 cells and shown them to activate genotoxic precursors and cause DNA damage (Yusuf *et al.* 2000).

Despite the presence of certain metabolizing enzymes, HepG2 cells are still considered to be poor substitutes for fresh human hepatocytes due to a reported lack of major CYP activities. There are, however, little published data on just *how* low Phase I and II drug-metabolizing enzymes are and whether they differ between HepG2 cells from different sources. A more comprehensive approach would be to establish the metabolic competence of HepG2 cells so that results from such toxicity assays can be fully interpreted.

HepG2 cells are routinely used from two different sources (M1 and M2) in assays measuring gene induction. M1 HepG2 cells were from a source at Merck KgaA and these cells are used in the present gene array assay. M2 HepG2 cells were from Xenometrix, Inc. (Boulder, CO, USA) and used in the Xenometrix reporter gene CatTox(L)[®] assay (Todd *et al.* 1995). The cells were cultured in accordance with the respective assays. M1 HepG2 cells were cultured in complete RPMI medium and M2 HepG2 cells were cultured in complete minimal essential medium (MEM). It was decided that the HepG2 cells should be grown in the medium normally used in the gene array assays because the gene array assays were being compared with each other, not the cells. The original aim of this work was to find out if the difference in gene expression caused by a test compound could be due to a difference in the metabolism. If metabolism was the same, this aspect could have been ruled out of the comparison. Enzyme activities were measured at confluence, since this is the point at which the cell number in each assay was comparable because the entire flask is covered with cells. Also, it was thought that confluent cultures with good cell-cell contact would be more likely to have detectable enzyme activities. Another reason for measuring activities in cultures was that we wanted to measure activities in cells that were not freshly thawed so that differences in metabolism could not be attributed to recovery from cryopreservation. Moreover, confluent monolayers were used in the respective gene assays and the desire was to have the conditions to be as similar as possible.

Since these assays are meant to represent human primary cells, the activities in HepG2 cells with fresh and/or cryopreserved human hepatocyte (CPH) data taken from the literature were also compared. It is known that fresh and cryopreserved human hepatocytes are comparable in their enzyme activities (Li *et al.* 1999a, b), thus they have been used interchangeably to give an idea of the rates of metabolism that one expects from human hepatocytes. The reason why the HepG2 cells are compared with freshly isolated or freshly thawed hepatocytes (rather than cultures) is because these activities will most closely resemble that in the intact liver.

The effect of selective inducers on various Phase I and II enzyme activities was assessed, since this is one of the applications of HepG2 cells for high throughput screening (Allen *et al.* 2001). Also measured was the metabolism of a

pharmaceutical compound, EMD68843, which has been previously characterized in CPHs and *in vivo* (Hewitt *et al.* 2001). The main metabolic pathways involved in its metabolism *in vivo* are hydrolysis, N-dealkylation, hydroxylation and subsequent glucuronidation. The enzymes involved in EMD68843 metabolism include CYP3A4, CYP2D6, CYP2C9, CYP2C19 and UDP-glucuronyl *S*-transferases. In CPHs, hydrolysis was the major pathway and N-dealkylation was absent. Metabolism of this compound was also determined in HepG2 cells, which had been pre-induced with three different inducers.

Materials and methods

Chemicals

Verapamil, *p*-nitrophenyl sulfate potassium salt, tolbutamide, phenacetin, acetaminophen, ethoxycoumarin, chlorzoxazone, dextromethorphan and foetal calf serum were from Sigma-Aldrich (Taufkirchen, Germany). Midazolam solution was from Merckle GmbH (Blaubeuren, Germany). MEM with Glutamax I, RPMI medium with Glutamax I, non-essential amino acids ($\times 100$), sodium pyruvate solution ($\times 100$) and Hank's balanced salt solution with Ca^{2+} and Mg^{2+} ($\times 1$, HBSS) and trypsin ethylene glycol-bis-(β -amino-ethylether)tetra-acetic acid (EGTA) solution ($\times 10$, 2.5% trypsin) were from GibcoBRL (Paisley, UK, or Eggenstein, Germany). Dextrorphan, 3-methylmorphinan hydrochloride, 1'-hydroxymidazolam, 4'-hydroxymidazolam and hydroxytolbutamide were from Ultrafine Chemicals Ltd (Manchester, UK). Testosterone, hydroxycoumarin, *p*-nitrophenyl glucuronide and all other chemicals were from Merck KGaA (Darmstadt, Germany).

Media

M1 HepG2 cells were cultured in RPMI medium (with Glutamax I) supplemented with 10% foetal calf serum, 100 U ml^{-1} penicillin and $100 \mu\text{g ml}^{-1}$ streptomycin. M2 HepG2 cells were cultured in MEM (with Glutamax I) containing 10% foetal calf serum, 2 mM sodium pyruvate, non-essential amino acids, 100 U ml^{-1} penicillin and $100 \mu\text{g ml}^{-1}$ streptomycin. Supplemented media are referred to as 'complete' media.

Cell culture

M1 HepG2 cells are routinely used in in-house studies at Merck KGaA (the source is proprietary) and M2 HepG2 cells are from Xenometrix, which obtained them from the American Type Culture Collection (Bethesda, MD, USA). HepG2 cells from one vial (containing approximately 10^6 cells) were thawed rapidly by immersing in a 37°C water bath. The cells were transferred to a 50-ml Falcon tube containing 1 ml complete medium, which was placed on ice. After 5 min, a further 1 ml medium was added. After a further 5 min, 20 ml complete medium were added and the cells resuspended by gentle aspiration with a pipette. After centrifugation for 5 min at $168g$ and 4°C , the supernatant was removed and the cells resuspended in 5 ml complete medium. Viability and cell density were determined by Trypan blue exclusion. The cells were diluted to a density of $0.1 \times 10^6 \text{ cells ml}^{-1}$ in complete medium and then 10 ml were placed into a T-75 culture flask (Becton Dickinson, Heidelberg, Germany). The cells were allowed to attach for 4 h (M1 HepG2) to 24 h (M2 HepG2), after which the medium was changed with fresh complete medium. The medium was changed every 48 h until the cells reached confluence.

Splitting and cryopreservation of HepG2 cells

HepG2 cells were removed from T-75 flasks by trypsinization. Briefly, the medium was removed and the cells washed twice with phosphate-buffered saline before the addition of 3 ml trypsin solution ($\times 1$). The flask was manipulated to ensure all the cells were covered by trypsin, which was then immediately removed. Another 3 ml trypsin ($\times 1$) were added to the flasks, which were placed in a 37°C humidified incubator for 5 min. The trypsin was deactivated by adding 10 ml complete medium. The cells were gently dislodged by tapping the flask. The resultant cell suspension was then centrifuged at $168g$ for 5 min at room temperature. After removal of the medium, the cells were resuspended in 5 ml complete medium. Cell viability and density were determined by Trypan blue exclusion.

Cells passaged further were diluted to $0.1 \times 10^6 \text{ cells ml}^{-1}$ and 10 ml were added to each T-75 flask. Cells cultured for test compound incubation were diluted to $0.066 \times 10^6 \text{ cells ml}^{-1}$ and 5 ml were added to each T-25 flask. The cultures were treated in the same way as described above.

Cells prepared for storage were diluted to $1.11 \times 10^6 \text{ cells ml}^{-1}$ in complete medium. Dimethyl sulfoxide was added dropwise over 5 min to give a final concentration of 10%. Cell suspensions were aliquoted in to cryotubes (1 ml/vial) and frozen in a Cryoson programmable freezer according to Swales and Utesch (1998) and then stored in liquid nitrogen until required.

Incubation of substrates

The incubation buffer was HBSS ($\times 1$, with Ca^{2+} and Mg^{2+}) supplemented with 0.5% glucose, 0.5% bovine serum albumin and 25 mM HEPES, pH 7.4. For midazolam, the incubation buffer contained no bovine serum albumin. Metabolism in HepG2 cells was always measured at confluence, which was achieved after 5 and 10 days for M1 and M2 cells, respectively. Enzyme activities were measured in passages 2–6 in M1 cells and in passages 1–4 in M2 cells, after receipt of the stock cells. Cultures were washed twice with HBSS (warmed to 37°C) before the addition of incubation medium containing the appropriate substrate. The substrate concentrations, incubation times and metabolite analysis methods are summarized in table 1. The number of cells in each T-25 was determined at the end of each incubation by trypsinizing the cells and then counting them by Trypan blue exclusion. Enzyme activities were expressed as $\text{pmol min}^{-1} 10^{-6}$ cells.

Induction incubations

M1 HepG2 cells were split and plated in T-25 flasks as described. The inducers were added in serum-free medium 48 h after plating, when the cells had reached about 40% confluence. The inducers were: 100 μM phenobarbitone (PB), 50 μM β -naphthoflavone (BNF), 50 μM dexamethasone (DEX) and 0.5% ethanol. A pilot study was run to test the cytotoxicity of each inducer in M1 HepG2 cells using a concentration range 0–1000 μM (data not shown). Medium (containing the respective inducers) was replaced every 24 h. Inducers were present for 72 h. After this time, the cells were washed with warm (37°C) HBSS and then incubated with selected substrates or 20 μM EMD68843 in incubation buffer as described above. The analysis of EMD68843 is described by Hewitt *et al.* 2001. None of the inducers caused cytotoxicity or decreased the growth of the cells.

Statistics

Results were compared using the Student's unpaired *t*-test. $p < 0.05$ was considered significant. 'n' refers to the number of different batches of cells frozen on different days.

Results

Phase I activities

Table 2 summarizes Phase I activities measured in M1 and M2 HepG2 cells and compares them with those reported to be present in human hepatocytes. Enzyme activities were very different between M1 and M2 HepG2 cells. Some activities, namely CYP1A1/2 (ethoxyresorufin only), CYP2C9 and CYP2E1, were present in

Table 1. Substrate incubation conditions and metabolite analysis methods.

Substrate	Enzyme (CYP)	Final concentration (μM)	Incubation times (h)	Method reference
Phase 1				
Ethoxyresorufin	1A1/2	10	0, 1	Lubet <i>et al.</i> (1985)
Ethoxycoumarin	2B6, 1A2, 2E1	500	0, 0.5, 1, 2, 4	Ekins <i>et al.</i> (1995)
Testosterone	3A, 2C, 2B	500	0, 0.25, 0.5, 1, 2	Arlotto <i>et al.</i> (1991)
Tolbutamide	2C9	500	0, 1, 2, 4, 6, 24	Miners <i>et al.</i> (1988)
Chlorzoxazone	2E1	50	0, 0.25, 0.5, 1, 2	Hickman <i>et al.</i> (1998)
Dextromethorphan	2D6, 3A	500	0, 0.25, 1, 2, 4	Chen <i>et al.</i> (1990)
				Witherow and Houston (1999)
Phenacetin	1A2	500	0, 0.5, 1, 2, 4, 6, 24	Stevens and Gill (1986)
Midazolam	3A4/5	50	0, 0.5, 1, 2, 4	Fabre <i>et al.</i> (1988)
Phase 2				
Hydroxycoumarin	UGT, ST	500	0, 0.5, 1, 2, 4	Ekins <i>et al.</i> (1995)
Acetaminophen	UGT, ST	500	0, 0.5, 1, 2, 4, 6, 24	Stevens and Gill (1986)
<i>p</i> -Nitrophenol	UGT, ST	12.5	0, 0.25, 0.5, 1, 2, 4, 6	Kern <i>et al.</i> (1997)
CDNB	GST	2000	10 min	Habig <i>et al.</i> (1974)

UGT, glucuronosyl *S*-transferase; ST, sulfotransferase; CDBN, 1-chloro 2,4-dinitrophenol.

Table 2. Phase I activities in M1 and M2 HepG2 cells and human hepatocytes.

Substrate	Enzyme ^a (CYP)	M1 HepG2	M2 HepG2	Human hepatocytes ^b	Reference
Phase 1					
Ethoxyresorufin	1A1/2	0.26 ± 0.22	0 ± 0*	0.01–0.36	Li <i>et al.</i> (1999)
Phenacetin	1A2	6.0 ± 2.8	14.7 ± 4.8*	0–64	IVT
Ethoxycoumarin	1A2, 2E1, 2B6	285.7 ± 60.4	1.5 ± 2.4*	27 ± 21; 7–84	Kern <i>et al.</i> (1997), IVT
Tolbutamide	2C9	7.8 ± 1.2	0 ± 0*	1.8–37	Li <i>et al.</i> (1999)
Testosterone	2C (16 α -OHT)	1.4 ± 0.9	3.9 ± 3.4	17.5 ± 10.5	Kern <i>et al.</i> (1997)
Testosterone	2C, 2B, CDH (ASD)	2.2 ± 0.4	77.3 ± 28.7*	138.1 ± 17.3	Kern <i>et al.</i> (1997)
Dextromethorphan	2D6 (DOR)	2.0 ± 0.7	3.6 ± 3.5	1.5–77	Li <i>et al.</i> (1999)
Dextromethorphan	3A (MEM)	20.8 ± 13.8	16.0 ± 2.3	n.r.	
Dextromethorphan	2D6/3A (HOM)	1.7 ± 1.6	2.3 ± 3.2	n.r.	
Chlorzoxazone	2E1	5.6 ± 1.1	0 ± 0*	2.9 ± 0.3; 18–69	Li <i>et al.</i> (1999), IVT
Testosterone	3A4/5 (6 β -OHT)	0 ± 0	0 ± 0	3.3–190	Li <i>et al.</i> , (1999)
Midazolam	3A4/5(1'-OHM)	40.5 ± 9.8	2.7 ± 4.6*	58 ± 6.2	Ledirac <i>et al.</i> (2000)
	3A4/5(4'-OHM)	19.7 ± 12.3	0.7 ± 0.7	n.r.	
	3A4/5(1'4'-OHM)	47.3 ± 41.3	0.4 ± 0.3	n.r.	

Activities are expressed as pmol min⁻¹ 10⁻⁶ cells (mean ± SD, $n = 3-8$ for M1 (except for ethoxycoumarin, which was $n = 2$), and $n = 3-4$ for M2).

^aEnzyme by which the substrate is metabolised in man.

^bHuman (fresh or cryopreserved) data are taken from the references listed.

CDH, cytosolic dehydrogenases; ASD, Androstene dione; OHT, hydroxytestosterone; DOR, dextrophan; MEM, 3-methoxymorphinan; HOM, 3-hydroxymorphinan; OHM, hydroxymidazolam; IVT, In Vitro Technologies website. (www.invitrotech.com); n.r., no reference. *Significant difference between M1 and M2 HepG2 cells ($p < 0.05$).

M1 but absent in M2. Despite the CYP3A-mediated hydroxylation of midazolam and N-demethylation of dextromethorphan by M1 and M2, 6 β -testosterone hydroxylase activity, also a marker of CYP3A4/5, was absent in both HepG2 cell lines. Midazolam was also metabolized by both M1 and M2 to two other metabolites, 4'-hydroxymidazolam (4'-OHM) and 1',4'-hydroxymidazolam (1',4'-OHM). The average rates of 1'-OHM, 4'-OHM and 1',4'-OHM production were higher in M1 than in M2, although only 1'-OHM reached statistical significance. Activities of CYP1A (ethoxyresorufin and phenacetin), CYP2C9, CYP2E1 and CYP3A (midazolam only) in M1 HepG2 cells were in the ranges reported in human hepatocytes. In contrast, only CYP1A2 (phenacetin only), CYP2C (testosterone 16 α -hydroxylation) and cytosolic dehydrogenases Androstene dione (ASD formation) in M2 HepG2 cells were within the ranges reported in human hepatocytes. Ethoxycoumarin-o-deethylase (ECOD) activity (reflective of 1A2, 2B6 and 2E1 activities) was higher in M1 HepG2 cells than in M2 and human hepatocytes. CYP2D6 activities in M1 and M2 were at the lower end of the range of activities in human hepatocytes.

Phase II activities

There was a difference in *p*-nitrophenol metabolism by M1 and M2 HepG2 cells. The only metabolite formed by M1 was the glucuronide, whereas M2 only produced the sulfate. There was a time lag of at least 30 min before any conjugation occurred in either cells, after which, the rate of glucuronidation was higher than sulfation in M2 (figure 1). The rate of conjugation of *p*-nitrophenol by both

M1 and M2 was lower than *p*-nitrophenol glucuronidation or sulfation in human hepatocytes (table 3).

The major metabolite of ethoxycoumarin was hydroxycoumarin, which was exclusively glucuronidated by M1 and sulfated by M2 HepG2 cells (figure 2). When hydroxycoumarin was incubated as the substrate with HepG2 cells, M1 still produced only a glucuronide but M2 produced both a sulfate and glucuronide, in equal amounts (figure 2).

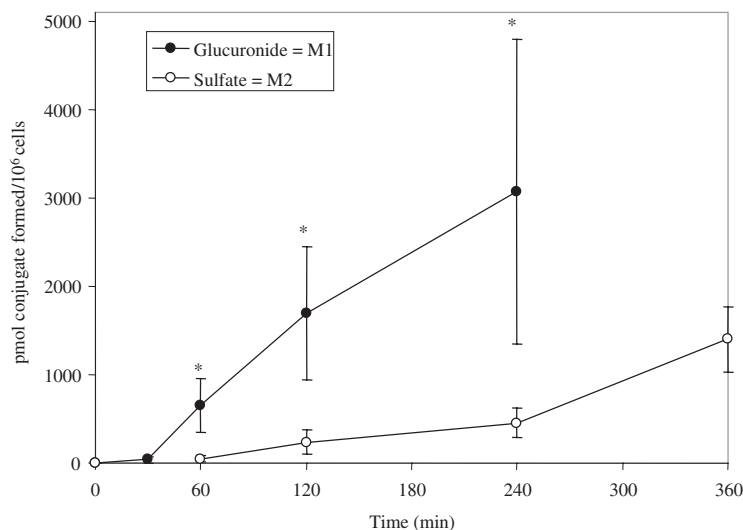


Figure 1. Time-dependent conjugation of *p*-nitrophenol in M1 and M2 HepG2 cells (mean SD, $n=3-5$). Confluent cultures were incubated with $12.5\ \mu\text{M}$ *p*-nitrophenol for up to 4 h (M1) or 6 h (M2). Results are pmol product formed per million cells. *Significant difference between M1 and M2 HepG2 cells ($p < 0.05$).

Table 3. Phase II activities in M1 and M2 HepG2 cells and human hepatocytes.

Substrate	Enzyme ^a	M1 Hep G2	M2 Hep G2	Human hepatocytes ^b	Reference
Phase 2					
Ethoxycoumarin	UGT	14.3 ± 17.1	$0 \pm 0^*$	21 ± 18	Kern <i>et al.</i> (1997)
Ethoxycoumarin	ST	0 ± 0	0.4 ± 0.7	3 ± 3	Kern <i>et al.</i> (1997)
Hydroxycoumarin	UGT	39.4 ± 17.5	$5.5 \pm 4.2^*$	$23.6-825$	Li <i>et al.</i> (1999)
Hydroxycoumarin	ST	0.2 ± 0.0	$5.2 \pm 4.0^*$	$2.5-39$	Li <i>et al.</i> (1999)
Phenacetin	UGT	2.1 ± 1.2	$6.4 \pm 2.9^*$	51% of the dose	Veroneses and McClean (1991)
Phenacetin	ST	3.8 ± 1.5	2.8 ± 1.7	30% of the dose	Veroneses and McClean (1991)
Acetaminophen	UGT	1.8 ± 1.9	$5.7 \pm 2.5^*$	16 ± 3	Kane <i>et al.</i> (1995)
Acetaminophen	ST	1.8 ± 1.1	$25.0 \pm 7.6^*$	4 ± 1	Kane <i>et al.</i> (1995)
<i>p</i> -Nitrophenol	UGT	15.1 ± 6.8	$0 \pm 0^*$	68.5 ± 35.9	Kern <i>et al.</i> (1997)
<i>p</i> -Nitrophenol	ST	0 ± 0	$4.4 \pm 1.4^*$	26.2 ± 11.7	Kern <i>et al.</i> (1997)
CDNB	GST	0.034 ± 0.014	$0.004 \pm 0.002^*$	27 ± 7.8	Hengstler <i>et al.</i> (2000)

Activities are expressed as $\text{pmol min}^{-1} 10^{-6}$ cells (mean $\pm$ SD, $n=3$) unless otherwise stated.

^aEnzyme by which substrate is metabolized in man.

^bHuman (fresh or cryopreserved) data are taken from the references listed.

UGT, UDPGA glucuronyl *S*-transferases; ST, sulfotransferases; GST, glutathione *S*-transferases. *Significant difference between M1 and M2 HepG2 cells ($p < 0.05$).

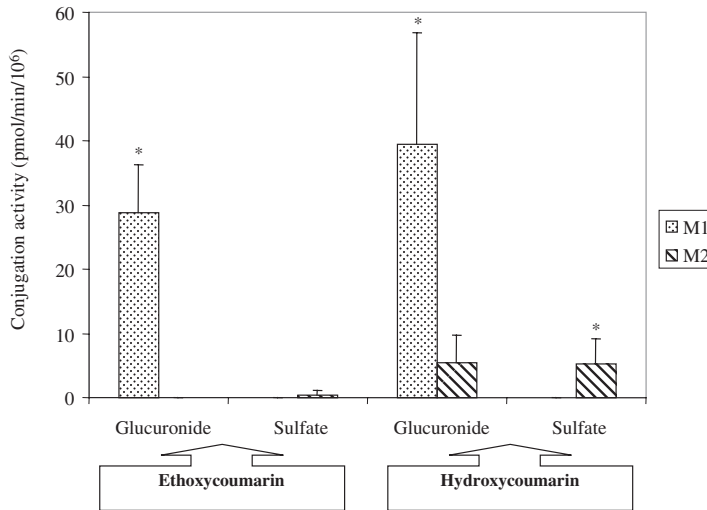


Figure 2. Conjugation of ethoxycoumarin and hydroxycoumarin by M1 and M2 HepG2 cells (mean $\pm$ SD, $n=3$). Confluent cultures were incubated with 500 μ M ethoxycoumarin or 500 μ M hydroxycoumarin for to 4 h. The highest rate of change was determined for each activity and values are expressed as pmol product formed per million cells min^{-1} . *Significant difference between M1 and M2 HepG2 cells ($p < 0.05$).

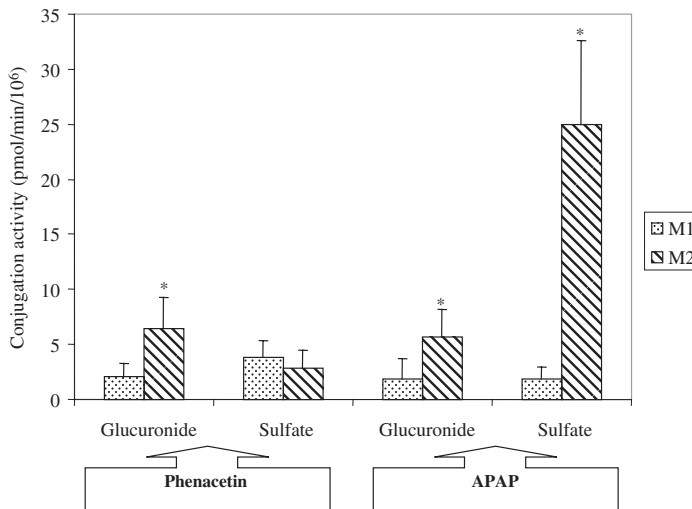


Figure 3. Conjugation of phenacetin and acetaminophen by M1 and M2 HepG2 cells (mean $\pm$ SD, $n=3$). Confluent cultures were incubated with 500 μ M phenacetin or 500 μ M acetaminophen for up to 4 h. The highest rate of change was determined for each activity and values are expressed as pmol product formed per million cells min^{-1} . *Significant difference between M1 and M2 HepG2 cells ($p < 0.05$).

Acetaminophen (APAP), the Phase I metabolite of phenacetin, was detected mainly as a glucuronide and sulfate in both M1 and M2 HepG2 cells, with little unconjugated APAP detected. The amount of sulfate formed was markedly increased in M2 HepG2 cells, from 2.8 ± 1.7 to 25.0 ± 7.6 $\text{pmol min}^{-1} 10^{-6}$ cells when APAP was incubated instead of phenacetin (figure 3). In contrast, the rate of glucuronide production was the same, whether incubated as APAP or its precursor, phenacetin.

Table 3 shows Phase II activities in M1 and M2 HepG2 cells and compares them with those reported to be present in human hepatocytes. All substrates (apart from CDNB) were predominantly glucuronidated in hepatocytes (between 70 and 90% glucuronides). Glucuronidation and sulfation activities were generally lower in HepG2 cells than in hepatocytes, with the exception of M2 HepG2 cells, which extensively sulfated acetaminophen.

Glutathione *S*-transferase activities were 10-fold higher in M1 than in M2 HepG2 cells. However, these activities were still much lower than in human hepatocytes (table 3).

Induction of CYP activities

A number of substrates were chosen to determine the induction potential of four inducers on M1 HepG2 cells. Although rifampicin is a classic inducer of CYP3A4 in human hepatocytes (and *in vivo*), it inhibited the growth of the HepG2 cells in culture (at 25 μ M) and therefore could not be used in this dosing regimen. The cells would not grow to confluence and this would also decrease the sensitivity of the assay. As an alternative, a high concentration of dexamethasone (50 μ M) was used, which did not have this effect. Figure 4 summarizes the effect of 100 μ M PB,

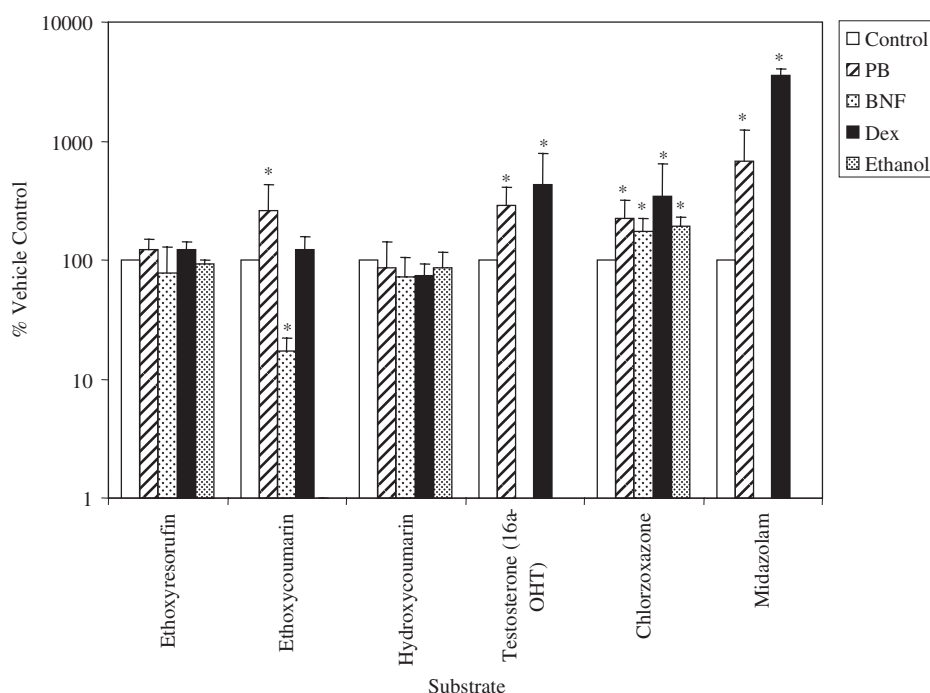


Figure 4. Induction of enzyme activities in M1 HepG2 cells (mean $\pm$ SD, $n = 3$). The inducers, 100 μ M phenobarbitone (PB), 50 μ M β -naphthoflavone (BNF), 50 μ M dexamethasone (DEX) or 0.5% ethanol were added to the culture medium when the cells were about 40% confluent. The total induction time was 72 h. Enzyme activities were determined at confluence and expressed as a percentage of vehicle control. Control activities were: EROD = 0.26 ± 0.22 pmol min⁻¹ 10⁻⁶ cells, ECOD = 285.7 ± 50.4 pmol min⁻¹ 10⁻⁶ cells, HC glucuronidation = 39.4 ± 17.5 pmol min⁻¹ 10⁻⁶ cells, testosterone 16 α -hydroxylation = 1.4 ± 0.9 min⁻¹ 10⁻⁶ cells, chlorzoxazone 4-hydroxylation = 5.6 ± 1.1 pmol min⁻¹ 10⁻⁶ cells and midazolam total hydroxylation = 107.6 ± 58.3 pmol min⁻¹ 10⁻⁶ cells. *Significant difference between M1 and M2 HepG2 cells ($p < 0.05$).

50 μ M BNF, 50 μ M DEX and 0.5% ethanol on the metabolism of six different substrates, ethoxyresorufin, ethoxycoumarin, hydroxycoumarin, testosterone, chlorzoxazone and midazolam. All four inducers had no effect on ethoxyresorufin (CYP1A1/2) *O*-deethylation EROD, however, ECOD (CYP2B6, CYP1A and CYP2E) was induced by PB (260% of control) and decreased by BNF (17% of control). Incubation of HepG2 cells with BNF did not cause obvious signs of cytotoxicity, as determined by visual microscopy and metabolism of other substrates (hydroxycoumarin, ethoxyresorufin and chlorzoxazone). The rates of 16 α -hydroxytestosterone (CYP2C) and midazolam hydroxylation (total OHM metabolites, CYP3A) production were both significantly induced by PB and DEX, the effect being more potent on CYP3A (682 and 3559% of control, respectively) than on CYP2C (287 and 433% of control, respectively). The observed increase in CYP3A-mediated midazolam hydroxylation was not reflected in a concomitant appearance of testosterone 6 β -hydroxylation (data not shown). Chlorzoxazone hydroxylation (CYP2E1) was induced by all four inducers. Phase II glucuronidation and sulfation of hydroxycoumarin was unaffected by all four inducers.

Metabolism of a pharmaceutical drug

The metabolism of EMD68843 (20 μ M) *in vivo*, in CPHs and M1 HepG2 cells (20 μ M) is compared in figure 5. In both CPHs and HepG2 cells, the hydrolysis product was the major metabolite (68% of the total metabolites in CPHs and

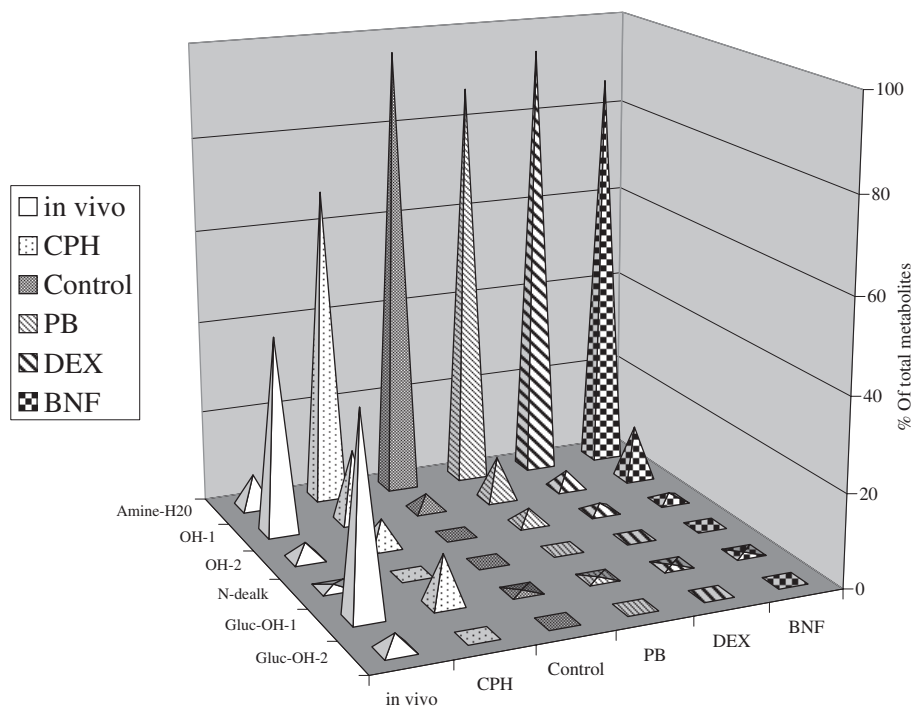


Figure 5. EMD68843 metabolites produced in control and induced M1 HepG2 cells, cryopreserved human hepatocytes and human *in vivo* (mean, $n=3$). Cultures were treated with inducers as described in figure 4. At confluence, the cultures were incubated with 20 μ M EMD68843 for 4 h. Results are the per cent of the total metabolites produced.

87–96% of the total metabolites in HepG2 cells). Control HepG2 cells produced only one hydroxide (OH-1) and its glucuronide conjugate. Only HepG2 cells induced with PB, BNF or DEX could produce the second hydroxide (OH-2). Like CPHs, HepG2 cells did not metabolize EMD68843 to its N-dealkylation product or the glucuronide conjugate of the second hydroxide. Metabolism of OH-1 was increased by PB and BNF pre-induction (271 and 341% of control in PB- and BNF-treated cells, respectively, $p < 0.05$). The production of the glucuronide of OH-1 was the same in control and pre-induced cells.

Discussion

Publications using HepG2 cells often use the phrases ‘HepG2 cells have been well characterized with respect to their cytochrome P450 expression and activity’ or ‘these cells express little endogenous P450 function’ and then refer to a cited reference. However, how many of these authors characterize the actual activities in their HepG2 cells they use? Can one expect a source of HepG2 cells to be the same as another? And, more importantly, if these cells have Phase I and II activities, how does this compare with that measured in human hepatocytes? These data demonstrate that two sources of HepG2 cells can have very different enzyme characteristics and that the belief they are devoid of CYP activity is not always true. In the present authors’ hands, two sources of HepG2 cells had significant Phase I and II drug-metabolizing activities, some of which were comparable with those in human hepatocytes. There were significant qualitative and quantitative differences between the two sources of HepG2 cells, which could have been due to the original source or, very likely, to the culture conditions in which these cells were grown. However, it is noteworthy that the two sources showed such marked differences in metabolism.

M1 HepG2 cells had a more complete set of Phase I enzymes than M2 HepG2 cells, substrates metabolized by CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A were all metabolized at rates similar to human hepatocytes. All these enzymes play an important role in drug metabolism, thus M1 HepG2 cells are more comparable with human primary hepatocytes than M2. Corresponding activities in M2 were either lower or absent. However, when M1 HepG2 cells were incubated with a pharmaceutical compound, namely EMD68843, metabolism was not exactly the same as in CPHs. A missing hydroxide was only produced in HepG2 cells pre-induced with PB, DEX or BNF. Thus, the presence of a number of drug-metabolizing enzymes does not necessarily imply that these cells have comparable drug metabolism to primary human hepatocytes.

The failure of both M1 and M2 to produce 6 β -hydroxytestosterone was notable, especially since both could metabolize midazolam, a CYP3A4/5 substrate (Fabre *et al.* 1988). It is known that CYP3A7 is a major P450 isoenzyme in foetal liver and liver tumour cells, the adult CYP3A4 being absent (Schuetz *et al.* 1993). Thus, CYP3A7 may have been responsible for midazolam metabolism but was unable to hydroxylate testosterone. PB and DEX both increased midazolam hydroxylation but not testosterone 6 β -hydroxylation, suggesting that CYP3A7 but not CYP3A4/5 was induced in these cells. Others have also demonstrated CYP3A7 induction in HepG2 cells treated with rifampicin (Pascussi *et al.* 1999,

Schuetz *et al.* 1993). PB is known to induce a broad spectrum of CYP isoenzymes in human hepatocytes, including CYP1A2, CYP2B6, CYP2C19, CYP2C9 and CYP3A4 (Meunier *et al.* 2000, Gerbal-Chaloin *et al.* 2001). It is reported that both rifampicin and PB act via the two nuclear receptors, pregnane X receptor (PXR) and constitutively activated receptor (CAR) to induce CYP3A4 to 3A7, CYP2C9 and CYP2B6 (Gerbal-Chaloin *et al.* 2001, Dvorak *et al.* 2003). In the present study, PB also resulted in an induction of CYP3A, CYP2C and CYP2B6/2E1 (ethoxycoumarin *O*-deethylation) in M1 HepG2 cells. Likewise, DEX induced both CYP3A and CYP2C in these cells. CAR and PXR are regulated, at least in part, by the glucocorticoid receptor (GR) and it has been hypothesized that a signal transduction cascade GR-[CAR/PXR]-P450 exists (Dvorak *et al.* 2003). DEX can induce CYP3A indirectly at submicromolar concentrations by activating GR and subsequently up-regulating PXR, CAR and the retinoic acid receptor (RXR) or at supramicromolar concentrations through direct activation of PXR (Pascussi *et al.* 2000a, b). It is also possible that in these cells, CYP3A7 could have been induced by GR directly rather than via PXR if this is absent or at low levels (Pascussi *et al.* 1999). HepG2 cells are considered to be classic models for the study of GR (Dondi *et al.* 2001) and since induction of CYP3A4 and CYP2C was evident in M1 HepG2 cells, it is pertinent to conclude that GR was also present in these cells. Whether or not these cells possess PXR is not known since, despite PXR normally being required as part of the cascade for CYP3A induction by DEX and PB, it can be that in the absence of PXR, GR responses may dominate.

Others have measured the effects of inducers on enzyme levels in transformed (Allen *et al.* 2001) and non-transformed HepG2 cells (Gross-Steinmeyer *et al.* 2001). Gross-Steinmeyer *et al.* measured induction by BNF and omeprazole of mRNA, protein and enzyme activity levels in non-transformed HepG2 cells. They showed that EROD activity was induced over 72 h by 20 μ M BNF, concomitant with increases in both mRNA and CYP1A protein levels. In contrast, the present results showed that EROD was not increased by 50 μ M BNF incubated for 72 h, which suggests that CYP1A was uninducible in M1 HepG2 cells. Interestingly, unlike in primary hepatocytes, CYP2E was induced not only by ethanol, but also by PB, BNF and DEX. The reason for this is uncertain but this reiterates the difference between using primary and continuous cultures of human hepatocytes.

Both M1 and M2 HepG2 cells could sequentially metabolize substrates by Phase I and II enzymes, an observation also made by Yoshitomi *et al.* (2001). glutathione *S*-transferase activity was common to both M1 and M2 but was 10-fold higher in M1 than in M2. M1 and M2 also both had the capacity for glucuronidation and sulfation, however the extent to which this occurred was substrate specific. *p*-Nitrophenol was entirely glucuronidated by M1 HepG2 cells and entirely sulphated by M2 HepG2 cells. The extent of Phase II conjugation in M2 HepG2 cells (but not M1) depended on substrate availability, since hydroxycoumarin was equally sulfated and glucuronidated when hydroxycoumarin was the substrate but when incubated as its precursor, ethoxycoumarin, only the sulfate was produced. The ratio of glucuronidation to sulfation was different between hydroxycoumarin and acetaminophen, suggesting the involvement of different glucuronyl *S*-transferases and *S*-transferases in their metabolism. Human tissue is known to contain many glucuronyl *S*-transferase isoforms (Chiu and Huskey 1998) and at least two forms of phenol *S*-transferases (Reiter and Weinshilboum 1982, Cambell *et al.* 1987). Phase II conjugation in

human hepatocytes was best reflected in M1, which preferentially glucuronidated the substrates. However, these activities, including glutathione *S*-transferases, in HepG2 cells were generally lower than in human hepatocytes.

In summary, these data demonstrate that HepG2 cells can undergo Phase I and II metabolism, but which activities and at what levels will depend on the source and culture conditions. Therefore, HepG2 cells routinely used in *in vitro* assays should be characterized for their drug-metabolizing capabilities. Moreover, it is advisable to check test compound metabolism in the appropriate incubation conditions before any results with HepG2 cells can be fully interpreted.

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Biotransformation of chlorpropham (CIPC) in isolated rat hepatocytes and xenoestrogenic activity of CIPC and its metabolites by *in vitro* assays

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1. The metabolism and action of chlorpropham (isopropyl *N*-(3-chlorophenyl)carbamate; CIPC, a post-harvest agent) were studied in freshly isolated rat hepatocytes, and the oestrogen-like activity of CIPC and its metabolites was assessed by *in vitro* assays. The exposure of hepatocyte suspensions to CIPC caused concentration- (0.25–1.0 mM) and time- (0–3 h) dependent cell death, which was assessed by Trypan blue exclusion, accompanied by losses of cellular adenosine triphosphate and adenine nucleotide pools, and formation of cell bleb.

2. CIPC at a weakly toxic level (0.25 or 0.5 mM) was metabolized to isopropyl *N*-(3-chloro-4-hydroxyphenyl)carbamate (4OH-CIPC) and subsequently to its glucuronide and sulfate conjugates (major metabolites) or alternatively to the minor metabolites 3-chloroaniline (3CA) and 3-chloroacetanilide. CIPC (0.25 mM) added to hepatocyte suspensions was distributed equally between hepatocytes and the extracellular medium during the incubation. The glucuronide rather than the sulfate conjugate of 4OH-CIPC predominantly increased in the medium with time, while the amount of unconjugated free 4OH-CIPC in the extracellular medium increased by approximately threefold compared with the amount in the cell fraction after 0.5 h and then decreased rapidly accompanied by increases in the conjugates. This indicates that unconjugated free 4OH-CIPC produced in hepatocytes was temporarily excreted in the extracellular medium and subsequently converted to the conjugates via re-influx into hepatocytes.

3. Diethylstilbestrol (DES), bisphenol A (BPA) and 4-hydroxybenzoic acid butyl ester (butylparaben), which are known xenoestrogenic compounds, competitively displaced 17 β -oestradiol bound to the oestrogen receptor- α (ER α) in a concentration-dependent manner; IC₅₀ values of DES, BPA, butylparaben and its derivative 3-chloro-4-hydroxybenzoic acid butyl ester (3-chloro-butylparaben) were approximately 10⁻⁸, 10⁻⁵, 5 $\times$ 10⁻⁵ and 5 $\times$ 10⁻⁴ M, respectively. In contrast, neither CIPC nor 4OH-CIPC impaired the binding of 17 β -oestradiol to ER α at concentrations ranging from 10⁻⁹ to 10⁻⁴ M, whereas at concentrations of >5 $\times$ 10⁻⁴ M, the binding affinity of 4OH-CIPC was greater than that of CIPC. In a proliferation assay of MCF-7 cells, CIPC, 4OH-CIPC and 3CA did not increase cell numbers at concentrations ranging from 10⁻⁹ to 10⁻⁵ M, but these compounds at a concentration of 10⁻⁴ M induced a considerable decrease in cell numbers relative to the control. The results suggest that even if CIPC is metabolized to 4OH-CIPC by hepatocytes, the chlorine adjacent to the 4-hydroxy group added to the intermediate as well as 3-chloro-butylparaben obstructs the appearance of oestrogen-like effects via an interaction between the intermediate and the ER.

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Introduction

Chlorpropham (isopropyl *N*-(3-chlorophenyl)carbamate; CIPC) is widely used to control grassy weeds in various food and non-food crops, and as a sprouting suppressant and/or post-harvest treatment of potatoes during storage and/or transport. Because its use is so widespread, even if consumption in the diet is low, the potential toxicity of CIPC has been studied *in vivo* and *in vitro* to assess its various toxicological properties. Although CIPC is not potently toxic in genotoxic and mutagenic studies (Andersen *et al.* 1972, Dolara *et al.* 1994) and in some short- and/or long-term dietary studies (Larson *et al.* 1960, Boyd and Carsky 1969, Van Esch and Kroes 1972, Van Engelen 2001), the liver, kidney, spleen and erythrocytes have been identified as targets of CIPC-induced toxicity in rats. Oral administration of CIPC to rats or mice caused dose-related increases in liver and spleen weight, accompanied by the acceleration of haematopoiesis and/or haemosiderosis in both organs. The serum concentrations of bilirubin and cholesterol, as parameters of hepatic damage, were significantly increased at the highest dose (Van Engelen 2001). In the kidney, intracytoplasmic hyaline resorption bodies were found in the tubules, with the effect being more pronounced in males than in females (Fujitani *et al.* 1997, 2000, Van Engelen 2001). In addition, it has been reported that CIPC is well absorbed from the gastrointestinal tract when administered via the oral route in rats and mice, undergoes extensive metabolism, and is excreted in the urine, and that the metabolism of CIPC proceeds either via aromatic 4-hydroxylation followed by conjugation of the intermediate with glucuronic acid and sulfate as a major pathway, or via oxidation of the isopropyl side chain and carbamate hydrolysis, followed by conjugation of these intermediates as minor pathways (figure 10) (Fang *et al.* 1974, Carrera *et al.* 1998).

Although many natural and man-made compounds are ubiquitous in the environment, little is known about the potential risks to humans of exposure to known xenoestrogens. Consequently, considerable attention has focused on synthetic or natural aromatic compounds bearing hydroxyl groups as endocrine-disrupting chemicals exhibiting weak oestrogenic activity in *in vivo* (Nagel *et al.* 1997, Vom Saal *et al.* 1998) and *in vitro* (Gaido *et al.* 1997) bioassays. Recently, the present authors reported that oestrogenic activity is induced by biotransformation, either via the direct hydroxylation of the *para* (or 4)-position or via oxidative dealkylation of 4-substituted group in aromatic compounds such as benzophenones (Nakagawa *et al.* 2000, Nakagawa and Suzuki 2002) and *trans*-anethole (Nakagawa and Suzuki 2003).

The metabolic pathway and toxic effects of CIPC have been studied *in vivo* and/or *in vitro* (Fang *et al.* 1974, Carrera *et al.* 1998), whereas the relationship between metabolism and oestrogenic activity has not been clarified. The freshly isolated rat hepatocyte system retains intact membranes and has high levels of various drug-metabolizing enzymes and their cofactors associated with phase I and II reactions and is useful for the study of uptake, intracellular target sites, and temporal sequences leading to cell injury by chemicals and/or their metabolites via biotransformation (Moldéus *et al.* 1978). The present study first investigated the metabolism and action of CIPC in isolated rat hepatocytes, and then assessed the potential oestrogenic activities of the compound and its intermediates using a competitive binding assay for recombinant human oestrogen receptor- α (ER α) and a proliferative assay of MCF-7 cells, which are oestrogen-responsive human breast cancer cells.

Materials and methods

Materials

The chemical compounds used were obtained from the following companies: CIPC (purity > 99.7%) from Hodogaya Chemical Industry (Tokyo, Japan); 3-chloroaniline (3CA), 3-chloroacetanilide (3CAA), 3-chloro-4-hydroxyaniline, 4-hydroxybenzoic acid butyl ester (butylparaben) and bisphenol A (BPA) (purities > 98%) from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan); 17 β -oestradiol (E₂), diethylstilbestrol (DES), β -glucuronidase, sulfatase (type VI, β -glucuronidase-free *Aerobacter aerogenes*), N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES) and bovine serum albumin from Sigma Chemical Co. (St Louis, MO, USA); collagenase and isopropyl chlorocarbonate (purity > 95%) from Wako Pure Chemical Industries Ltd (Osaka, Japan); and 3-chloro-4-hydroxybenzoic acid from Avocado Research Chemicals Ltd (Heysham, UK). All other chemicals were of the highest purity commercially available.

Isopropyl N-(3-chloro-4-hydroxyphenyl)carbamate (4OH-CIPC) was synthesized by stirring and refluxing 3-chloro-4-hydroxyaniline (20 mM) and isopropyl chlorocarbonate (10 mM) in a mixture (200 ml) of anhydrous diethylether and dichloromethane (50:50, by volume) at room temperature for 6 h. After the reaction mixture was filtered and evaporated, the residue dissolved in benzene was purified by chromatography on silica gel column with same solvent as eluent. Finally, the product (4OH-CIPC) was recrystallized in benzene and its purity was > 98%. The compound was identified by ¹³C-nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC/MS). ¹³C-NMR spectra were obtained with a model JNM-A500 spectrometer (JOEL Ltd, Tokyo, Japan) operated at 125 MHz. Chloroform-*d*₁ was used as a solvent and all chemical shifts (ppm) were referenced to the intrinsic value for the solvent. The assignments for ¹³C were as follows: carboxyl carbon, 153.5 (-COO-); aromatic carbon, 131.4 (C₁), 120.1 (C₂), 119.9 (C₃), 147.6 (C₄), 116.2 (C₅), and 119.5 (C₆); alkyl carbon, 68.9 (-COO-CH₂-), and 22.0 (-CH₃). The negative electrospray ionization (-ESI)/MS spectrum of 4OH-CIPC showed a peak at *m/z* 228, corresponding to [M - H]⁻, and also showed a fragment ion at *m/z* 141 ([M - H-COOC₃H₇]⁻).

3-Chloro-4-hydroxybenzoic acid butyl ester (3-chloro-butylparaben) was synthesized by stirring 3-chloro-4-hydroxybenzoic acid (10 mmol) in *n*-butyl alcohol (100 ml) added with concentrated sulfuric acid (1 ml) at room temperature for 12 h. The reaction mixture was washed twice by water (100 ml) saturated with *n*-butyl alcohol and neutralized with sodium hydrogen carbonate. After dehydration with anhydrous sodium sulfate, the butyl alcohol was evaporated to dryness. The residue dissolved in benzene was purified by chromatography on a silica gel column with benzene, and the purity of the product (3-chloro-4-hydroxybutylparaben) was > 99%. The assignments for ¹³C were as follows: carboxyl carbon, 167.0 (-COO-); aromatic carbon, 123.7 (C₁), 132.5 (C₂), 121.7 (C₃), 158.9 (C₄), 117.1 (C₅), and 130.7 (C₆); alkyl carbon, 65.8 (-COO-CH₂-), 31.9 (-CH₂-), 20.3 (-CH₂-), and 114.6 (-CH₃). The -ESI/MS spectrum of 3-chloro-butylparaben showed a peak at *m/z* 227, corresponding to [M - H]⁻, and also showed fragment ions at *m/z* 126 ([M - H-COOC₄H₉]⁻) and at *m/z* 170 ([M - H-C₄H₉]⁻), respectively.

Isolation and incubation of hepatocytes

Male F344/DuCrj (220–250 g) rats were obtained from Charles River Japan, Inc. (Yokohama, Japan) and were housed in wire-bottom cages. The rats were allowed food (CE-2, Clea Japan, Inc., Tokyo) and water *ad libitum* before hepatocytes were prepared. Hepatocytes (10⁶ cells ml⁻¹) were isolated by collagenase perfusion of the liver and suspended in Krebs–Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and 0.1% albumin, as described (Moldéus *et al.* 1978). All incubations were performed in rotating, round-bottomed flasks at 37°C, under a constant flow of humidified carbogen (95% O₂/5% CO₂). Reactions were started by the addition of CIPC or 4OH-CIPC dissolved in dimethyl sulfoxide (DMSO; final concentration < 0.5%). Corresponding control groups received an equivalent volume of DMSO. Aliquots of the incubation mixture were taken at intervals to monitor cell death and the concentrations of intracellular adenine nucleotides, and CIPC and its metabolites.

Biochemical and morphological assays

Adenine nucleotides (ATP, ADP and AMP) in hepatocytes were determined by a modification of a high-performance liquid chromatography (HPLC) method, as described by Jones (1981). Hepatocyte viability was assessed by Trypan blue (0.16%, w/v) exclusion under a light microscope, and initial cell viability in each experiment was more than 85%. The number of blebbed hepatocytes was counted by light microscopy and expressed as the percentage of Trypan blue excluding cells, which exhibited multiple surface protrusions.

Determination of CIPC metabolites by HPLC

An equal volume of cold methanol was added to the cell suspension and the mixture was filtered through a membrane cartridge (pore size of cellulose membrane, 0.45 μ m; 13 mm i.d. $\times$ 20 mm; Millex-SLHV, Millipore Co., Bedford, MA, USA). The eluate (20 μ l) was injected onto the Atlantis dC18

column (4.6 i.d. $\times$ 250 mm, 5 μ m particle size, Waters Co., Milford, MA, USA) equipped with a ultraviolet light absorbance detector (260 nm). The mobile phase was methanol/0.1 M ammonium dihydrogen phosphate (60:40, by volume, pH 5.3), and the flow rate was 1.0 ml min⁻¹. CIPC, 4OH-CIPC, 3CA and 3CAA were identified by co-chromatography with these authentic compounds or by LC/-ESI/MS. The recoveries for these compounds were checked by the addition of known amounts of authentic compounds to hepatocyte suspensions, and were more than 85%.

In other experiments, the hepatocyte suspensions were centrifuged to separate the cell fraction (cell pellet) from extracellular medium for 8 min at 100g. The concentrations of CIPC and its metabolites in the cell pellet, which was once washed with Krebs-Henseleit buffer and resuspended in the buffer after centrifugation, and the medium fraction were determined by HPLC method described above.

Conjugates derived from CIPC in the cell suspensions were enzymatically hydrolysed with β -glucuronidase or β -glucuronidase-free sulfatase and the hydrolytic products were identified and/or determined by HPLC. Briefly, aliquots (0.5 ml) of cell suspensions were treated with a cell disrupter (Sonifier Branson Sonic Power Co., Danbury, CT, USA) in ice water for 15 s and then filtered through an ultrafiltration cartridge (limit of elution < molecular weight 5000; Ultrafree-MC centrifugal filter unit, Millipore). The eluate adjusted to pH 4.5 with sodium acetate buffer (0.2 M) was incubated with β -glucuronidase (about 400 U) or sulfatase (about 2.5 U) for 3 h at 37°C and then the mixture (20 μ l) was injected into the HPLC system.

Identification of metabolites derived from CIPC by mass spectroscopy coupled with HPLC

An equal volume of chilled methanol was added to the cell suspension (1 ml) and the mixture was centrifuged at 200g and 4°C for 5 min to obtain supernatant. The supernatant fraction was concentrated by solid-phase extraction using C₁₈ Sep-Pak cartridges (Waters), and the methanolic eluate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol and subjected to LC/-ESI/MS spectroscopy. The mass spectra of CIPC metabolites were obtained using a LC/MS system, consisting of a Waters mass spectrometer ZMD and a Waters Alliance 2690 pump (Waters). The column was eluted with a linear gradient of acetonitrile-water (solvents contained 1% v/v acetic acid); the gradient started at 20% acetonitrile and changed to 80% during 30 min and the flow rate was 0.2 ml min⁻¹. The MS spectrum by negative-ion mode was scanned from 100 to 500 daltons in 1.0 s and produced with a cone voltage of 40 or 50 V.

Oestrogen receptor competitive binding assay

Competitive binding between 17 β -oestradiol and various compounds was determined using an oestrogen-R(α) competitor screening kit (Wako Pure Chemical Industries Ltd, Osaka, Japan); the kit consists of recombinant human oestrogen receptor- α (ER α) coated on the bottom of 96-well multiwell plates and fluorescein-labelled 17 β -oestradiol as the competitor for the assay. DES and other compounds dissolved in DMSO were added to fluorescein-labelled 17 β -oestradiol, and the mixture (100 μ l) was added into each well. The concentrations of the various compounds used in this study ranged from 5 $\times$ 10⁻¹⁰ to 10⁻³ M. After 2 h of incubation at room temperature, the mixture, containing free compounds or the 17 β -oestradiol unbound to ER α , in wells was aspirated and exchanged with the assay solution (100 μ l). The concentration of fluorescein-labelled 17 β -oestradiol bound to ER α on the bottom was measured in a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA, USA) with filters set for 485 nm excitation and 535 nm emission. The results are expressed as percentages of the fluorescences for the reaction solution without test samples.

MCF-7 cell proliferation assay

MCF-7 cells (cultured human breast cancer cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in phenol red free-RPMI-1640 medium, supplemented with 5% foetal calf serum (FCS), 15 mM HEPES, 50 U ml⁻¹ penicillin, 50 μ g ml⁻¹ streptomycin and 10 ng ml⁻¹ insulin at 37°C with 5% CO₂ in air at saturating humidity, and were routinely precultured at approximately 80% confluence. Before initiating experiments, cells were seeded in and attached to 96-well multiwell plates at 4 $\times$ 10³/well in 0.3 ml RPMI-1640 medium, supplemented with 5% oestrogen-free FCS. After 24 h, the medium was replaced with the same volume of the above medium containing 17 β -oestradiol (1 nM) as a positive control, CIPC or its metabolites: the concentrations of compounds used in this study ranged from 1 nM to 100 μ M. Oestrogen-free FCS was prepared using a dextran-charcoal procedure (Soto *et al.* 1995). The cells were cultured for 6 days and cell numbers in each well were determined using a cell proliferation assay kit (cck-8; Dojindo Laboratories Co., Kumamoto, Japan); 20 μ l WST-8 solution, 5 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetra-zolium monosodium salt and 0.2 mM 1-methoxy phenazinium methyl-sulfate dissolved in 150 mM KCl were added to wells containing 0.3 ml medium with cells. After incubation for 2 h at 37°C in a humidified 5% CO₂ atmosphere, the absorbance of the well was read at 450 nm (reference wavelength, 650 nm) by a microculture plate reader (model 450; Bio-Rad Laboratories, New York, NY, USA). The assay, a modified tetrazolium colorimetric assay, was based

on metabolic reduction of WST-8 to its corresponding formazan and the absorbance obtained was directly proportional to the number of viable cells. The absorbance was linear for up to 120 min after addition of the dye-containing medium, with cell numbers exceeding 3×10^4 .

Statistical analysis

Statistical analysis was performed by one-way analysis of variance, followed by Dunnett's multiple comparison test. Statistical significance was assumed at $p < 0.05$.

Results

Toxic effects of CIPC on rat hepatocytes

The exposure of rat hepatocytes to CIPC caused concentration- (0.25–1.0 mM) and time-dependent (0–3 h) cell death accompanied by a depletion of intracellular ATP and total adenine nucleotides (the sum of ATP, ADP and AMP) and an increase in multiple surface protrusions, indicating cellular morphological injury (figure 1). Although the cells that formed blebs were not dead, as assessed by Trypan blue exclusion test, they appeared to be initiating a necrotic process (Thor *et al.* 1984, Nicotera *et al.* 1986, Lemasters *et al.* 1987). CIPC 1.0 mM elicited a rapid depletion of total nucleotide pools with abrupt lethality, whereas the loss of

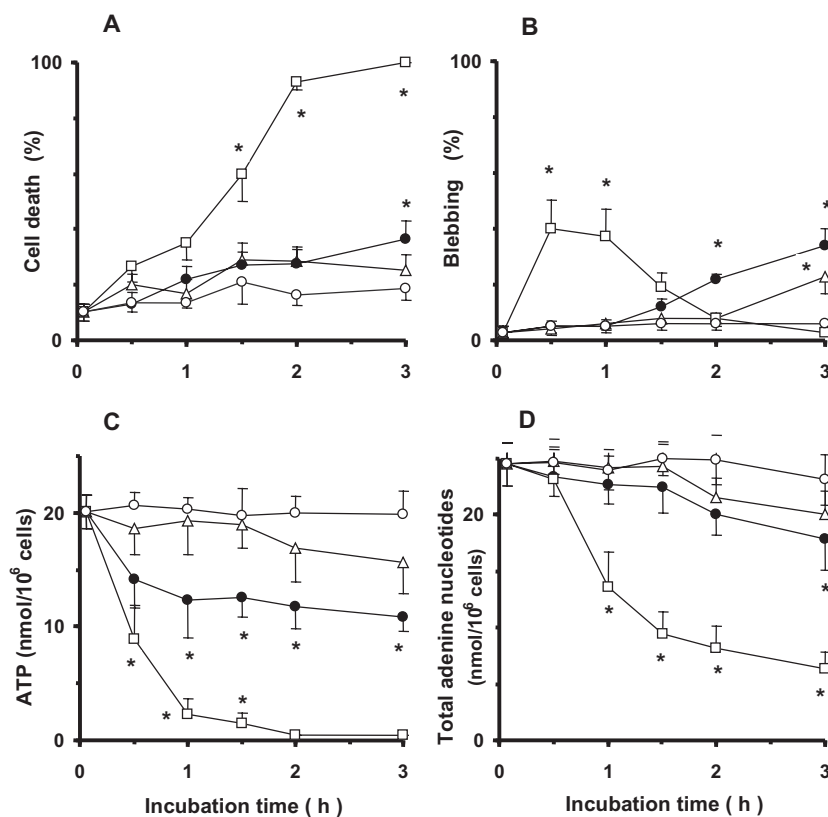


Figure 1. Effects of CIPC on cell death (A), blebbing (B), intracellular levels of ATP (C) and total adenine nucleotides (D) in isolated rat hepatocytes. Hepatocytes were incubated in 10^6 cells mL^{-1} in Krebs–Henseleit buffer, pH 7.4, with no addition (○) or 0.25 (△), 0.5 (●) and 1.0 mM (□), as described in the Materials and methods. Results are expressed as the means $\pm$ SE of three experiments. *Significant differences between the control group and treated groups ($p < 0.05$).

ATP was reflected by concomitant increases in the levels of ADP and AMP; at 1 h, the mean levels of intracellular ATP, ADP and AMP were 2.1, 5.9 and 5.6 nmol 10^{-6} cells, respectively.

Metabolism of CIPC in isolated rat hepatocytes

As shown by the HPLC elution profiles (figure 2), CIPC (retention time [R_t] approximately 21 min) at concentration of 0.25 mM was converted to several metabolites during a 1.5-h incubation. The metabolites eluted in peaks a, b and c were identified as 4OH-CIPC (R_t 7.5 min), 3CA (6.8 min) and 3CAA (7.9 min), which are known as metabolites of CIPC in rat urine and rat hepatocytes (Fang *et al.* 1974, Carrera *et al.* 1998), by co-chromatography with these authentic compounds or by LC/-ESI/MS. The metabolites eluted in peaks d (R_t 3.8 min) and e (4.6 min) were enzymatically hydrolysed with β -glucuronidase and sulfatase, respectively, to yield free 4OH-CIPC, indicating that the metabolites were a glucuronide (peak d) and a sulfate (e) of 4OH-CIPC. Further, the conjugates were identified as a monoglucuronide and a monosulfate by LC/-ESI/MS (figure 3); the-ESI/MS spectrum of peak d showed a peak at m/z 404, corresponding to $[M - H]^-$. The MS spectrum of peak d also showed a fragment ion at m/z 228, which

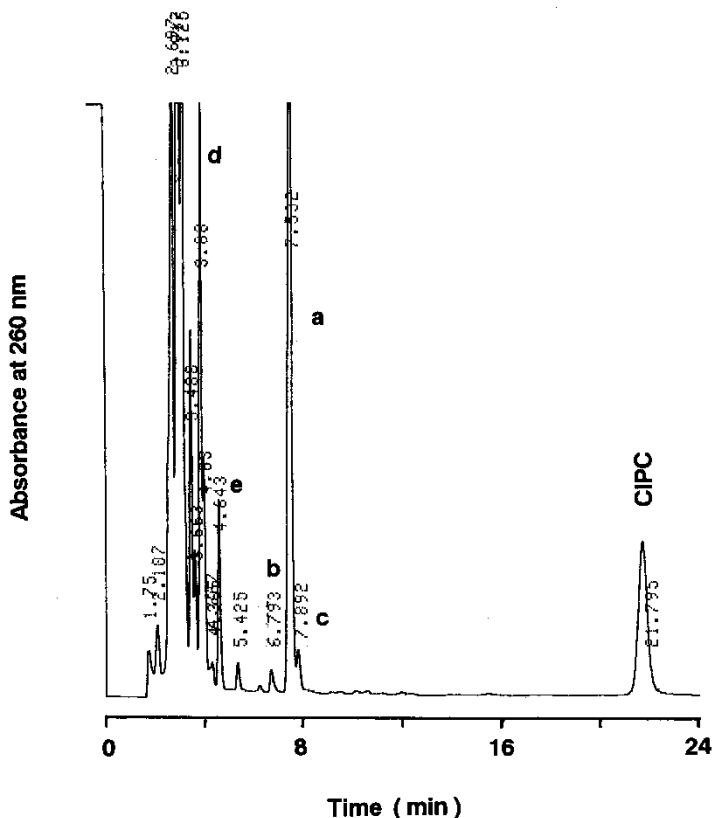


Figure 2. Typical HPLC elution profile of metabolites in hepatocyte (10^6 cells ml^{-1}) suspensions treated with 0.25 mM CIPC for 90 min. Peaks are the glucuronide of 4OH-CIPC (d; retention time [R_t], 3.8 min), sulfate of 4OH-CIPC (e; R_t , 4.6 min), 3CA (b; R_t , 6.8 min), 4OH-CIPC (a; R_t , 7.5 min), 3CAA (c; R_t , 7.9 min) and CIPC (R_t , 21.8 min).

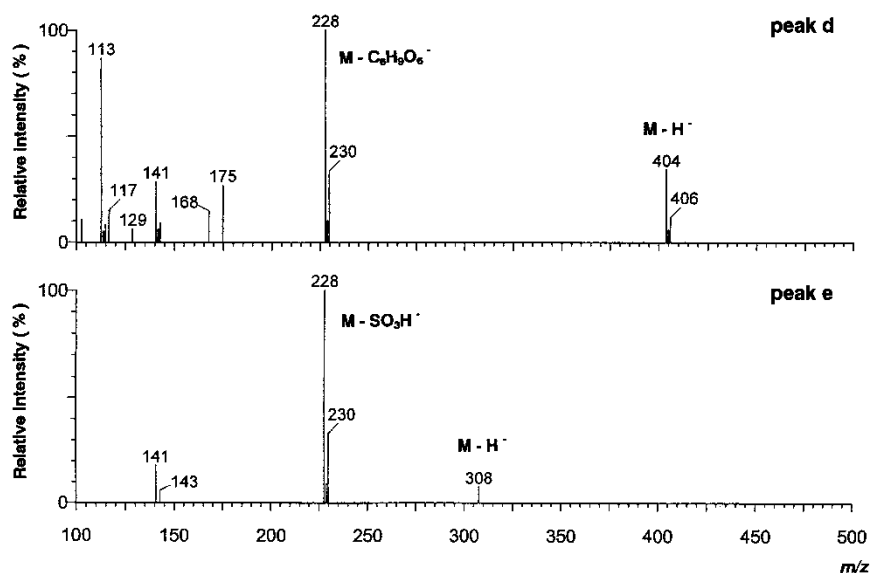


Figure 3. Typical -ESI mass spectra of peaks d and e in figure 2.

corresponds to a loss of 177 (glucuronyl moiety; 177 amu), $[M - C_6H_9O_6]^-$. The fragment ions at m/z 113 and 117 may be derived from glucuronyl moiety. In addition, the MS spectrum of peak e showed a base peak at m/z 228, which corresponds to a loss of 81 (sulfate moiety; 81 amu), $[M - SO_3H]^-$ and fragment ions at m/z 308 ($[M - H]^-$) and 141, which corresponds to a loss of 87 ($[M - SO_3H-COOCH(CH_3)_2]^-$). Based on these results, peaks d and e were tentatively identified as a monoglucuronide and a monosulfate conjugate of 4OH-CIPC, respectively.

To understand the metabolism of CIPC in rat hepatocytes, time courses for the change in levels of CIPC and its metabolites were investigated at a weakly toxic (0.25 or 0.5 mM) or a highly toxic (1.0 mM) level of CIPC (figure 4). At concentrations of 0.25 and 0.5 mM, the amounts of glucuronide and sulfate of 4OH-CIPC increased with time, followed by a loss of CIPC, whereas the amounts of conjugates derived from CIPC 1.0 mM were less than those from CIPC (0.25 and 0.5 mM). CIPC at a concentration of 0.25 mM was converted to free 4OH-CIPC, whose concentration temporarily reached a maximum level after 0.5 h and decreased thereafter. On the other hand, the intermediate derived from CIPC (0.5 mM or 1.0 mM) increased with time and reached an apparent steady state at approximately 120–130 μ M within 1–1.5 h. When CIPC (0.5 mM) was added to hepatocyte suspensions, approximately the majority (0.420 mM) disappeared by 3 h, total concentrations of these metabolites reached approximately 0.356 mM. Because CIPC (0.25 mM) added in Krebs–Henseleit buffer without hepatocytes was stable during a 3-h incubation, 3CA as a minor intermediate may be produced by enzymatic hydrolysis. These results indicate that in rat hepatocytes CIPC is readily metabolized, mainly by hydroxylation at the 4-position of the aromatic ring, and then converted to its glucuronide and/or sulfate conjugates. There were other minor or trace metabolites found in HPLC, and the materials ($RT < 3.8$ min) eluted before peak d resisted hydrolysis by sulfatase and/or β -glucuronidase (data not shown).

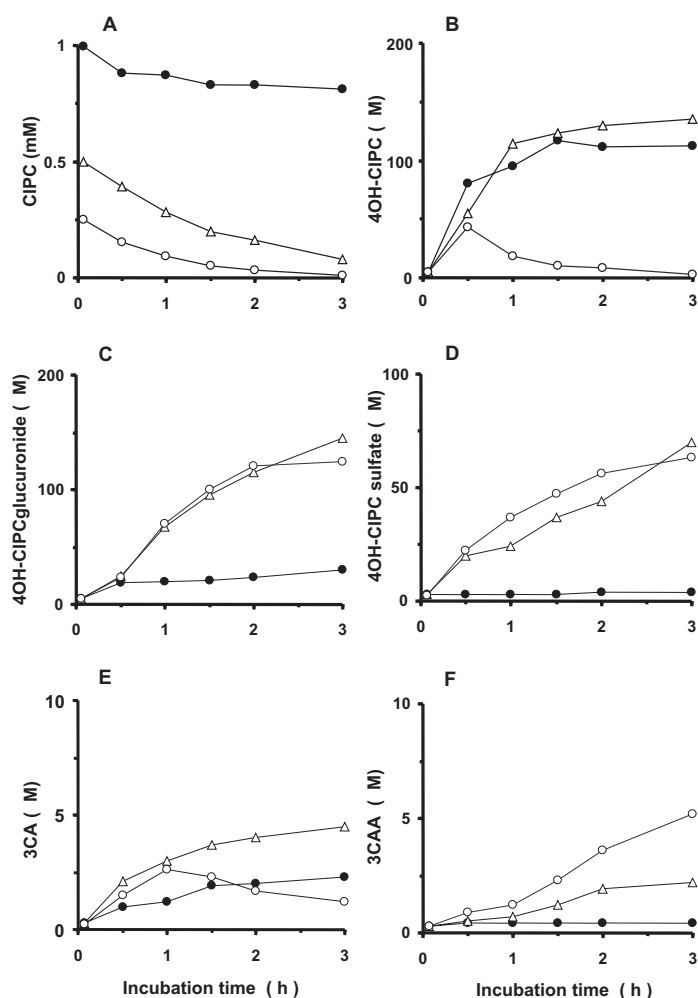


Figure 4. Changes in the levels of CIPC (A) and its metabolites, 4OH-CIPC (B), glucuronide of 4OH-CIPC (C), sulfate of 4OH-CIPC (D), 3CA (E) and 3CAA (F), in rat hepatocyte suspensions. Hepatocytes (10^6 cells ml^{-1}) were incubated with 0.25 (○), 0.5 (△) and 1.0 mM (●) CIPC for 3 h. Results are the means of two or three experiments.

Efflux and influx of metabolites of CIPC in hepatocytes

Distribution of CIPC and its metabolites between the hepatocyte fraction and the extracellular medium fraction is shown in figure 5. After the addition of CIPC (0.25 mM) to hepatocyte suspensions, the compound was distributed equally between hepatocytes and the medium and decreased at similar time-dependent rates. Although the glucuronide and/or sulfate of 4OH-CIPC predominantly increased in the medium fraction with time, the amounts of unconjugated 4OH-CIPC in the medium fraction temporarily reached about threefold that in the hepatocyte fraction after 0.5 h. The metabolism of 4OH-CIPC (0.25 mM) itself, which did not cause acute cell death during a 3-h incubation (cell death in untreated cells, $20 \pm 4\%$; cell death in treated cells, $22 \pm 5\%$), took place in hepatocytes, accompanied by rapid formation of glucuronide and sulfate (figure 6). 4OH-CIPC added in Krebs–Henseleit buffer without hepatocytes was stable

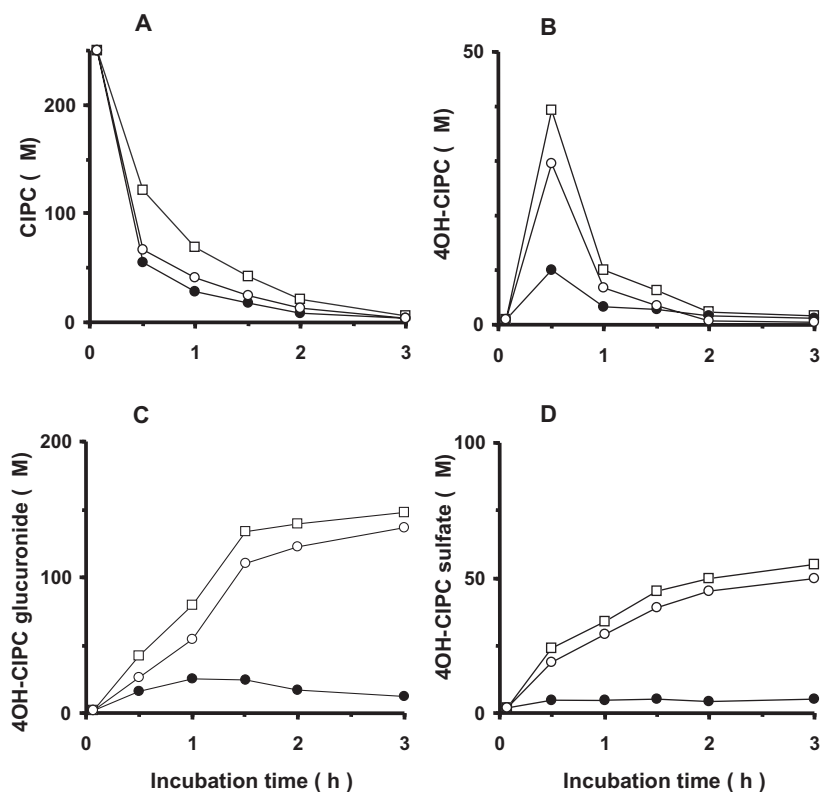


Figure 5. Distribution of CIPC (A) and its metabolites, 4OH-CIPC (B), glucuronide of 4OH-CIPC (C) and sulfate of 4OH-CIPC (D) between hepatocyte fraction and extracellular medium fraction. Hepatocytes (10^6 cells ml^{-1}) were incubated with 0.25 mM for 3 h. Results are the means of two or three experiments. Hepatocyte fraction ($\bullet$), extracellular medium fraction ($\circ$), total fraction ($\square$).

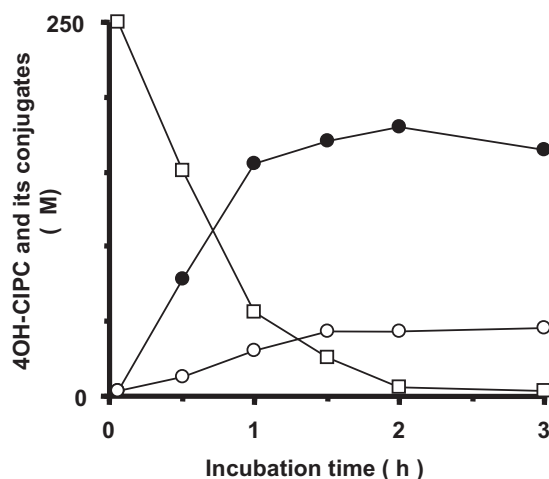


Figure 6. Changes in the levels of 4OH-CIPC ($\square$) and its metabolites, glucuronide of 4OH-CIPC ($\bullet$) and sulfate of 4OH-CIPC ($\circ$), in rat hepatocyte suspensions. Hepatocytes (10^6 cells ml^{-1}) were incubated with 0.25 mM 4OH-CIPC for 3 h. Results are the means of two experiments.

during a 2-h incubation. These results indicate that a substantial amount of 4OH-CIPC produced in hepatocytes was temporarily excreted in the medium and after re-influx, was conjugated to the glucuronide and sulfate derivatives.

Competitive binding assay of CIPC and its metabolites for oestrogen receptor α (ER α)

To compare affinities among CIPC, 4OH-CIPC, 3CA and known xenoestrogenic compounds, DES, BPA and butylparaben, for binding to the ER, the ability of these compounds to bind to ER α was assayed and compared with that of fluorescein-labelled 17 β -oestradiol (figure 7). Chemical structures of butylparaben, 3-chloro-butylparaben, BPA and DES are shown in figure 8. Butylparaben as

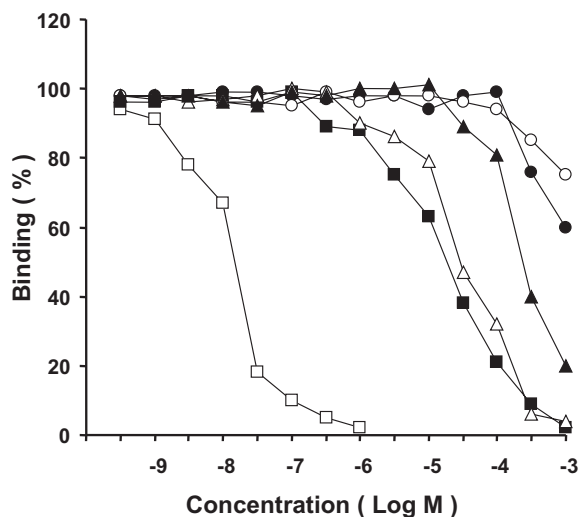


Figure 7. Competitive binding assay of CIPC, its metabolites and known xenoestrogenic compounds, diethylstilbestrol (DES), bisphenol A (BPA) and butylparaben, for human recombinant oestrogen receptor- α (ER α). The ability of these compounds to bind to ER α was via competition with that of fluorescein-labelled 17 β -oestradiol. Results are means from three determinations. CIPC (○), 4OH-CIPC (●), DES (□), BPA (■), butylparaben (△), 3-chloro-butylparaben (▲).

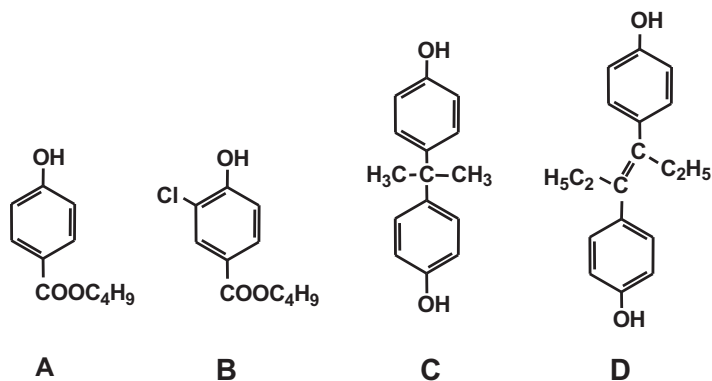


Figure 8. Chemical structures of butylparaben (A), 3-chloro-butylparaben (B), bisphenol A (C) and diethylstilbestrol (D).

well as CIPC are aromatic compounds with a long side chain. DES displaced fluorescein-labelled 17β -oestradiol bound to $ER\alpha$ with a high affinity, while BPA, butylparaben and its chlorinated derivative 3-chloro-4-hydroxybenzate butyl ester (3-chloro-butylparaben) displaced 17β -oestradiol bound to the receptor in a competitive manner. Although neither CIPC nor 4OH-CIPC at concentrations from 10^{-9} to 10^{-4} M impaired the binding of 17β -oestradiol to $ER\alpha$, at $> 5 \times 10^{-4}$ M the binding affinity of 4OH-CIPC was greater than that of CIPC. 3CA did not impair the binding at concentrations ranging from 10^{-9} to 10^{-3} M (data not shown). Fifty per cent inhibitory values (IC_{50}) of DES, BPA, butylparaben and 3-chloro-butylparaben were approximately 10^{-8} , 10^{-5} , 5×10^{-5} and 5×10^{-4} M, respectively. The binding affinity of butylparaben was higher than that of its chlorinated derivative.

Proliferative effects of CIPC and its metabolites on MCF-7 cells

Figure 9 shows the effects of CIPC and its metabolites, 4OH-CIPC and 3CA, on MCF-7 cells derived from oestrogen-responsive human breast cancer cells. The proliferative values were compared with those for untreated control cells. Relative to control (approximately 13.8×10^3 cells per well) on day 6, neither CIPC nor its metabolites at concentrations ranging from 10^{-9} to 10^{-5} M affected cell proliferation. The concentration of 17β -oestradiol is typical of a positive control in assays evaluating the oestrogenicity of the above compounds, and the proliferation induced by oestradiol in this study is consistent with previous studies (Soto *et al.* 1997, Breinholt and Larsen 1998). On the other hand, all compounds at a concentration of 10^{-4} M induced a considerable decrease in cell number relative to the control; viable percentages of MCF-7 cells exposed to CIPC, 4OH-CIPC and 3CA were approximately 52, 51 and 54% of untreated cells, respectively. Thus, this indicates that these compounds at a higher concentration caused toxic and/or growth inhibitory effects on MCF-7 cells.

Discussion

Cytotoxicity and metabolism of CIPC in rat hepatocytes

The results show that in isolated rat hepatocytes, CIPC induced a concentration- and time-dependent loss of cell viability, followed by decreases in intracellular levels of ATP and total adenine nucleotide pools. In addition, a high-concentration of CIPC (1.0 mM) caused an abrupt increase in multiple surface protrusions: bleb formation appears to be a prominent feature of most forms of liver injury, regardless of origin, and has already been described in hepatic injury (Thor *et al.* 1984). The cytotoxicity of CIPC may depend on the initial concentration rather than on that of its major intermediate 4OH-CIPC, because (1) there is no good correlation between cytotoxicity and 4OH-CIPC levels (figures 1 and 4), (2) the exposure of hepatocytes to 4OH-CIPC at concentration of 0.25 mM did not elicit acute cell death during a 3-h incubation (figure 6) and (3) at a concentration of 0.5 mM, intracellular ATP levels in CIPC-treated hepatocytes were lower than those in 4OH-CIPC- and 3CA-treated hepatocytes in a 3-h incubation (data not shown). In connection with this, Cerrera *et al.* (1998) also reported a similar result showing that inhibition of either sulfation or glucuronidation, which was accompanied by the loss of unconjugated free 4OH-CIPC, did not affect the cytotoxicity

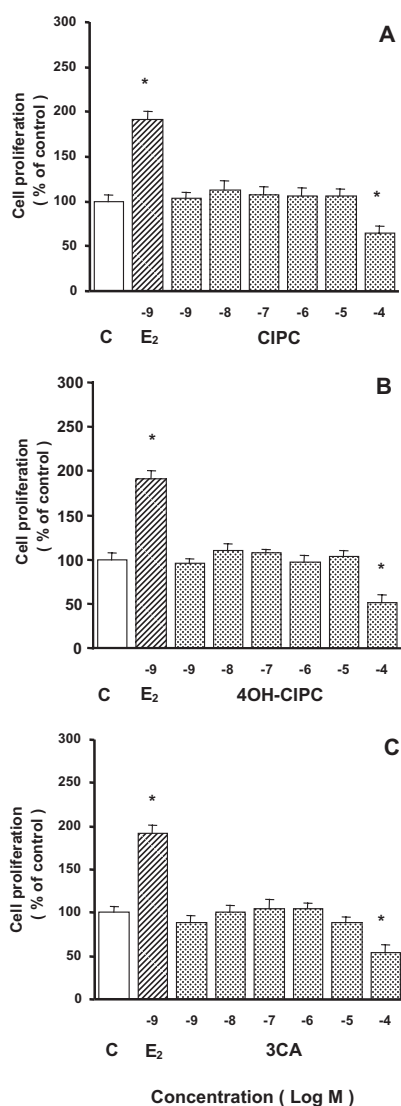


Figure 9. Effects of CIPC (A) and its metabolites, 4OH-CIPC (B) and 3CA (C), on the growth of MCF-7 cells. These compounds were tested at concentrations ranging from 10^{-9} M (−9) to 10^{-4} M (−4) for 6 days. Results are the means $\pm$ SE of three or four determinations. C and E₂ indicate untreated control and 17 β -oestradiol (10^{-9} M)-treated cells, respectively. *Significant differences between the control group and treated groups ($p < 0.05$).

of CIPC. On the other hand, it is interesting that at a concentration of 0.5 mM, CIPC was slightly cytotoxic at later time points, which suggests that cellular interactions due to its initial concentration, even if some of it was detoxified by phase I and II reactions, play an important role in the onset of irreversible lethal alteration of cell function. These results and findings confirm, at least in part, that the liver is a target organ of CIPC in rats (Fujitani *et al.* 2000, Van Engelen 2001).

At weakly toxic levels (<0.5 mM), CIPC is rapidly converted to at least three major metabolites: 4OH-CIPC via hydroxylation at the 4-position of the aromatic ring and conjugates of 4OH-CIPC via glucuronidation or sulfation in rat

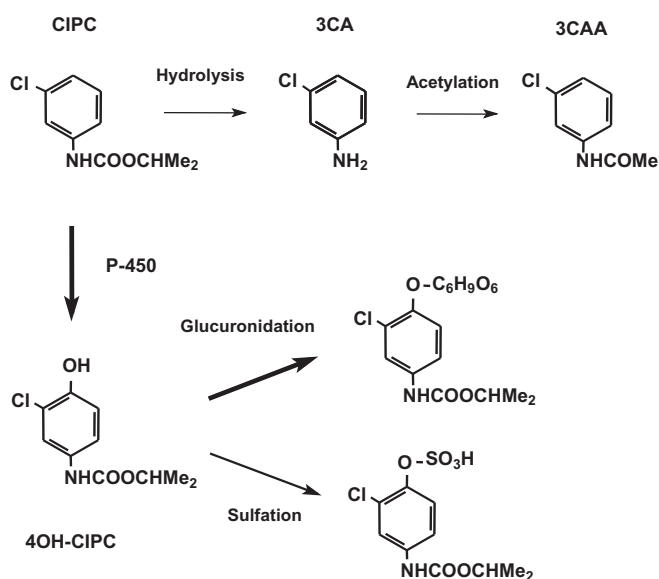


Figure 10. Proposed metabolism of CIPC in rat hepatocytes. Me, methyl group.

hepatocytes (figure 10). Glucuronide or sulfate conjugation is a major metabolic pathway in the biotransformation and detoxification of xenobiotics such as phenols and related compounds, and endobiotics in mammals (Jakoby *et al.* 1980, Kasper and Henton 1980). CIPC, which is more lipophilic than 4OH-CIPC, when added to hepatocyte suspensions was almost equally distributed between the extracellular medium fraction and the hepatocyte fraction. At a low concentration of CIPC (0.25 mM), the compound in the medium fraction was taken up by hepatocytes over time, and a large portion of unconjugated 4OH-CIPC as well as 4OH-CIPC-glucuronide and -sulfate was temporarily discharged from hepatocytes to the medium during initial 30 min (figure 5). However, all of the intermediate in the medium was effectively and rapidly conjugated by glucuronide and/or sulfate via re-influx. With the addition of 4OH-CIPC (0.25 mM) to hepatocyte suspensions, it was converted rapidly to its glucuronide and sulfate forms (figure 6). The results indicate that although the pass-through mechanism of 4OH-CIPC by either specific transporters or passive diffusion is not clear, 4OH-CIPC as well as the parent compound may easily permeate the cell membrane. On the other hand, 3CA and its acetylated derivative 3CAA were identified as minor metabolites. Because CIPC itself was stable in Krebs–Henseleit buffer without hepatocytes under carbogen during a 3-h incubation, it appears that 3CA is produced by enzymatic hydrolysis of the NHCO-linkage and further converted to 3CAA. Further studies are necessary to determine the mechanisms of CIPC-induced cytotoxicity.

Oestrogen-like activity of CIPC and its metabolites

It is well established that hydrophobicity, as demonstrated by its close correlation with the partition coefficient of xenobiotics, is often associated with biological action, which is expressed as a structure–toxicity or –activity relationship (Hansch *et al.* 1995). The *n*-octanol/water partition coefficients ($\log p$), a physical property used extensively to describe the lipophilicity or hydrophobicity

of chemicals, of DES, BPA, and butylparaben are approximately 5.07, 3.64 and 3.20, respectively (Tang-Liu *et al.* 1992, Meylan and Howard 1995, Interactive LogKow n.d.). Addition of chlorine at the 3-position on the aromatic ring of butylparaben increases its log *p* by about +0.64, whereas chlorination decreases the oestrogenic potency, as assessed by competitive binding assay (figure 7). Although it is apparent that the existence of a hydroxyl group in the aromatic ring is necessary for the onset of oestrogenic effects (White *et al.* 1994), the hydroxylation at the 4-position on the aromatic ring of CIPC did not produce any marked increase in oestrogenic potency to increase markedly (figures 7 and 9) accompanying a decrease in its hydrophobicity (log *p*, about -0.46). Thus, these results suggest that even if the hydrophobicity of 4-hydroxyphenyl with a side chain such as alkyl or carbamoyl group increases due to chlorination at the 3-position, the reaction product decreases the oestrogenic potency. Based on the relative IC₅₀ values, the binding potency for ER α is DES $\gg$ BPA, butylparaben > 3-chlorobutylparaben > 4OH-CIPC, CIPC.

Recently, the xenoestrogenicity of BPA, alkylphenols, and other phenolic compounds has been demonstrated in a number of *in vitro* and *in vivo* assays (Gaido *et al.* 1997, Vom Saal *et al.* 1998). In particular, it is known that phenolic compounds with an alkyl group at the *para* (or 4)-position on the aromatic ring stimulate the proliferation of MCF-7 cells (Routledge and Sumpter 1997). For example, acetamidophenol (paracetamol or acetaminophen) significantly increases the proliferation of MCF-7 cells, and the relative potency of its isomers in stimulating the proliferation of cells is *p*->*m*->*o*-acetamidophenol, indicating that the position of the hydroxy group on the aromatic ring influences the oestrogenic effect (Routledge *et al.* 1998, Harnagea-Theopilus *et al.* 1999). In addition, the onset of oestrogenic effects of monophenyl and biphenyls is associated with hydroxylated intermediates produced by microsomal cytochrome P450 monooxygenase, since the present authors have reported that the oestrogenic effects of *trans*-anethole (1-methoxy-4-(1*E*)-1-propenylbenzene), benzophenone and 4-methoxy-2-hydroxy-benzophenone were produced by their 4-hydroxylated intermediates, 4-hydroxy-1-propenylbenzene, 4-hydroxybenzophenone and 2,4-dihydroxy-benzophenone, respectively (Nakagawa *et al.* 2000, Nakagawa and Tayama 2001, Nakagawa and Suzuki 2002, 2003). All of these compounds as well as DES, BPA and butylparaben have a common structure with units of a 4-mono- or 4,4'-di-hydroxy group in the aromatic rings. Despite this, neither 4OH-CIPC nor the parent compound in concentrations ranging from 10⁻⁹ to 10⁻⁴ M exhibited a proliferative effect on MCF-7 human breast cancer cells with ER (figure 9). In a study of a structure-activity relationship, Miller *et al.* (2001) suggested that the major criteria for oestrogenic activity appear to be the presence of an unhindered phenolic OH group in the 4-position, because 3-methoxy-4-hydroxyallylbenzene (eugenol), an analogue of 4-hydroxy-1-propenylbenzene, did not exhibit oestrogenic activity in an *in vitro* yeast bioassay. This finding suggests that chlorine as well as a bulky moiety such as methoxy group adjacent to the 4-hydroxy group obstructs the interaction between xenobiotics and the ER and the affinity between ER and their compounds, and that these substitutions affect the onset of oestrogenic effects of a 4-hydroxylated aromatic compound. While experimental data on the oestrogenic potential of 4OH-CIPC-sulfate or -glucuronide were not obtained in this study, it can be assumed that these conjugates would not have the capability to interact with the oestrogen receptor, because neither

glucuronide nor sulfate derived from xenoestrogenic compounds bisphenol A and alkylphenols interacts with the ER and affects oestrogenic activity in MCF-7 cells (Certa *et al.* 1996, Matthews *et al.* 2001).

In conclusion, the results of present study show that the incubation of hepatocytes with CIPC causes a concentration- and time-dependent decrease in cell viability, accompanied by losses of intracellular ATP, adenine nucleotide pools, and that CIPC is converted enzymatically to the metabolites 4OH-CIPC and its glucuronide/sulfate, and 3CA. In *in vitro* oestrogenic assays, neither 4OH-CIPC nor CIPC affects the competitive binding to ER α at concentrations from 10^{-9} to 10^{-4} M and the proliferation of MCF-7 cells at concentrations from 10^{-9} to 10^{-5} M, respectively.

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Pharmacokinetics of *S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide in rats, a non-steroidal selective androgen receptor modulator

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1. *S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide (also known as *S*-4) is a non-steroidal selective androgen receptor modulator demonstrating tissue-selective androgenic and anabolic effects. The purpose of the present study was to examine the systemic pharmacokinetics, elimination and oral bioavailability of *S*-4 in rats.

2. Thirty-five male Sprague–Dawley rats weighing approximately 250 g were randomly assigned to one of seven treatment groups. Intravenous doses of 0.5, 1, 10, and 30 mg kg⁻¹ were given via a jugular catheter. Oral doses of 1, 10 and 30 mg kg⁻¹ were administered via gavage. Plasma concentrations were determined using a validated high-performance liquid chromatography or by a high-performance liquid chromatography/mass spectrometry method.

3. Clearances ranged between 1.0 and 2.1 ml min⁻¹ kg⁻¹ and varied with dose. The volume of distribution was approximately 0.448 l kg⁻¹ in all treatment groups. Oral bioavailability was also dose dependent, with the lower doses showing complete oral bioavailability. The half-life of *S*-4 over the dose range tested was between 2.6 and 5.3 h.

4. It was demonstrated that *S*-4 is rapidly absorbed, slowly cleared, and has a moderate volume of distribution in rats. The pharmacokinetics and oral bioavailability of *S*-4 indicate that it is an excellent candidate for clinical development.

Introduction

Synthesized steroidal androgens are used to treat a wide variety of conditions resulting from endogenous androgen deficiencies in both male and female patients. Unfortunately, virtually all current formulations have severe limitations (Wu 1992, Bhasin and Bremner 1997). Testosterone, in its pure form, suffers from low systemic bioavailability when administered orally (Handelsman *et al.* 1990). Testosterone esters (e.g. testosterone propionate, testosterone enanthate, testosterone cypionate and testosterone buciclate) were developed in order to achieve a more desirable pharmacokinetic profile. They are usually administered intramuscularly in oil. Since the esters are less polar than free testosterone, they are absorbed more slowly and therefore offer a prolonged duration of action (Wilson 1996). However, the esters produce erratic plasma concentrations of testosterone.

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17-Alkylated testosterone derivatives (e.g. methyltestosterone and oxandrolone) are orally bioavailable, but they cause unacceptable hepatic toxicity, are less efficacious and are, therefore, not recommended for long-term administration (Heywood *et al.* 1977, Ishak and Zimmerman 1987, Velazquez and Bellabarba Arata 1998). Another drawback of testosterone administration is the cross-reactivity of both testosterone and its metabolites with other steroid hormone receptors (Wilson *et al.* 1980, Bhasin and Bremner 1997).

The present authors' laboratory was the first to report the discovery of a series of non-steroidal agonists for the androgen receptor (AR). These ligands bind the AR with an affinity similar to that of testosterone, and they mimic the effects of known steroidal agonists on receptor-mediated transcriptional activation (Dalton *et al.* 1998). However, extensive hepatic metabolism and poor oral bioavailability severely limit the *in vivo* androgenic activity of these compounds (Yin *et al.* 2003).

It was recently demonstrated that a series of structurally optimized non-steroidal AR agonists elicit *in vivo* androgenic and anabolic effects upon parenteral administration to rats (Yin *et al.* 2003). *S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide (denoted S-4 herein and by Yin *et al.* 2003) emerged as the lead compound in this novel class of drugs. The structure of S-4 is shown in figure 1.

S-4 is a ligand for the AR with potent binding affinity that exhibits tissue-selective androgenic and anabolic effects in rats, identifying it as one of the first members of a new class of drugs known as selective AR modulators (SARMs). In castrated male rats, S-4 shows dose-dependent effects in the levator ani muscle. These effects are similar in potency and efficacy to those of testosterone propionate. However, S-4 is only a partial agonist in the prostate and seminal vesicles, restoring them to 33.8 and 28.2% of intact animals, respectively (Yin *et al.* 2003). These results clearly show that compound S-4 exhibits tissue-specific pharmacological activity and is a strong candidate for clinical development as the first non-steroidal SARM. Negro-Vilar (1999, p. 3460) defined the ideal anabolic SARM as 'orally active, ideally with a pharmacokinetic profile consistent with once a day administration, with anabolic effects on muscle and bone, but lesser activity in the prostate and seminal vesicles'. An orally active anabolic SARM would be a valuable therapeutic agent for a variety of indications including muscle wasting diseases, male hypogonadism, male fertility, and osteoporosis. Given its non-steroidal structure, it was hypothesized that S-4 would be rapidly and completely absorbed after oral administration and avoid some of the historical drawbacks of testosterone administration. Yin *et al.* (2003) administered S-4 via subcutaneous osmotic pumps. Therefore, the purpose of the present study was to examine the pharmacokinetics and oral bioavailability of S-4 in rats, and further to demonstrate the promising preclinical properties of a representative SARM.

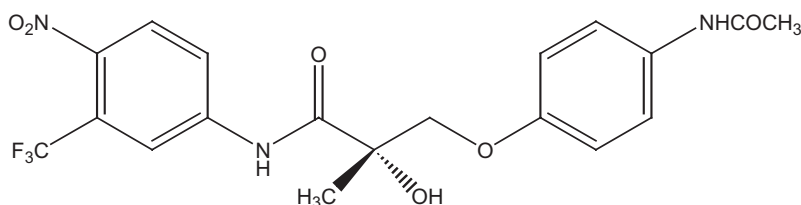


Figure 1. Structure of S-4.

Materials and methods

Animals

The Institutional Laboratory Animal Care and Use Committee (ILACUC) of The Ohio State University approved the study. Thirty-five male Sprague–Dawley® rats, weighing approximately 250 g, were purchased from Harlan (Indianapolis, IN, USA). Eighteen hours before dosing, the animals were catheterized in the right jugular vein, and food (Harlan Teklad 22/5 rodent diet) was removed. The animals were provided water *ad libitum* and weighed immediately before dose administration. Food was returned 12 h after dosing.

Chemicals and formulations

S-4 was synthesized using the methods of Marhefka *et al.* (2003). Chemical purity was confirmed using elemental analysis, mass spectrometry and proton nuclear magnetic resonance. Dosing solutions were prepared immediately before administration and consisted of a co-solvent mixture of polyethylene glycol-300 (Sigma Chemical Co., St Louis, MO, USA; Lot 30K0174) and ethanol (Pharmco Products, Inc., Brookfield, CT, USA; Lot T022001).

Study design

Animals were randomized into seven groups, with five animals per group. Intravenous (i.v.) doses (0.5, 1, 10, 30 mg kg⁻¹) were administered via the jugular catheter. Dosing solutions were prepared at an appropriate concentration to deliver the dose in a final volume of 0.2–0.3 ml. A 1 ml syringe graduated to 0.1 ml was used to deliver the dose volumetrically. After dose administration, the catheters were flushed with an aliquot (three times the volume of the administered dose) of sterile heparinized saline. Oral (p.o.) doses (1, 10, 30 mg kg⁻¹) were introduced directly into the stomach via oral gavage in 0.2–0.3 ml. These doses were chosen to represent the range of S-4 doses used during preclinical pharmacology, safety and toxicology studies.

Sample collection and preparation

Blood samples (about 250 µl each) were withdrawn from the jugular vein catheter before each dose and at 5, 10, 20, 30, 60, 120, 240, 480, 720 and 1440 min after i.v. doses of 0.5, 1 and 10 mg kg⁻¹, and before and at 30, 60, 90, 180, 240, 360, 480, 720, 1440 and 2160 min after dose administration for the 1 and 10 mg kg⁻¹ p.o. doses. For the 30 mg kg⁻¹ i.v. and p.o. dose groups, blood samples were drawn immediately before and at 20, 40, 60, 90, 120, 240, 360, 480, 720 and 1440 min after dose administration. A volume of heparinized saline (100 units ml⁻¹) equal to the volume of blood removed was infused following each blood draw. Blood samples were collected into 1.5-ml heparinized microcentrifuge tubes and placed on ice until the plasma was separated by centrifugation at 800g for 10 min at 4°C. Plasma samples were stored at -20°C until analysis. Metabolic cages were used to collect urine samples from the high-dose group over the 0–24 and 24–48 h following administration of the dose.

Plasma extraction for high-performance liquid chromatography (HPLC)

S-4 was extracted from plasma using a liquid/liquid extraction method. Aliquots of plasma (100 µl) were spiked with internal standard (2-ethyl-2-hydroxy-N-(4-nitro-3-trifluoromethyl-phenyl)-butyramide, MW 320, a structural analogue of S-4) and vortexed briefly. Plasma proteins were precipitated by the addition of acetonitrile (500 µl) and separated from the solution by centrifuging at 9300g for 2 min. The supernatant was aspirated into 12-ml glass extraction tubes and 1.0 ml phosphate buffered saline, pH 7.4, and 7.0 ml ethyl acetate were added. Extraction tubes were shaken horizontally at 180 oscillations min⁻¹ for 40 min on an Eberbach (Ann Arbor, MI, USA) reciprocating shaker. The mixture was then centrifuged at 1540g for 10 min to facilitate phase separation. The organic phase was removed, placed in a glass centrifuge tube and evaporated to dryness under nitrogen. The residue was reconstituted in 150 µl mobile phase, centrifuged at 9300g for 2 min to separate any residual particulate matter and 100 µl were injected into the HPLC.

HPLC plasma analysis

Plasma concentrations for the 10 mg kg⁻¹ i.v. dose group and the 30 mg kg⁻¹ i.v. and p.o. dose groups were determined using a validated HPLC method. HPLC analysis was performed on a Waters Chromatographic System (Waters Corporation, Milford, MA, USA) consisting of a model 510 pump, Nova-Pak® C₁₈ 3.9 × 150 mm × 4 µm column model 717 autosampler, and a model 486 ultraviolet light detector (Waters Corporation, Milford, MA, USA). The mobile phase consisted of acetonitrile:water (40:60) with 30 mM ammonium acetate at a flow rate of 1 ml min⁻¹. The eluent was monitored for ultraviolet light absorbance at 270 nm. Standard curves were linear ($R^2 \geq 0.989$) over the concentration range 1–150 µg ml⁻¹, and all quality control standards predicted values were within 85–115% of the actual concentration. Intra- and interday variabilities were less than 14.9 and 8.2%, respectively, at the lower limit of quantitation.

HPLC/mass spectrometry (MS) plasma and urine analysis

Plasma concentrations for the 0.5 and 1 mg kg⁻¹ i.v. doses and the 1 and 10 mg kg⁻¹ p.o. dose were determined using a validated HPLC/MS method. S-4 was extracted from plasma aliquots using a liquid/liquid extraction method. Aliquots of plasma (100 µl) were spiked with internal standard (deuterated compound S-4, MW 444) and vortexed briefly. Plasma proteins were precipitated by the addition of acetonitrile (500 µl) and separated from the solution by centrifuging at 16 100 g for 30 min. The supernatant was aspirated into 1.5-ml Eppendorf® tubes and evaporated to dryness under nitrogen. The residue was reconstituted in 150 µl mobile phase, centrifuged at 16 100 g for 5 min to separate any residual particulate matter, and 20 µl were injected into the HPLC/MS system column. HPLC analysis was performed on an Agilent 1100 Series System (binary pump, G1312A; degasser, G1379A; autosampler, G1367A; autosampler thermostat, G1330B; thermostatted column compartment, G1316A; Palo Alto, CA, USA) with a 2.1 × 50 mm, 5-µm particle size, Zorbax® SB-phenyl column (Agilent). The mass spectrometer was a single quadrupole mass selective detector (Agilent SL G1946D). Liquid chromatography (LC)/MS analyses were performed using an electrospray ionization source and the following conditions: negative single-ion mode; dry gas flow 101 min⁻¹; capillary voltage 2500 V; nebulizer pressure 25 psig; drying gas temperature 350°C; fragmentor voltage 174 V; peak width 0.15 min; time filter on; run time 12.3 min; post-run time 6 min; column temperature 28°C; flow rate 0.15 ml min⁻¹. A gradient mobile phase consisting of two solutions was used. Solution A was an aqueous solution containing 5% acetonitrile, 10 mM ammonium formate and 0.1% formic acid. Solution B contained 95% acetonitrile, 5% water, 10 mM ammonium formate and 0.1% formic acid. The percentage of solution B in the mobile phase increased linearly from 40 to 100% during the first 6 min of each chromatographic run, was maintained at 100% from 6 to 12.0 min, and then decreased linearly to 40% at 12.2 min for the remainder of the run. S-4 was identified as the ion with 440.3 *m/z* while the deuterated compound S-4 was identified as the ion with 443.3 *m/z*. Data acquisition was performed with Agilent ChemStation Software (Version A.08.04). Standard curves for plasma were linear ($R^2 \geq 0.984$) over the concentration range 0.005–0.050 µg ml⁻¹, and all quality control standards predicted values were within 85–115% of the actual concentration. Rat urine samples were centrifuged at 800 g for 10 min to separate particulates, and then filtered through a 0.22-µm syringe filter. Filtered rat urine samples were diluted 200 times with HPLC-grade water and a 15-µl aliquot was injected into the LC/MS. The running conditions for the urine samples were identical to those used for plasma analysis. The standard curve for urine specimens was linear over the range 0.005–0.5 µg ml⁻¹. Intra- and interday variabilities were less than 12 and 11%, respectively, at the lower limit of quantitation for these assays.

Pharmacokinetic data analysis

The plasma concentration–time data were analysed by non-compartmental methods using WinNonlin® (Version 3.1, Pharsight Corporation, Mountain View, CA, USA). The area under the plasma concentration–time curve from time zero to infinity (AUC) was calculated by the trapezoidal rule with extrapolation to time infinity. The terminal half-life ($T_{1/2}$) was calculated as $0.693 \lambda_z^{-1}$, where λ_z was the terminal phase rate constant. The plasma clearance (CL) was calculated as $CL = \text{dose}_{i.v.} / \text{AUC}_{i.v.}$, where $\text{dose}_{i.v.}$ and $\text{AUC}_{i.v.}$ are the i.v. dose and corresponding area under the curve from time 0 to infinity, respectively. The maximal plasma concentration ($C_{\max}$) and time at which it occurred ($T_{\max}$) for the p.o. doses were determined by visual inspection of the concentration–time profiles. The apparent volume of distribution at equilibrium (V_{ss}) was calculated using:

$$V_{ss} = \text{dose} \text{AUMC}_{0-\infty} (\text{AUC}_{0-\infty})^{-2}.$$

where $\text{AUMC}_{0-\infty}$ is the area under the first moment of the plasma concentration–time curve extrapolated to infinity. The mean residence time (MRT) was calculated using:

$$\text{MRT} = \text{AUMC}_{0-\infty} (\text{AUC}_{0-\infty})^{-1}.$$

Oral bioavailability (*F*) for each dose was calculated using:

$$F = \text{AUC}_{p.o.} \text{dose}_{i.v.} (\text{AUC}_{i.v.} \text{dose}_{p.o.})^{-1}$$

where $\text{dose}_{p.o.}$, $\text{dose}_{i.v.}$, $\text{AUC}_{i.v.}$ and $\text{AUC}_{p.o.}$ are the mean oral dose, mean i.v. dose, and the corresponding mean areas under the curve from time 0 to infinity, respectively. Oral bioavailability for the 300 mg kg⁻¹ p.o. dose was calculated using the CL of the 100 mg kg⁻¹ i.v. dose.

Statistical analysis

Statistical analyses were performed by single-factor analysis of variance (ANOVA). $p < 0.05$ was considered as statistically significant differences.

Results

Pharmacokinetics of compound S-4 after i.v. doses

S-4 achieved average maximal plasma concentrations of 1.6, 2.3, 28 and 168 $\mu\text{g ml}^{-1}$ following i.v. doses of 0.5, 1, 10 and 30 mg kg^{-1} , respectively (figure 2). The average steady-state volume of distribution for S-4 (0.45 l kg^{-1}) was slightly less than total body water (0.67 l kg^{-1}) (Davies and Morris 1993). CL remained relatively constant for the 0.5, 1 and 10 mg kg^{-1} doses at 1.92, 2.12 and 1.52 $\text{ml min}^{-1} \text{ kg}^{-1}$, respectively. However, the CL of S-4 was lower ($1.00 \text{ ml min}^{-1} \text{ kg}^{-1}$, $p < 0.05$) at the 30 mg kg^{-1} dose. Accordingly, the area under the plasma concentration-time curve increased proportionally with dose up to the 10 mg kg^{-1} dose, 0.24 mg min ml^{-1} at the 0.5 mg kg^{-1} dose, 0.46 mg min ml^{-1} at the 1 mg kg^{-1} dose, and 6 mg min ml^{-1} at the 10 mg kg^{-1} dose. However, at an i.v. dose of 30 mg kg^{-1} , the AUC increased disproportionately to 29 mg min ml^{-1} . Urinary excretion data showed that less than 0.15% of the drug was excreted unchanged, indicating that renal clearance of S-4 was negligible. The $T_{1/2}$ of S-4 was 154, 182, 223 and 316 min after doses of 0.5, 1, 10 and 30 mg kg^{-1} , respectively. MRT increased from 222 and 240 min at the 0.5 and 1 mg kg^{-1} doses to 305 and 423 min following the 10 and 30 mg kg^{-1} doses, respectively. Pharmacokinetic parameters for the i.v. doses are summarized in table 1.

Pharmacokinetics of S-4 after oral doses

S-4 achieved average maximal plasma concentrations of 1.4, 11 and 20 $\mu\text{g ml}^{-1}$ following p.o. doses of 1, 10 and 30 mg kg^{-1} , respectively (figure 3). The time to reach the maximal plasma concentration (T_{max}) was 48, 84, and 336 min for the 1, 10 and 30 mg kg^{-1} doses, respectively. S-4 was completely bioavailable for the 1 and 10 mg kg^{-1} doses. However, following the 30 mg kg^{-1} dose, the bioavailability of S-4 decreased to 57%. The $T_{1/2}$ of S-4 was 203, 173 and 266 min after doses of 1,

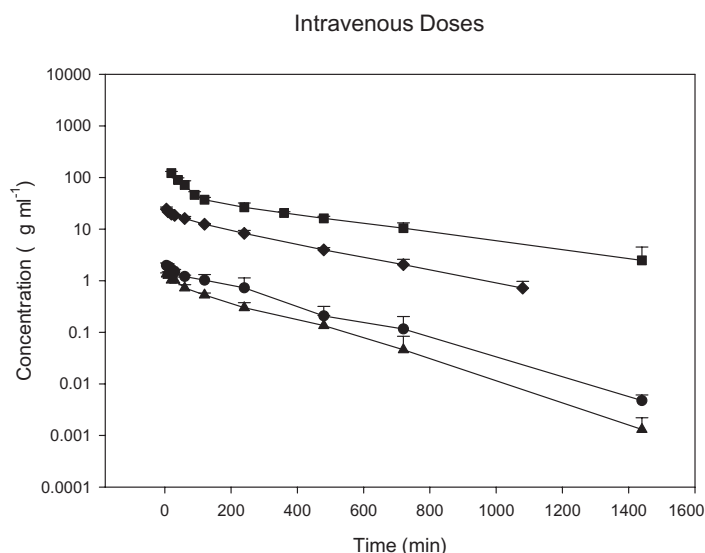


Figure 2. Mean plasma concentration-time profiles following intravenous doses of S-4 in male rats. Square, 30 mg kg^{-1} ; diamond, 10 mg kg^{-1} ; circle, 1 mg kg^{-1} ; triangle, 0.5 mg kg^{-1} . Data are the mean $\pm$ SD for $n = 5$ in each group.

Table 1. Pharmacokinetics of S-4 in rats after intravenous administration.

	0.5 mg kg ⁻¹	1 mg kg ⁻¹	10 mg kg ⁻¹	30 mg kg ⁻¹
CL (ml min ⁻¹ kg ⁻¹)	1.92 ± 0.20	2.12 ± 0.07	1.52 ± 0.12	1.00 ± 0.08
V _{ss} (l kg ⁻¹)	0.42 ± 0.06	0.48 ± 0.08	0.44 ± 0.04	0.42 ± 0.14
λ _z (min ⁻¹)	0.0045 ± 0.00046	0.0038 ± 0.00077	0.0031 ± 0.00044	0.0022 ± 0.0008
MRT (min)	222 ± 43	240 ± 45	305 ± 32	423 ± 158
AUC (mg min ml ⁻¹)	0.24 ± 0.03	0.46 ± 0.16	6.0 ± 0.5	28 ± 3

Data are mean ± SEM, with five animals per dose group.

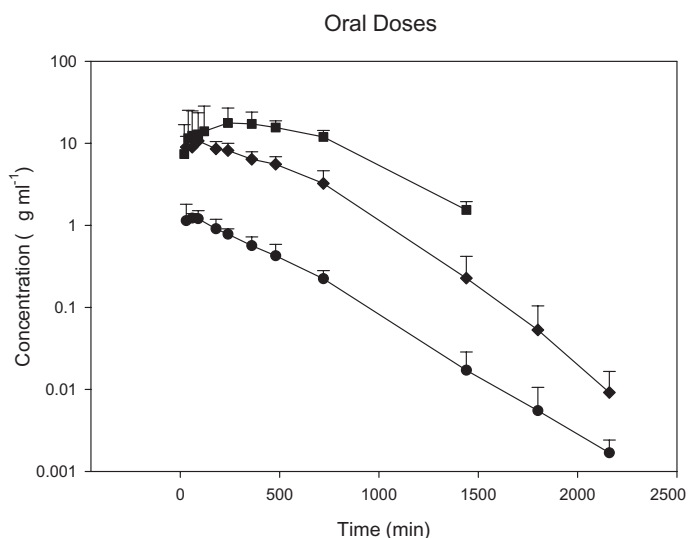


Figure 3. Mean plasma concentration-time profiles following oral doses of S-4 in male rats. Square, 30 mg kg⁻¹; diamond, 10 mg kg⁻¹; circle, 1 mg kg⁻¹. Data are the mean ± SD for *n* = 5 in each group.

Table 2. Pharmacokinetics of S-4 in rats after oral administration.

	1 mg kg ⁻¹	10 mg kg ⁻¹	30 mg kg ⁻¹
λ _z (min ⁻¹)	0.0034 ± 0.00032	0.004 ± 0.0004	0.0026 ± 0.00021
C _{max} (μg ml ⁻¹)	1.39 ± 0.49	11.32 ± 3.7	20 ± 11
T _{max} (μg ml ⁻¹)	48 ± 16	84 ± 61.5	336 ± 156
AUC (mg min ml ⁻¹)	0.55 ± 0.08	6.0 ± 1.6	16 ± 6
<i>F</i> (%)	120	100	57

Data are mean ± SEM, with five animals per dose group.

10 and 30 mg kg⁻¹, respectively. Pharmacokinetic parameters for the p.o. doses are summarized in table 2.

Discussion

Analysis of variance showed no significant difference in the CL of S-4 at doses of 0.5, 1 and 10 mg kg⁻¹ (*p* > 0.05). Previous *in vivo* studies in the present authors' laboratory showed that the dose required to restore the levator ani muscle weight in castrated animals, an indicator of anabolic activity, compared with that of intact

animals was less than $4 \text{ mg kg}^{-1} \text{ day}^{-1}$ (Yin *et al.* 2003). Thus, S-4 demonstrates linear pharmacokinetics within the dose range needed to exert maximal pharmacological effects.

The lack of parent drug in the urine suggests that S-4 is extensively metabolized. Assuming a hepatic blood flow of 13.8 ml min^{-1} in the rat (Davies and Morris 1993), the hepatic extraction ratio of S-4 would be less than 0.05. Based on this hepatic extraction ratio, a greater than 95% bioavailability (i.e. less than 5% of the drug would be removed by first-pass metabolism) is predicted. The present results confirmed this prediction, as S-4 was completely bioavailable following pharmacologically relevant doses (i.e. doses $\leq 10 \text{ mg kg}^{-1}$).

Although S-4 is structurally similar to bicalutamide, a clinically used anti-androgen, its total body clearance was quite different. In rats, bicalutamide is eliminated with a half-life of 17–21 h (Boyle *et al.* 1993). S-4 is cleared much more rapidly with an average half-life of about 4 h. The p.o. bioavailability of bicalutamide (61%) following a 25 mg kg^{-1} p.o. dose (Boyle *et al.* 1993) is nearly identical to the p.o. bioavailability of S-4 (57%) following the 30 mg kg^{-1} dose. Like bicalutamide, a very small percentage (0.15%) of S-4 is excreted unchanged in the urine. As a whole, these data suggest that the primary difference in pharmacokinetic parameters for S-4 and bicalutamide is a result of differences in metabolism and not other factors such as drug absorption, distribution or renal excretion.

The observed decrease in p.o. bioavailability and the prolonged time to peak plasma concentration observed after 30 mg kg^{-1} p.o. doses of S-4 were most likely due to the poor aqueous solubility of the compound (about $80 \mu\text{g ml}^{-1}$). Similar behaviour was noted for bicalutamide during preclinical pharmacokinetic studies (Boyle *et al.* 1993). Decreased p.o. bioavailability at higher doses may serve as a protective mechanism given the potential for abuse of an anabolic drug such as S-4. However, it is important to note that S-4 demonstrated high p.o. bioavailability and a short time to peak plasma concentration at doses capable of eliciting a maximal pharmacological effect (Yin *et al.* 2003).

A similar trend was observed in half-life following i.v. administration. As the dose was increased, λ_z decreased ($p < 0.001$) and the half-life of S-4 increased ($p < 0.01$). This observation also supports the conclusion that the CL of the drug was diminished at the highest doses. The CL of S-4 at a dose of 0.5 mg kg^{-1} ($1.92 \text{ ml min}^{-1} \text{ kg}^{-1}$) was significantly ($p < 0.001$) greater than that observed for the 30 mg kg^{-1} dose ($1.00 \text{ ml min}^{-1} \text{ kg}^{-1}$). These data suggest that saturation of the drug-metabolizing enzymes might be occurring at this higher dose. Therefore, one would expect to see further suppression of CL following doses greater than 30 mg kg^{-1} . However, due to the potency of S-4, the authors do not anticipate the need for such high doses during clinical use. Forthcoming data from the present authors' laboratory will provide needed information about the hepatic metabolism and pharmacokinetics of S-4 in this and other species.

The pharmacological activity and pharmacokinetics of S-4 in rats suggest that this compound has the properties of an ideal SARM as defined by Negro-Vilar (1999). It is rapidly absorbed following p.o. doses (t_{max} , 48–84 min), and it exerts tissue-specific anabolic effects *in vivo*, with anabolic effects in muscle and bone but lesser effects in the prostate and seminal vesicles (Kearbey *et al.* 2003, Yin *et al.* 2003). These properties coupled with forthcoming reports from the present authors' laboratory about the pharmacological effects of S-4 in other pertinent

animal models and its pharmacokinetics and metabolism in dogs and humans, favour the continued development of S-4 as an orally bioavailable non-steroidal SARM.

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Use of ^{19}F -nuclear magnetic resonance and gas chromatography-electron capture detection in the quantitative analysis of fluorine-containing metabolites in urine of sevoflurane-anaesthetized patients

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1. The use of fluorine-19 nuclear magnetic resonance (^{19}F -NMR) and gas chromatography-electron capture detection (GC-ECD) in the analysis of fluorine-containing products in the urine of sevoflurane-exposed patients was explored.

2. Ten patients were anaesthetized by sevoflurane for 135–660 min at a flow rate of 6 l min^{-1} . Urine samples were collected before, directly after and 24 h after discontinuation of anaesthesia.

3. ^{19}F -NMR analysis of the urines showed the presence of several fluorine-containing metabolites. The main oxidative metabolite, hexafluoroisopropanol (HFIP)-glucuronide, showed two strong quartet signals in the ^{19}F -NMR spectrum. HFIP concentrations after β -glucuronidase treatment were quantified by ^{19}F -nuclear magnetic resonance. Concentrations directly after and 24 h after discontinuation of anaesthesia were 131 ± 41 (mean $\pm$ SEM) and $61 \pm 19\text{ mol mg}^{-1}$ creatinine, respectively. Urinary HFIP excretions correlated with sevoflurane exposure.

4. Longer scanning times enabled the measurement of signals from two compound A-derived metabolites, i.e. compound A mercapturic acid I (CAMA-I) and compound A mercapturic acid II (CAMA-II), as well as products from β -lyase activation of the respective cysteine conjugates of compound A. The signals of the mercapturic acids, 3,3,3-trifluoro-2-(fluoromethoxy)-propanoic acid and 3,3,3-trifluorolactic acid were visible after combining and concentrating the patient urines. CAMA-I and -II excretions in patients were completed after 24 h.

5. Since ^{19}F -nuclear magnetic resonance is not sensitive enough, urinary mercapturic acids concentrations were quantified by gas chromatography-electron capture detection. CAMA-I and -II urinary concentrations were 2.3 ± 0.7 and $1.4 \pm 0.4\text{ mol mg}^{-1}$ creatinine, respectively. Urinary excretion of CAMA-I showed a correlation with sevoflurane exposure, whereas CAMA-II did not.

6. The results show that ^{19}F -nuclear magnetic resonance is a very selective and convenient technique to detect and quantify HFIP in non-concentrated human urine. ^{19}F -nuclear magnetic resonance can also be used to monitor the oxidative biotransformation of sevoflurane in anaesthetized patients. Compound A-derived mercapturic acids and 3,3,3-trifluoro-2-(fluoromethoxy)-propanoic acid and 3,3,3-trifluorolactic acid, however, require more sensitive techniques such as gas chromatography-electron capture detection and/or gas chromatography-mass spectrometry for quantification.

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Introduction

^{19}F -nuclear magnetic resonance (^{19}F -NMR) spectroscopy has been used extensively for the identification of fluorine-containing drugs and metabolites in biological media (reviewed by Martino *et al.* 2000). The advantages of ^{19}F -NMR include: (1) the high NMR sensitivity for the ^{19}F nucleus, (2) the broad ppm-scale and high resolution of ^{19}F -NMR, enabling the simultaneous analysis of fluorine-containing compounds of complex mixtures and (3) the lack of interfering background compounds, enabling analyses of biological samples without any sample treatment. The present study evaluated the potential of ^{19}F -NMR for quantitative analysis of fluorine-containing metabolites in the urine of patients exposed to the anaesthetic sevoflurane (fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl)ethyl-ether) and its alkaline degradation product, 2-[fluoromethoxy]-1,1,3,3,3-pentafluoro-1-propene (compound A).

The alkaline degradation of sevoflurane in the anaesthesia machine and further biotransformation is shown in figure 1. Cytochrome P450 catalyses sevoflurane oxidation at the fluoromethoxy-carbon to produce a transient intermediate that decomposes into the free fluoride and hexafluoroisopropanol (HFIP), which accounted for 82% of the organic fluorinated metabolites (Holaday and Smith 1981). Cytochrome P4502E1 appears to be the major isoform involved in sevoflurane oxidation (Kharasch *et al.* 1995a). Several analytical techniques have been developed to quantify free HFIP and HFIP-glucuronide in blood and urine. These include headspace gas chromatography for HFIP after hydrolyse by β -glucuronidase (Martis *et al.* 1981, Morgan *et al.* 1994) or by acid (Kharasch *et al.* 1995b) treatment, and direct ion-exchange chromatography for HFIP-glucuronide (Jiaxiang *et al.* 1993). Peak urinary excretion of HFIP-glucuronide, which has an excretion half-life of 55 h, occurs in the first 12 h after discontinuation of sevoflurane anaesthesia (Kikuchi *et al.* 1987, Jiaxiang *et al.* 1993).

It is now well established that sevoflurane is partially degraded by strong base in the carbon dioxide absorbents of anaesthesia machines, resulting in formation of compound A (Baxter *et al.* 1998). Concentrations of compound A during low-flow sevoflurane anaesthesia are 20–40 ppm (Bito and Ikeda 1994, Frink *et al.* 1994). During surgery, the inhaled concentrations of compound A are significantly higher than the exhaled concentrations, indicative for uptake by the patient (Frink *et al.* 1992, 1994). It has been shown that compound A is mainly metabolized to several glutathione (GSH) conjugates by conjugation with GSH. Following intraperitoneal injection of compound A, two alkane and two alkene glutathione conjugates were identified in bile. These GSH conjugates are subsequently degraded enzymatically to the corresponding cysteine *S*-conjugates (Jin *et al.* 1995, 1996). Subsequent N-acetylation, resulting in corresponding mercapturic acids, has recently been demonstrated in rats treated with compound A (Uttamsingh *et al.* 1998, Kharasch *et al.* 1999a), also in humans anaesthetized with sevoflurane (Kharasch and Jubert 1999b, Iyer *et al.* 1998). Furthermore, bioactivation of cysteine *S*-conjugates via renal cysteine conjugate β -lyase afford highly reactive, electrophilic species, which may bind covalently to cellular macromolecules or undergo hydrolysis to TFPA and partially to TFLA (Iyer and Anders 1996, 1997, Spracklin and Kharasch 1996).

Iyer *et al.* (1998) were the first to apply ^{19}F -NMR for the qualitative analysis of urines of humans anaesthetized with sevoflurane. Both HFIP and compound A-related metabolites were identified in urine of human volunteers exposed to

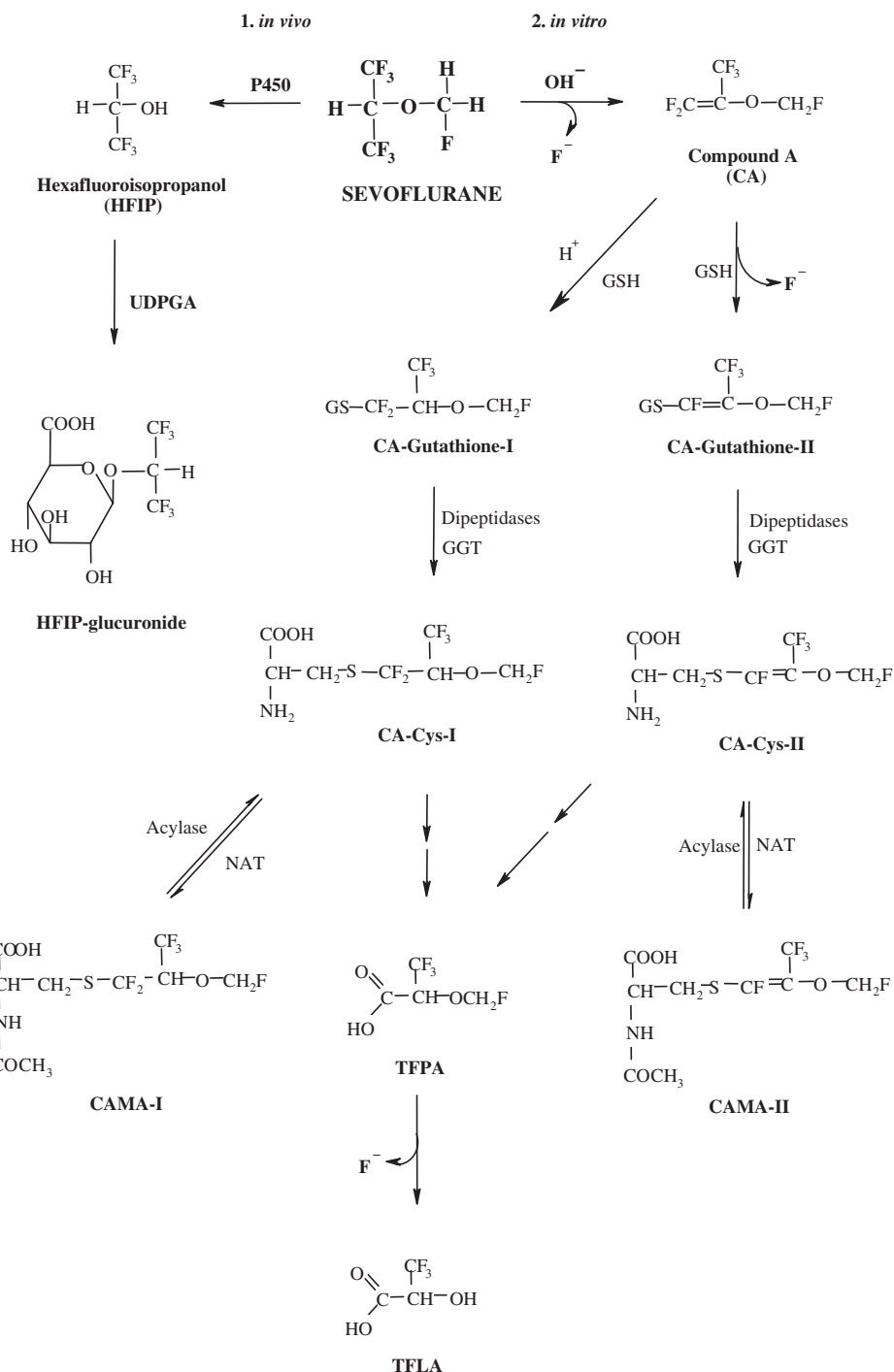


Figure 1. Sevoflurane P450-mediated biotransformation and alkaline degradation in the anaesthesia circuit and subsequent biotransformation via glutathione conjugation. CAMA-I, compound A mercapturic acid I; CAMA-II, compound A mercapturic acid II; CA-Cys-I, compound A cysteine conjugate I; CA-Cys-II, compound A cysteine conjugate II; NAT, N-acetyl transferase; TFLA, 3,3,3-trifluorolactic acid; TFPA, 3,3,3-trifluoro-2-(fluoromethoxy)-propanoic acid; UDPGA, uridinediphosphoglucuronic acid.

sevoflurane. The present study evaluated the use of ^{19}F -NMR to identify fluorine-containing metabolite products in the urine of patients exposed to sevoflurane and to quantify relative metabolite excretion to establish dose–excretion relationships. The applicability of gas chromatography-electron capture detection (GC-ECD) and gas chromatography-mass spectrometry (GC-MS) to analyse compound A-related metabolites was also evaluated.

Material and methods

Chemicals

Sevoflurane was supplied by Abbott Laboratories (Abbott Park, IL, USA). HFIP was obtained from Lancaster (Eastgate, UK). N-acetyl-L-cysteine and benzyltrimethylammonium hydroxide (40 wt% solution in water) were purchased from Aldrich Co. (Stenheim, Germany). β -glucuronidase (Type H-1 from *Helix pomatia*) was obtained from Boehringer (Mannheim, Germany). N-acylase, uridine diphosphoglucuronic acid (UDPGA), triethylamine and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dimethylsulphoxide (DMSO) and ethyl acetate were supplied from Janssen Chimica (Beerse, Belgium). All the other chemicals were of analytical grade.

Phenyldiazomethane and diazomethane were synthesized as described (Hinsberg 1890, Fieser and Fieser 1967).

Methods

Urine samples from sevoflurane-anaesthetized patients. Ten patients were included in the randomized study. They were informed of the study and its purpose. The demographic data of the patients are summarized in table 1. Patients had no history of renal disease and they were chosen from a non-renal surgery group. Anaesthesia was induced by propofol 2 mg kg^{-1} , and was maintained with sevoflurane at a flow rate of 6 l min^{-1} , and with nitrous oxide (50% in O_2).

Duration of sevoflurane anaesthesia ranged from 135 to 660 min (table 1). Spot urine samples (5 ml) were collected 24 h before, directly after discontinuation of anaesthesia and 24 h after discontinuation of anaesthesia. Urine samples were frozen (-35°C) until analyses.

Synthesis of compound A-derived mercapturic acids. Compound A-derived mercapturic acids were synthesized similar to the procedure of Orhan *et al.* (2001). Briefly, ice-cold sevoflurane (40 g, 0.2 mol) was added to a round-bottomed flask containing potassium hydroxide (KOH) (11.2 g, 0.2 mol) pulverized under nitrogen. A total of 1 ml benzyltrimethylammonium hydroxide was added as the phase transfer reagent and the mixture was refluxed at 80°C while being stirred vigorously for 30 min. Subsequently, the gas phase/head space was transferred with the aid of a slow nitrogen stream to a mixture 5 g N-acetyl-L-cysteine and 2 ml triethylamine in 50 ml DMSO. The reaction was preceded for 4 h. Subsequently, DMSO phase was diluted fivefold with water; the pH was adjusted to 1–2 by 2 N HCl, and extracted twice with 2 vols ethyl acetate. Ethyl acetate phases were combined and washed several times with acidic (pH 2.0) water in order to remove unreacted N-acetyl-L-cysteine and traces of DMSO. The ethyl acetate phases were evaporated *in vacuo* and yielded a light yellow solid. Products were characterized by GC-MS after methylation by ethereal diazomethane. The minor product formed

Table 1. Individual patient demographics.

Patient no.	Age (years)	Height (cm)	Weight (kg)	Sex	Duration of anaesthesia (min)	Sevoflurane in gas mixture (%)	Surgical procedure
1	45	178	68	m	660	1.5	supratentorial tumour
2	19	167	68	m	425	2.0	craniopharyngioma
3	21	173	65	f	265	2.0	bimaxillary osteotomy
4	37	163	60	f	385	2.0	craniotomy
5	40	178	80	m	300	2.0	supratentorial tumour
6	42	181	75	m	230	1.2	posterior fossa tumour
7	36	172	82	m	270	1.5	supratentorial tumour
8	44	160	55	f	180	1.2	supratentorial tumour
9	48	152	58	f	135	1.5	vaginal hysterectomy
10	44	155	70	f	135	2.0	vaginal hysterectomy

(20%) was *S*-[1,1,3,3,3-pentafluoro-2-(fluoromethoxy)propyl]-*N*-acetyl-L-cysteine (compound A mercapturic acid I; CAMA-I), whereas 80% was identified as *S*-[1-fluoro-2-(fluoromethoxy)-2-(trifluoromethyl)vinyl]-*N*-acetyl-L-cysteine (compound A mercapturic acid II; CAMA-II). The ratio of the products was determined by ^{19}F -NMR. Electron impact mass spectra of methyl esters of mercapturic acids and ^{19}F -NMR spectra were identical to those described previously (Uttamsingh *et al.* 1998, Iyer *et al.* 1998, Kharasch *et al.* 1999a). No attempts were undertaken to separate the saturated and unsaturated products. Stock solutions (pH 7.0) were calibrated by ^{19}F -NMR using a known concentration of TFA as internal standard.

S-[1-bromo-1-chloro-2,2-difluoroethylene]-*N*-acetyl-L-cysteine (BCDFE-Nac) was used as an internal standard for GC-MS and GC-ECD analyses of compound A-derived mercapturic acids and was synthesized in the same way as described above, using halothane instead of sevoflurane. The mass spectrum and the ^1H -NMR spectrum were identical to those described previously (Cohen *et al.* 1975, Wark *et al.* 1990).

Biosynthesis of glucuronic acid conjugate of HFIP. The glucuronic acid conjugate of HFIP was generated biosynthetically. A total of 3.52 mM commercial HFIP was incubated in 100 mM Tris buffer (pH 7.15) in the presence of 2.86 mM UDPGA, 2 mg ml $^{-1}$ rat liver microsomes, 0.14% Triton X-100, and 14% D $_2$ O. After 45 min of incubation at 37°C, incubation media were analysed directly by ^{19}F -NMR. Control incubations were performed in the absence of UDPGA. Rat liver microsomes were prepared from phenobarbital-induced rats as described (Orhan *et al.* 2001, Rooseboom *et al.* 2001).

Biosynthesis of compound A cysteine conjugates and β -lyase products. To obtain β -lyase-dependent metabolites of compound A, TFPA and TFLA, the reference mercapturic acid mixture was treated with *N*-acylase. For this, a synthetically mixture of 0.6 mM (final) CAMA-I and 3 mM (final) CAMA-II was incubated in 50 mM phosphate buffer, pH 6.0, in the absence or presence of 0.5 mM amino-oxyacetic acid, 1 mg ml $^{-1}$ *N*-acylase and 15% D $_2$ O. After 60 min of incubation at room temperature and 60 h of incubation at 37°C, incubation media were analysed directly by ^{19}F -NMR. These analyses showed that subsequent β -lyase treatment did not appear to be necessary to obtain the products as they were already formed during *N*-acylase incubation. Subsequently, incubation media were acidified to pH 2.0, extracted by ethyl acetate, derivatized either with ethereal diazomethane or phenyldiazomethane, and monitored by GC-SIM for the ions of the methyl esters of TFPA (m/z 131, 112 and 142) and TFLA (m/z 80, 91 and 99) (Iyer *et al.* 1998), and for the ions of the phenyl esters of TFPA (m/z 131, 217 and 266) and TFLA (m/z 99 and 234). TFLA was identified by ion chromatograms of m/z 99 and 234 (retention time 14.80 min), and TFPA was identified by ion chromatograms of m/z 131, 217 and 266 (retention time 15.07 min; data not shown).

^{19}F -NMR analyses of urine of patients anaesthetized with sevoflurane. Before analysis, urine samples were centrifuged at 3000g for 15 min to remove precipitates. A total of 1 ml of the supernatant and 100 μ l deuterium oxide were transferred to 5 mm NMR tubes. Alternatively, urine samples of 1 ml, directly after ($n=10$) and 24 h after discontinuation of anaesthesia ($n=10$), were combined, individually concentrated by freeze-drying and reconstituted with 1 ml nanopure water and ± 100 litres D $_2$ O. ^{19}F -NMR spectra were recorded on a Bruker MSL 400 spectrometer operating at 376 MHz or Bruker Avance 250 spectrometer operating at 235 MHz for ^{19}F at room temperature. The number of scans necessary to obtain a good signal-to-noise ratio ranges from 270 scans (HFIP and HFIP-glucuronide) to 60 000 scans (compound A-related metabolites).

For quantitative analysis of total HFIP excretion (free HFIP plus HFIP-glucuronide), 1 ml urine samples were treated for 2 h with 100 000 U ml $^{-1}$ β -glucuronidase (EC 3.2.1.31) at 37°C. At the end of the incubation, all standards and samples were spiked with TFA as internal standard (final concentration 0.2 mM) and analysed by ^{19}F -NMR.

A calibration curve was obtained by spiking blank urine with known concentrations of HFIP (ranging from 0 to 5 mM) and 0.2 mM TFA. Ratios of peak area of HFIP relative to that of TFA were used to quantify HFIP concentrations in urine.

GC-MS and GC-ECD analysis of compound A-derived metabolites. A total of 1 ml centrifuged urine samples was spiked with 50 nmol BCDFE-Nac as an internal standard. The urine samples were acidified to pH 1–2 by 2 N HCl and extracted three times with 2 ml ethyl acetate. The organic phases were combined, concentrated to 0.5 ml and treated with an excess of freshly prepared ethereal diazomethane or phenyldiazomethane. After 30 min at room temperature, the samples were evaporated to dryness under nitrogen and redissolved in 200 μ l ethyl acetate. The samples were analysed by GC-MS and GC-ECD. Calibration curves for compound A mercapturic acids were prepared by spiking blank urine samples with 50 nmol BCDFE-Nac (internal standard) and different concentrations of compound A mercapturic acids, ranging from 0 to ± 200 M.

Samples were also analysed for β -lyase products by GC-MS in full-scan mode by the ions described above.

Instruments. GC-MS analyses were carried out using a Hewlett-Packard 5890/MSD system. An ID-BPX5 capillary column (25 m) was obtained from SGE Ltd (Ringwood, Victoria, Australia). The operation temperatures were 270°C (split injector and transfer line) and 280°C (ion source, electron impact ionization, electron energy of 70 eV). Column head pressure was 29 psi (195 kPa).

For the analysis of the CAMA-I and -II, the column temperature was programmed from 80°C (2.5 min) to 280°C at 20°C min⁻¹ (method I). The retention times were 9.95 min (CAMA-II) and 10.00 min (CAMA-I).

For β -lyase degradation products, the operation temperatures were 150 and 200°C (split injector and transfer line, respectively) and 270°C (ion source, electron impact ionization, electron energy of 70 eV). Column temperature was programmed from 32°C (1 min) to 200°C at 10°C min⁻¹ for methyl esters, and programmed from 35°C (5 min) to 250°C at 20°C min⁻¹, then to 280°C at 10°C min⁻¹ (10 min), for phenyl esters.

GC-ECD measurements were carried out using a Hewlett-Packard 5890 series II gas chromatograph coupled to a Hewlett-Packard 15 mCi ⁶³Ni electron capture detector; the injector and the transfer-line temperatures were 150 and 200°C, respectively. Oven temperature was programmed from 50°C (1 min initial time) to 280°C by a linear gradient of 20°C min⁻¹. Retention times of the reference compounds were: CAMA-I, 10.32 min, CAMA-II, 10.37 min and BCDFE-Nac 11.98 min (method II).

Results

¹⁹F-NMR analyses of compound A cysteine conjugates

Figure 2A shows a ¹⁹F-NMR spectrum of the reference mercapturic acids. After 1 h incubation with N-acylase at room temperature, formation of the unsaturated cysteine conjugate of compound A (CA-Cys-II) was observed (figure 2B). CAMA-I was not converted to the corresponding cysteine conjugate (CA-Cys-I), since it is resistant to N-acylase (Uttamsingh *et al.* 1998). After 60 h, the signal intensity of CA-Cys-II was diminished, while signals corresponding to TFPA and TFLA emerged (figure 2C). A similar pattern can be seen in the -OCFH₂ signals of the corresponding compounds in the small panels of each figure. The formation of TFLA and TFPA by N-acylase was also confirmed by performing the incubations in presence of a β -lyase inhibitor, amino-oxyacetic acid, which is indicative for non-enzymatic degradation of the cysteine conjugate. The small acids formed were analysed by GC-MS after phenyldiazomethane derivatization. TFLA was identified by ion chromatograms of *m/z* 99 and 234 (retention time 14.80 min), and TFPA by ion chromatograms of *m/z* 131, 217, and 266 (retention time 15.07 min) (data not shown).

¹⁹F-NMR analyses of urine of patients anaesthetized by sevoflurane

¹⁹F-NMR analyses of urine of sevoflurane-anaesthetized patients showed the presence of several fluorine-containing metabolites. After collecting 270 scans (equivalent to 15 min of measuring time), two multiplet major signals centred at 1.73 and 1.82 ppm in a ratio of approximately 2:1 were observed (figure 3A). β -Glucuronidase treatment of the urine sample resulted in the transformation of these signals to a doublet centred at -1.80 ppm (figure 3B), which was identical to the signal obtained with commercially available HFIP (figure 3C). HFIP-glucuronide conjugate formed by rat liver microsomes also resulted in the same multiplets at 1.73 and 1.82 ppm (data not shown), and could also be hydrolysed back to HFIP by β -glucuronidase treatment. According to these results, HFIP is excreted in urine exclusively as a glucuronide. The complexity of the spectrum of HFIP-glucuronide can be explained by second-order spectral interactions (Fischer *et al.* 1984).

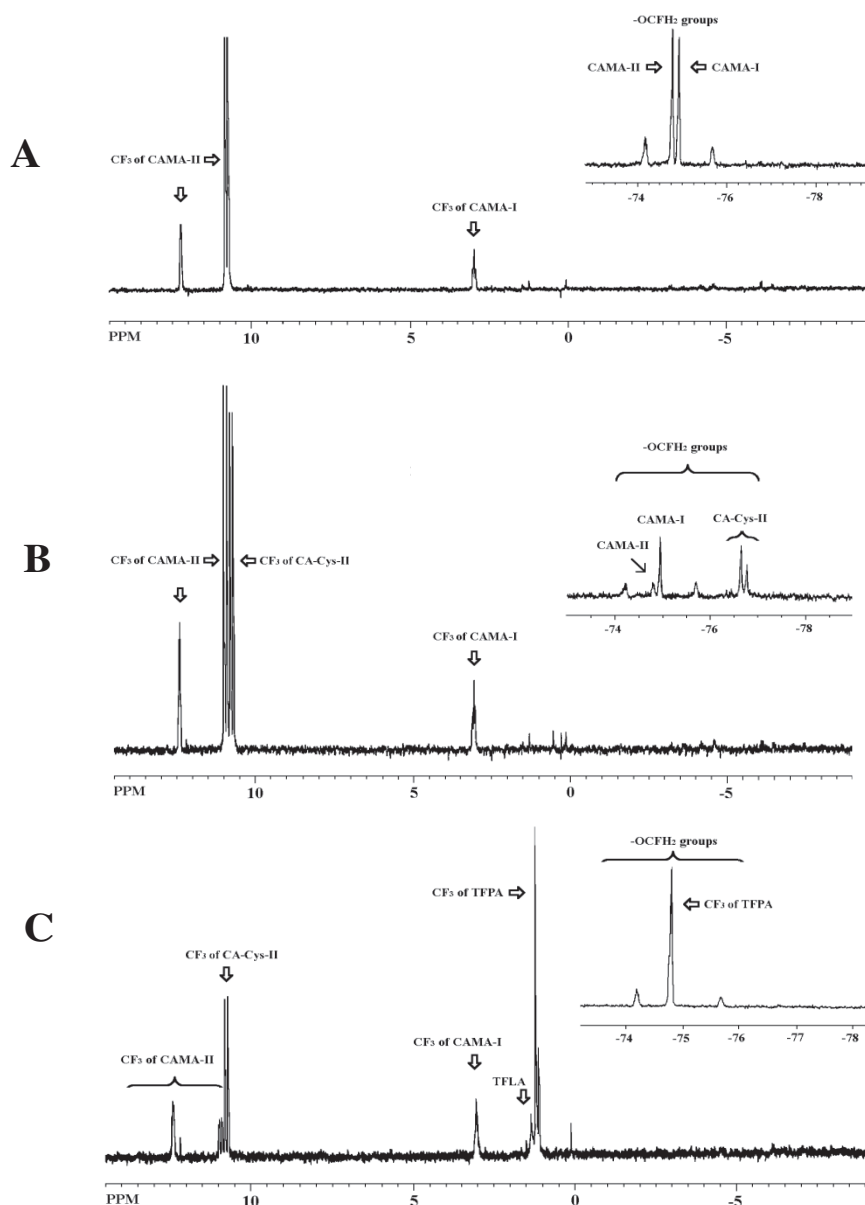


Figure 2. ^{19}F -NMR spectra of the reference CAMA-I and -II. (A) CAMA-I and -II in deuterium oxide; (B) after 1-h incubation with N-acylase at 37°C ; (C) after 60-h incubation with N-acylase at 37°C . Small panels in each spectrum represent the low region for $-\text{OCFH}_2$ groups. CAMA-I, compound A mercapturic acid I; CAMA-II, compound A mercapturic acid II; CA-Cys-II, compound A cysteine conjugate II; TFPA, 3,3,3-trifluoro-2-(fluoromethoxy)-propanoic acid; TFLA, 3,3,3-trifluorolactic acid.

To obtain signals from compound A-derived metabolites, much longer measuring times appeared to be necessary. Figure 4A shows the ^{19}F -NMR-spectrum of non-concentrated urine containing the highest HFIP-concentration after 60 000 scans, which is equivalent to a 50-h measuring time. A number of the signals observed could be attributed to the mercapturic acids, CAMA-I and -II, and

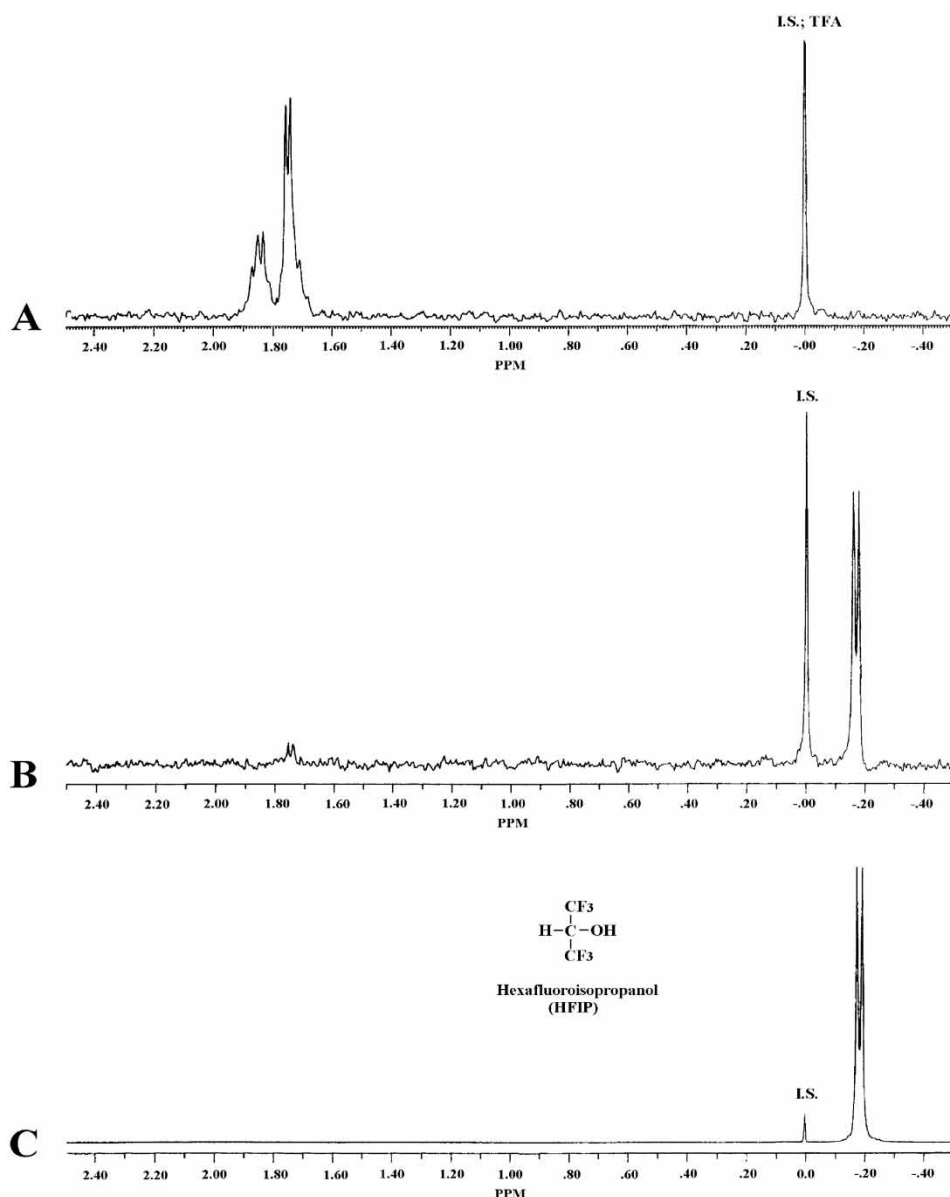


Figure 3. ^{19}F -NMR-spectra of non-concentrated urine of a patient anaesthetized with sevoflurane. (A) Before β -glucuronidase treatment; (B) after β -glucuronidase treatment; (C) reference HFIP spectrum in blank urine. The complexity of the glucuronide multiplet is attributed to second-order spectral effects (Fischer *et al.* 1984).

products from β -lyase-activation of compound A, TFPA and TFLA, consistent with previous observations (Spracklin and Kharasch 1996). This observation was confirmed by combining all patients' urine samples directly after discontinuation of anaesthesia and by concentrating them 12-fold (figure 4B). Signals of mercapturic acids and TFPA and TFLA were visible. In addition, a number of signals of as yet unidentified fluorine-containing compounds were also observed. CAMA-I and -II signals were not detectable in the combined and concentrated urine

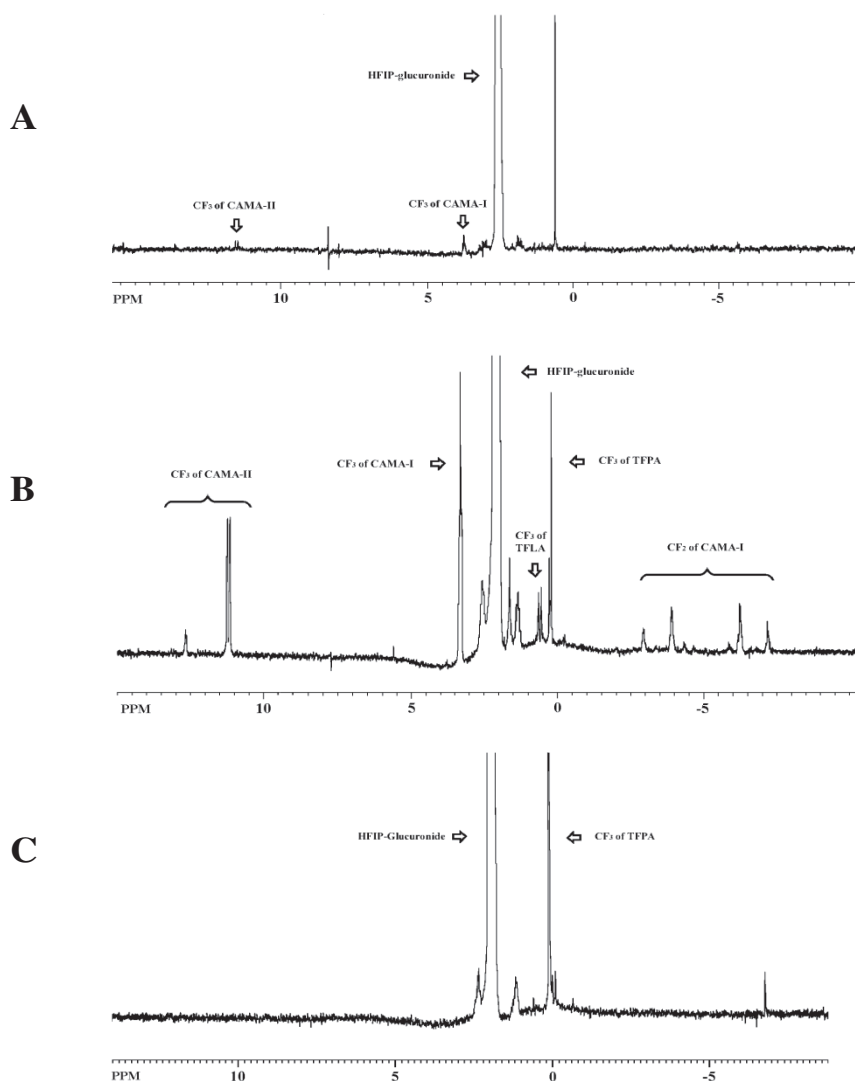


Figure 4. ^{19}F -NMR spectra of the urine samples of a sevoflurane-anaesthetized patient directly after and 24 h after discontinuation of anaesthesia. (A) 60 000 scans (50 h) of one patient's urine; (B) 16 000 scans (13 h) of the combined and concentrated urines directly after discontinuation of anaesthesia; (C) 16 000 scans (13 h) of the combined and concentrated urines 24 h after discontinuation of anaesthesia. CAMA-I, compound A mercapturic acid I; CAMA-II, compound A mercapturic acid II; TFPA, 3,3,3-trifluoro-2-(fluoromethoxy)-propanoic acid; TFLA, 3,3,3-trifluorolactic acid.

samples obtained 24 h after discontinuation of anaesthesia (figure 4C). GC-ECD and GC-MS analyses confirmed this observation.

Quantitative analysis of HFIP-excretion by ^{19}F -NMR

For quantitative analysis of urinary HFIP excretion by ^{19}F -NMR, all urine fractions were treated with β -glucuronidase to obtain free HFIP. The less complex coupling pattern and higher signal-to-noise ratios allowed detection of

low concentrations in relatively short measuring times. The detection limit based on the signal-to-noise ratio ($=3$) for quantitative analysis of HFIP, using a measuring time of 15 min (270 scans), was about $10\text{ }\mu\text{M}$. The standard curve ($y = 8.04x - 0.29$) of varying concentrations of HFIP revealed a very good linearity ($r^2 = 0.998$) over the range of 0 to 5 mM.

The sensitivity of ^{19}F -NMR, using 15-min measuring time per sample, was high enough to quantify HFIP in all urines analysed. The levels of HFIP-excretion directly after and 24 h after discontinuation of anaesthesia are summarized in table 2. The highest levels were observed directly after discontinuation of anaesthesia, whereas the levels decreased two- to 30-fold 24 h after discontinuation of anaesthesia. HFIP was not detected in urine samples obtained before anaesthesia.

HFIP excretion levels directly after and 24 h after discontinuation of anaesthesia correlate reasonably well with the exposure to sevoflurane ($r^2 = 0.79$ and 0.74 , respectively), as estimated from the duration of anaesthesia and the sevoflurane concentration used (figure 5A, B).

GC-ECD and GC-MS analyses of compound A-derived metabolites excretion

Figure 6 shows representative GC-ECD chromatograms obtained from methylated extracts of patient urine fractions. Figure 6A shows the GC-ECD chromatogram obtained from blank urine spiked with a mixture of the synthetic CAMA-I and -II (retention times 10.32 and 10.37 min), and BCDFE-Nac (retention time 11.98 min) as internal standard. The mercapturic acid at 10.32 min corresponds to CAMA-II, resulting from addition/elimination of compound A to glutathione (Jin *et al.* 1996). The second peak at 10.37 min corresponds to CAMA-I, resulting from addition reaction of compound A to glutathione.

Analysis of urine samples obtained before anaesthesia, directly after and 24 h after discontinuation of anaesthesia showed that the compound A-mercapturic acids were only detectable directly after discontinuation of anaesthesia. CAMA-II was excreted at slightly higher concentrations of CAMA-I. Sevoflurane exposure

Table 2. Urinary excretion of CAMA-I, CAMA-II and HFIP in sevoflurane-anaesthetized patients.

Patient no.	Exposure factor (dose $\times$ time)	CAMA-I ($\mu\text{mol mg}^{-1}$ creatine)	CAMA-II ($\mu\text{mol mg}^{-1}$ creatine)	HFIP 0 h ^a ($\mu\text{mol mg}^{-1}$ creatine)	HFIP 24 h ^b ($\mu\text{mol mg}^{-1}$ creatine)	HFIP total ($\mu\text{mol mg}^{-1}$ creatine)
1	990	4.90 (2.3) ^c	1.68 (0.8)	203.3 (96.8)	119.5 (100.0)	322.8
2	850	3.94 (1.9)	4.35 (2.1)	200.3 (96.0)	101.5 (100.0)	301.8
3	530	2.09 (1.1)	1.69 (0.9)	182.3 (98.0)	97.0 (100.0)	279.3
4	770	3.39 (1.9)	2.26 (1.3)	169.7 (97.0)	74.6 (100.0)	244.3
5	600	3.17 (2.5)	1.36 (1.1)	123.1 (96.4)	70.2 (100.0)	193.3
6	276	0.59 (0.5)	0.27 (0.2)	110.4 (99.2)	63.3 (100.0)	173.7
7	405	0.44 (0.5)	0 (0)	87.8 (99.5)	60.4 (100.0)	148.2
8	216	0 (0)	0 (0)	63.9 (100.0)	5.5 (100.0)	69.2
9	202	3.09 (2.9)	1.36 (1.3)	103.0 (95.8)	19.4 (100.0)	122.4
10	270	1.72 (2.4)	1.05 (1.5)	68.9 (96.1)	2.4 (100.0)	71.3
Mean $\pm$ SEM		2.3 $\pm$ 0.8 (1.6 $\pm$ 0.5)	1.4 $\pm$ 0.4 (0.9 $\pm$ 0.3)	131 $\pm$ 43 (97.0 $\pm$ 32)	61 $\pm$ 20(100.0)	192 $\pm$ 64

^aDirectly after discontinuation of anaesthesia.

^bTwenty-four hours after discontinuation of anaesthesia.

^cPercentages of total urinary fluorine-containing metabolites of sevoflurane are presented in parentheses.

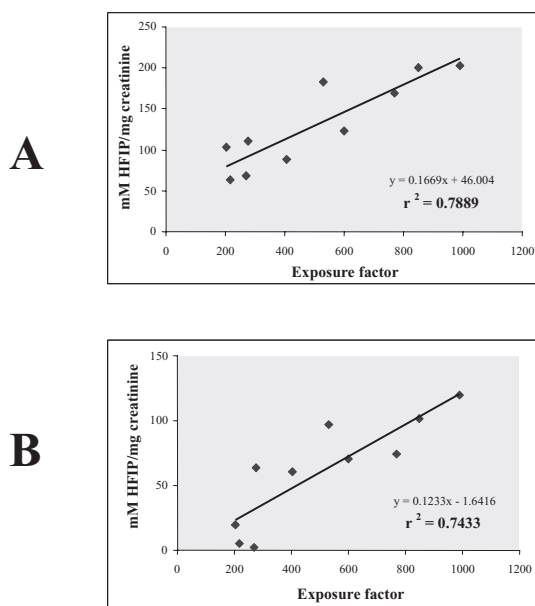


Figure 5. Correlations of urinary excretion of HFIP with exposure to sevoflurane. (A) HFIP directly after discontinuation of anaesthesia; (B) HFIP 24 h after discontinuation of anaesthesia.

and CAMA-I urinary excretion poorly correlated ($r^2 = 0.63$; figure 7A), whereas CAMA-II excretion did not correlate with sevoflurane exposure at all ($r^2 = 0.49$; figure 7B).

The presence of the two compound A mercapturic acids in the urines of the patients was confirmed by GC-MS, using selected ion monitoring of characteristic ions of methylated mercapturic acids (figure 8A, B). CAMA-I was positively identified by ion chromatograms of m/z 176, 256, 298 and 357, and CAMA-II by ion chromatograms of m/z 144, 236 and 278. The presence of methyl or phenyl esters of the β -lyase-mediated metabolites TFLA and TFPA were analysed by selected ion monitoring as described for methyl esters (Iyer *et al.* 1998) and for phenyl esters of reference compounds as obtained from *in vitro* incubation of mercapturic acids with N-acylase, and derivatized with phenyldiazomethane. However, both small molecule acids could not be detected in patients' urines, probably due to the too low concentrations.

Discussion

The objective of the present study was to explore the applicability of ^{19}F -NMR for the qualitative and quantitative analysis of fluorine-containing urinary metabolites after sevoflurane anaesthesia. Urines of 10 patients exposed to sevoflurane were analysed. According to the present results, HFIP-glucuronide was the only fluorine-containing metabolite that could be measured by ^{19}F -NMR with relatively short measuring times (15 min). Only after a 50-h measuring time, however, CAMA-I, CAMA-II, TFPA and TFLA signals corresponding to those reported by Iyer *et al.* (1998) were also obtained. That group, however, obtained signals

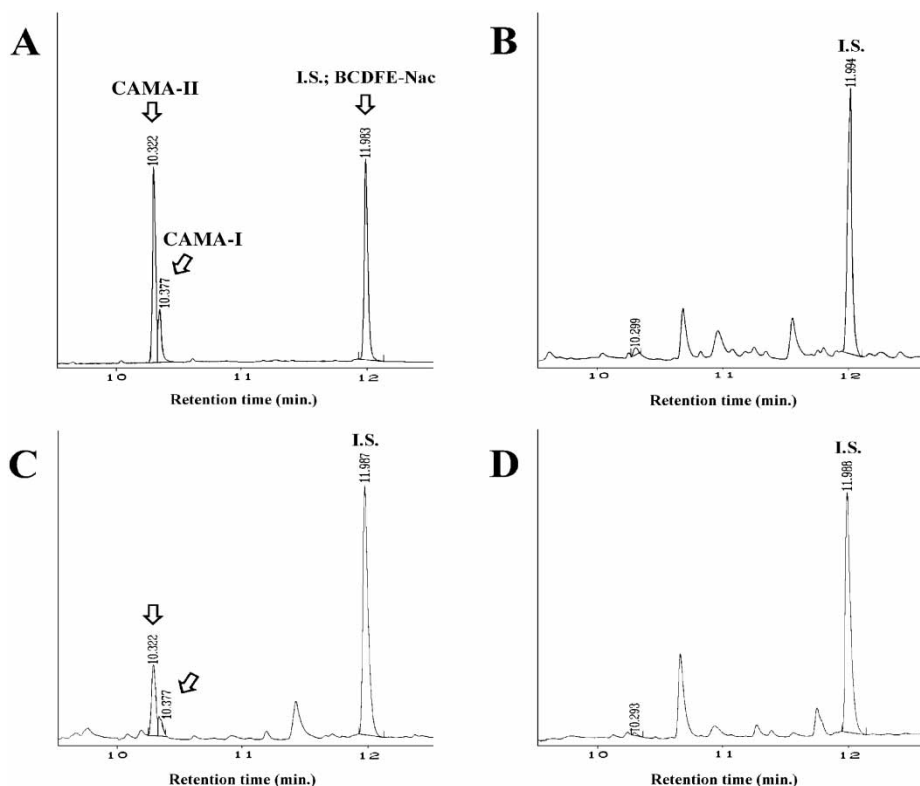


Figure 6. GC-ECD chromatograms of a non-concentrated urine sample of a sevoflurane-anaesthetized patient. (A) Reference chromatograms of CAMA-I and -II; (B) before operation; (C) directly after discontinuation of anaesthesia; (D) 24 h after the discontinuation of anaesthesia.

after scanning 50-fold concentrated urines. The ^{19}F -NMR signals were obtained with 1 ml non-concentrated urine samples. Although not reported previously, urinary HFIP-glucuronide appeared to result in two quartets centred at 1.73 and 1.82 ppm (figure 3). Identical signals were obtained when HFIP-glucuronide was prepared biosynthetically. The reason why two signals are observed is not yet fully clear. Intramolecular rearrangement of the glucuronide, similar to that of acyl glucuronides, does not seem likely, since both signals disappeared on β -glucuronidase treatment. The complexity of the spectrum can be explained by second-order spectral interactions, however. Such effects were previously observed with proton NMR spectrum of 2,2,2-trifluoroethyl glucuronide, which bears aglycon with C-1 hydrogen (Fischer *et al.* 1984), and with ^{19}F -NMR spectrum of 2-chloro-2,2-difluoroethyl glucuronide (Harris and Anders 1991).

After hydrolyses of the conjugate by β -glucuronidase, the two quartets transformed to a doublet centred at -1.80 ppm (figure 3B), identical to that of free HFIP (figure 3C). Because of an improved signal-to-noise ratio, subsequent quantification of HFIP-glucuronide was performed when treating urine samples with β -glucuronidase. As seen in table 2, urinary excretion of HFIP is higher in all patients directly after discontinuation of anaesthesia when compared with 24 h after discontinuation of anaesthesia. This observation corresponds well with the literature, as reported both free HFIP and HFIP-glucuronide were already

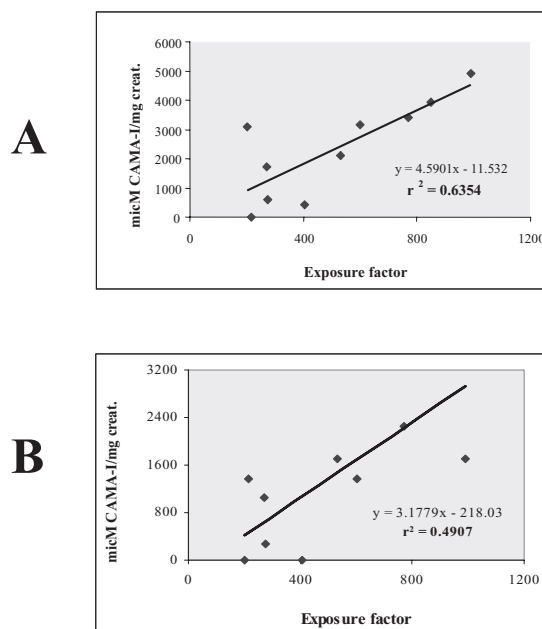


Figure 7. Correlations of urinary excretion of compound A mercapturic acids with exposure to sevoflurane directly after discontinuation of anaesthesia. (A) CAMA-I; (B) CAMA-II.

present in blood 5 min after the start of sevoflurane anaesthesia (Kharasch *et al.* 1995b). Maximum excretion of both compounds occurred in the first 12 h (Martis *et al.* 1981).

HFIP represents approximately $97\% \pm 30$ (mean $\pm$ SEM) of the total amount of the urinary fluorine-containing metabolites directly after discontinuation of anaesthesia (table 2), whereas CAMA-I represents $1.6\% \pm 0.5\%$ and CAMA-II represents $0.9\% \pm 0.3\%$ of the metabolites detected in urine. This is, to our knowledge, the first report of the compound A mercapturic acids ratio over HFIP in humans. Holaday and Smith (1981) reported that 82% of the urinary organic fluorinated metabolites is HFIP.

Previous attempts to measure HFIP in urine have involved extensive extraction and/or complicated derivatization steps. The present ^{19}F -NMR technique does not require any sample preparation except β -glucuronidase treatment. Moreover, it was possible to detect signals both from conjugated as well as free HFIP, and to check the efficiency of enzymatic conjugate hydrolysis.

According to the present results, CAMA-I and -II were completely excreted within 24 h after discontinuation of anaesthesia. This is in accordance with the literature, where the urinary half-life of mercapturic acids was reported to be 1.5–9 h (Van Welie *et al.* 1992). However, Kharasch and Jubert (1999b) observed that both sevoflurane mercapturic acids were detectable for 2 days postoperatively in patients anaesthetized with sevoflurane. In the present study, the mercapturic acid concentrations were below the limit of quantification in samples obtained 24 h after the discontinuation of anaesthesia of all patients (beginning of day 2). Table 2 summarizes the excretion of both CAMA-I and -II. It has been reported that CAMA-I was excreted at slightly lower concentrations of CAMA-II (Jin *et al.*

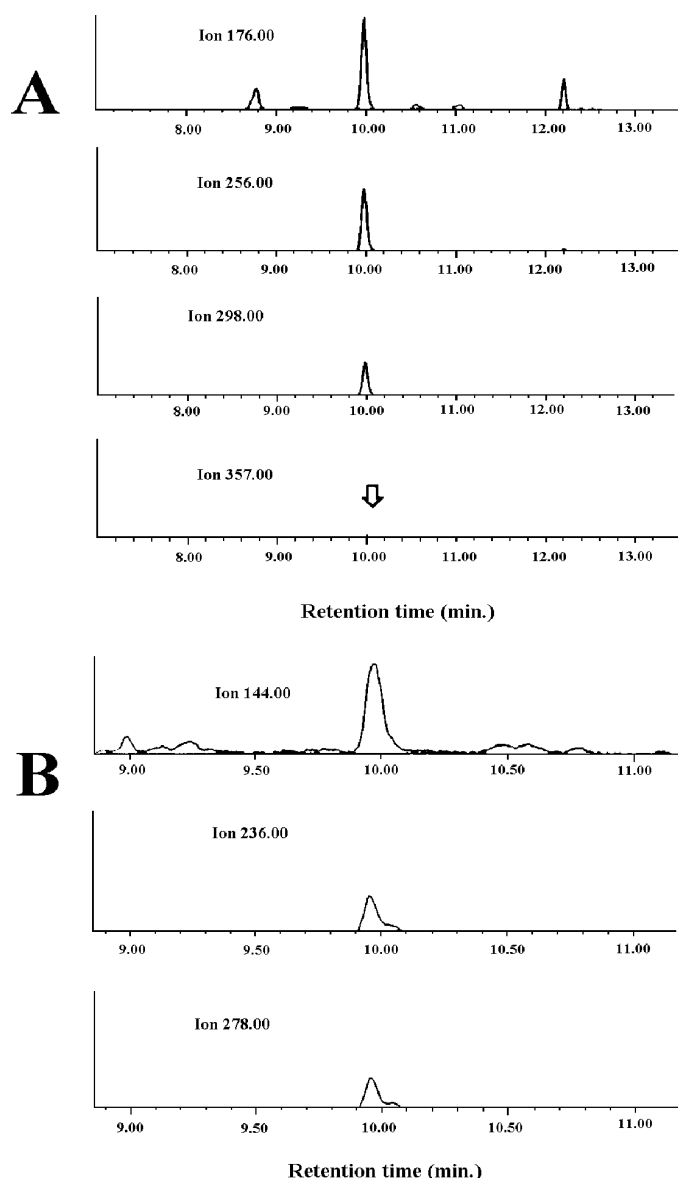


Figure 8. GC-MS ion chromatograms of a non-concentrated urine sample of a sevoflurane-anaesthetised patient. (A) Main fragmentations of CAMA-I; (B) main fragmentations of CAMA-II. The arrow indicates the position of the very weak molecular ion of CAMA-I.

1996, Uttamsingh *et al.* 1998, Kharasch and Jubert 1999b). This was observed in the present study as well, except in one patient (second patient in table 2; also figure 6C).

Several groups (Iyer *et al.* 1996, Spracklin and Kharasch 1996, Iyer and Anders 1997, Iyer *et al.* 1998, Uttamsingh *et al.* 1998, Kharasch *et al.* 1999a) reported that compound A-derived cysteine conjugates undergo β -lyase-mediated bioactivation to TFPA, and decompose partially to TFLA. Indeed, we observed the signals of these two acids by ^{19}F -NMR in combined and concentrated urines

directly after discontinuation of anaesthesia (figure 4B). The $-\text{CF}_3$ signal of TFPA was still present in combined and concentrated urines 24 h after discontinuation of anaesthesia, whereas the TFLA signals disappeared. On the other hand, these two small acids could not be detected in non-concentrated urines (1 ml) by GC-MS. Owing to the too low concentrations, probably higher volumes of urine are needed to detect these metabolites.

Interestingly, it was found that N-acylase caused formation of TFPA and TFLA directly from CAMA-I and -II after 60-h incubation at 37°C (figure 2C). To avoid β -lyase-mediated degradation of the formed cysteine conjugates to TFLA and TFPA, which might be present as a contaminant in commercial N-acylase, incubations were performed in the presence of amino-oxyacetic acid, a potent β -lyase inhibitor. This finding may have an impact on the interpretation of sevoflurane toxicity; toxic intermediates might be formed in the presence of N-acylase, without β -lyase activity. However, this needs also to be proved *in vivo*.

In conclusion, the present results show that ^{19}F -NMR can be used to identify and quantify HFIP in non-concentrated urines of sevoflurane-anaesthetized patients. Since HFIP is the main metabolite of sevoflurane (95–99%) and since its urinary excretion showed a good correlation with exposure, sevoflurane disposition can easily be monitored by ^{19}F -NMR analysis of HFIP. The method can detect as low as $\pm 10\text{ M}$ levels. For minor other metabolites, either longer scanning times are necessary or the concentration of the samples is required. Alternatively, the minor fluorine-containing metabolites, CAMA-I, CAMA-II, TFPA and TFLA, require GC-based techniques for quantification. GC-ECD is a very sensitive detection technique and sample clean-up is not necessary to identify/quantify CAMA-I and -II, indicating that this technique may also be useful for monitoring compound A-related metabolites.

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Transport, transformation and distribution space of propofol in the rat liver studied by means of the indicator-dilution technique

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1. The transport, transformation and distribution space of the endovenous anaesthetic propofol in the isolated perfused rat liver were investigated by using the multiple-indicator dilution technique with constant infusion (step input).
2. The behaviour of propofol in the liver was described by a space-distributed variable transit-time model. The drug permeated the cell membrane at very high rates and its distribution into the cellular space was flow-limited. The apparent distribution space of propofol varied between 284 and 125 times the water space, and was inversely related to the tested portal concentrations (33–250 μM).
3. The corresponding ratios of intra- to extracellular concentration varied between 319 and 187, revealing a very high affinity of the liver for propofol. They most probably reflect binding to several cellular structures, including membranes and proteins.
4. The single-pass rate coefficients for biotransformation decreased with increases in the portal concentration of propofol. The liver released significant amounts of 4-hydroxypropofol, reaching 41.7% of the total single pass of 67.2 μM propofol biotransformation. These results disprove previous notions that hydroxylation is rate limiting for conjugation and suggest that the liver might function as a 4-hydroxypropofol source for conjugation to glucuronic acid or sulfate in other tissues.

Introduction

Propofol (2,6-diisopropylphenol) is an endovenous anaesthetic used for general anaesthesia or as a sedative during surgeries or in intensive care units (Mirenda and Broyles 1995). As so many drugs, propofol is intensely metabolized in the liver (Larijani *et al.* 1989, Court *et al.* 2001), but the contribution of several extrahepatic tissues (Shafer 1993), especially the kidney, seems significant (McGurk *et al.* 1998). Conjugation of propofol to glucuronic acid is an important biotransformation reaction (Simons *et al.* 1991, Raoof *et al.* 1996a, McGurk *et al.* 1998). Oxidation to 4-hydroxypropofol (2,6-diisopropyl-1,4-quinol), which is followed by conjugation with glucuronic acid and sulfate, has also been demonstrated (Vree *et al.* 1999).

In molecular terms, propofol is characterized by a high degree of lipophilicity (Tonner *et al.* 1992, Tsuchiya 2001). An octanol/water partition coefficient of 4300 has been reported (Tonner *et al.* 1992). The high affinity of biological membranes for lipophilic compounds generally confers on them the ability to affect membrane-bound biological activities. This is especially true for membrane-bound enzymes. For example, propofol inhibits both the plasma membrane and endoplasmic reticulum Ca^{2+} -ATPases as a result of entropy-driven interactions with these structures (Lopez and Kosk-Kosicka 1997). Propofol is also known

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to affect energy transduction in isolated mitochondria (Branca *et al.* 1991a, b). Consistently with the high affinity for lipophilic structures, the apparent distribution space of propofol in whole organisms is very high, generally exceeding the true physical limits (Kanto and Gepts 1989, Baroni *et al.* 2000, Weaver *et al.* 2001). Besides the high distribution space, the pharmacokinetics of propofol are quite complex. Analysis of the various blood concentration–time curves obtained to date do not allow a definitive conclusion about the model that describes the behaviour of propofol. Conclusions have oscillated between the two- and three-compartment models, sometimes within the same experimental series (Kanto and Gepts 1989, Zoran *et al.* 1993). It is certainly not fortuitous that standard deviations of the fitted parameters, such as half-life times, are generally very high (Kanto and Gepts 1989, Zoran *et al.* 1993, Baroni *et al.* 2000). All these characteristics reveal a very complex behaviour for propofol in the whole organism, which can be only described imprecisely by the current relatively simple compartmental models.

The whole distribution of propofol *in vivo* evidently results from a summation of transport and distribution phenomena in almost all tissues (Weaver *et al.* 2001). Since different tissues are likely to respond differently to propofol, specific studies are important for a complete understanding of the behaviour of this anaesthetic. In this respect, with the exception of biotransformation, the interactions of the liver with propofol have not yet been investigated in detail and the present study was undertaken to fill this gap. The isolated perfused rat liver was used as the experimental model. This experimental system is adequate for studying transport phenomena, distribution spaces as well as metabolic transformation and metabolic effects (Bracht *et al.* 1980, Kelmer-Bracht *et al.* 1993). Owing to its highly lipophilic nature, it is likely that propofol permeates the cell membrane very rapidly, possibly in a flow-limited fashion. Confirmation of this hypothesis, however, requires specific measurements that can be done by means of the multiple-indicator dilution technique (Goresky *et al.* 1970, Kelmer-Bracht *et al.* 1993). Furthermore, such experiments equally should allow quantification of the distribution space of propofol, its concentration dependence as well as the overall rate coefficient for hepatic biotransformation and its concentration dependence.

Material and methods

Material

The perfusion apparatus and rapid sampling apparatus were built in the workshops of the University of Maringá. Several peristaltic pumps used in the experiments and the precision infusion pumps were a gift of Dr Roland Scholz, Institute for Physiological Chemistry, University of Munich, Germany. Propofol (99% purity) was purchased from Acros (NJ, USA). Highly pure solvents (high-performance liquid chromatography [HPLC] grade) and reagent grade chemicals were from J. T. Baker (NJ, USA) and Labsynth (Sao Paulo, Brazil).

Liver perfusion and indicator-dilution experiments

Male albino rats (Wistar strain, 200–250 g) received a standard laboratory diet (Nuvilab) and water *ad libitum* before the surgical removal of the liver under thiopental anaesthesia (50 mg kg⁻¹). The perfusion technique described by Scholz and Bücher (1965) was used. The perfusion fluid was Krebs–Henseleit-bicarbonate buffer, pH 7.4, saturated with an oxygen:carbon dioxide mixture (95:5%) containing 3.4 µM fatty acid-free bovine serum albumin. The fluid was pumped through a temperature-regulated (37°C) membrane oxygenator before entering the liver via a cannula inserted into the portal vein. The flow rate through the liver was 30 ml min⁻¹.

Indicator-dilution experiments were performed using the constant infusion method (step input; Meier and Zierler 1954). In all experiments, [³H]water and propofol were infused simultaneously into the portal vein using precision infusion pumps. Propofol was infused for 90 min as a concentrated solution in dimethylsulfoxide, the concentration and the infusion rate being adjusted to obtain the

desired final concentration. During the first 90 s after the onset of the infusion, the perfusate was fractionated in 0.5–2.0-s fractions by means of a specially designed fraction collector (Bracht *et al.* 1980). After the first 90 s, the samples were taken manually at intervals ranging from 5 to 10 min. Aliquots of these samples were used for the assay of [^3H]water and for the extraction and assay of propofol.

Experiments with isolated mitochondria

Incubations of propofol with isolated mitochondria were done in order to evaluate binding to this organelle. Fed rats, weighing between 150 and 180 g, were decapitated and their livers removed immediately and cut into small pieces. These tissue fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 1.0 mM Tris-HCl (pH 7.4), 1 mM ethylene glycol-bis-(β -amino-ethyl-ether)tetra-acetic acid (EGTA) and 50 mg% fatty acid-free bovine serum albumin. Homogenization was carried out in the same medium by means of a Van Potter–Elvehjem homogenizer. After homogenization, the mitochondria were isolated by differential centrifugation (Voss *et al.* 1961, Bracht *et al.* 2003a) and suspended in the same medium, which was kept at 0–4°C.

Binding of propofol to mitochondria was measured at 37°C in 1.5 ml incubation medium containing 2 mg mitochondrial protein, 260 mM mannitol, 1 mM EGTA, 10 mM Tris-HCl (pH 7.4) and variable propofol concentrations in the range 25–300 μM . After 7 min of incubation, the samples were rapidly centrifuged for 5 min at 10 000g in a refrigerated centrifuge (2°C). To 1 ml of the supernatant, 3 ml ethyl acetate were added and the mixture vigorously shaken for 60 s. After 1 h, the organic phase was used for propofol assay.

Experiments with isolated microsomes

Microsomes were isolated by differential centrifugation (Moore *et al.* 1975, Bracht *et al.* 2003b). The rats were decapitated and their livers excised, cut into small pieces with scissors and washed with a cold (4°C) aqueous solution (isolation medium) containing 150 mM KCl, 100 μM phenylmethylsulfonyl-fluoride and 20 mM Tris-HCl (pH 7.4). After suspension in 10 vols of the isolation medium, the tissue was homogenized with a Dounce homogenizer. The homogenate was filtered through gauze and centrifuged at 2550g for 10 min in a refrigerated centrifuge. The supernatant was again centrifuged in two steps of 7080 and 12 434g for 10 min. Finally, the supernatant of the last centrifugation was collected and centrifuged at 105 000g for 1 h. The pellet containing the microsomal fraction was suspended in cold isolation medium at a final protein concentration of 20 mg ml $^{-1}$.

The incubation system for measuring 4-hydroxypropofol (2,6-diisopropyl-1,4-quinol) production from propofol was the following: 6 mM MgCl $_2$, 20 mM phosphate (pH 7.4), 1 mM NADPH, 25 μM propofol and 1 mg microsomal protein ml $^{-1}$. The temperature was 37°C and the final volume 1 ml. Closed flasks were used to avoid propofol evaporation (Court *et al.* 1999). The reaction was stopped at different times by the addition of 2 vols ethyl acetate. This addition was followed by vigorous mechanical shaking for 60 s. After 1 h, the supernatant was collected for chromatographic analysis.

Analytical

Tritiated water in the samples of the outflowing perfusate was assayed by means of liquid scintillation counting. The scintillation solution contained toluol/ethanol (10/2) and 5.0 g l $^{-1}$ 1,5-diphenyloxazole plus 0.2 g l $^{-1}$ 2,2-*p*-phenyl-bis-5-phenyleneoxazole. Tritiated water appearing in the outflowing perfusate was expressed as fraction of the portal concentration.

The ethyl acetate extracts from the supernatant of mitochondrial incubations were used for direct assay of propofol by means of spectrofluorometry, with excitation at 276 nm and emission at 310 nm. Standard curves were constructed with ethyl acetate extracts obtained from solutions containing known amounts of propofol. The amount of propofol in the supernatant of the mitochondrial incubations was considered as free propofol. Bound propofol was calculated as the difference between added propofol and free propofol.

The ethyl acetate extracts from perfusate and microsomal incubations were fractionated by means of HPLC. A HRC-ODS-Shimadzu column was used, with spectrofluorometric detection. Excitation at 276 nm and detection at 310 nm were used in most separation procedures. Alternatively, excitation at 288 nm and detection at 330 nm were used when trying to identify the propofol metabolite 4-hydroxypropofol. The mobile phase was composed of 70% acetonitrile plus 30% water containing 20 mM phosphate at pH 7.0. The flow rate was 1 ml min $^{-1}$. Temperature was kept at 25°C and the injection volume was always 20 μl .

The concentrations of propofol in each perfusate sample were calculated by means of the regression parameters obtained from a calibration curve. The calibration curve was constructed by separating chromatographically ethyl acetate extracts from standard Krebs–Henseleit-bicarbonate buffer solutions in the range 10–250 μM propofol. Within this range, a linear relationship was obtained between the concentration and the areas under the fluorescence curves. The linear regression coefficient was equal to 0.0002028 ± 0.00000459 and the correlation coefficient was 0.999. Propofol appearing in the outflowing perfusate was expressed as fraction of the portal concentration.

The protein content of mitochondrial and microsomal suspensions was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Histological examination

For histological examination, two lobules of perfused livers were fixed in 10% formaldehyde and embedded in paraffin. Slices obtained with a microtome were stained with haematoxylin and eosin for conventional microscopic examination. Three livers were examined for each of the following conditions: (1) 90 min of vehicle infusion (dimethylsulfoxide, 60 $\mu\text{l min}^{-1}$); and (2) 90 min of vehicle plus 250 μM propofol infusion.

Model analysis and calculations

The dilution curves of propofol were analysed by means of the space-distributed and variable transit-time model of Goresky *et al.* (1970, 1983a). This model describes the exchange between the cellular and extracellular space and metabolic transformation in the liver. For the case of flow-limited distribution in the whole liver space, the model of Goresky *et al.* yields the following equation for a step input (constant infusion) of a substance that undergoes irreversible transformation (Kelmer-Bracht *et al.* 1993):

$$Q_{\text{pro}}(t) = \int_{t_0}^t Q'_{\text{ref}}(t) \cdot e^{-k_3(t-t_0)} dt \quad (1)$$

where $Q_{\text{pro}}(t)$ is the normalized outflow profile of propofol (venous/portal concentration), t is the time after starting infusion and t_0 is the transit time in the large vessels plus catheters between the liver and collecting system. The measurable biotransformation rate parameter, k_3 , is a composite coefficient, a function of the kinetic constants of the enzymic systems involved in biotransformation (V_{max} and K_m), of the intracellular concentration and of the intracellular binding degree. The parameter k_3 has the dimensions of ml s^{-1} (ml total cellular space accessible to propofol) $^{-1}$. $Q'_{\text{ref}}(t)$ is the first derivative (i.e. $dQ_{\text{ref}}(t)/dt$) of the outflow profile of a hypothetical substance that undergoes flow-limited distribution within the same space as propofol but which does not undergo transformation. In practical terms, $Q'_{\text{ref}}(t)$ can be generated from the first derivative of a tritiated water curve (i.e. $Q'_{\text{water}}(t) = dQ_{\text{water}}(t)/dt$), provided that the tracer is infused simultaneously with propofol, by means of the following linear transformation (Kelmer-Bracht *et al.* 1993):

$$Q'_{\text{ref}}(t) = \frac{1}{1 + \Phi} Q'_{\text{water}}\left(\frac{t - t_0}{1 + \Phi} + t_0\right), \quad (2)$$

where $Q'_{\text{ref}}(t)$ is the first derivative of the [^3H]water curve at time $\left(\frac{t - t_0}{1 + \Phi} + t_0\right)$ and divided by the factor $(1 + \Phi)$. This factor is the ratio of the apparent distribution space of propofol to the distribution space of labelled water. When Φ is zero, the distribution space of propofol is equal to the water space. The parameter Φ is thus the ratio of the extra propofol space to the water space.

The cellular propofol concentration (C_i), including bound and free forms, was calculated according to the formula derived by Kelmer-Bracht *et al.* (1993):

$$C_i = (1 + \Phi + \Phi/\theta) \cdot C_e, \quad (3)$$

where θ is the ratio of intra- to extracellular water spaces and C_e is the mean extracellular propofol concentration. Equation (3) is valid under the assumption of a flow-limited distribution of propofol within the cellular space of the liver, a condition for which the rate coefficients for influx (k_1) and efflux (k_2) tend to infinity (Kelmer-Bracht *et al.* 1993). The mean extracellular propofol concentration was calculated from the portal (C_p) and venous (C_v) concentrations according to the formula proposed by Goresky *et al.* (1983b):

$$C_e = \frac{C_p - C_v}{\ln C_p - \ln C_v}, \quad (4)$$

where the parameters Φ , k_3 and t_0 are determined by fitting equations (1) and (2) to the experimental propofol curves employing a non-linear least-squares procedure (Björck and Dahlquist 1972). An iterative procedure that generates the appropriate reference curve according to equation (2) was used. Preliminary estimates of t_0 , Φ and k_3 were introduced and the iteration continued until the sum of squares of the differences between the experimental and calculated propofol curves (standard errors of the estimate) was minimal. Interpolation between the experimental points and calculation of the first derivative were done by means of a spline function. The trapezoid rule was used for numerical integration (Björck and Dahlquist 1972).

Binding of propofol to isolated mitochondria was analysed by means of the classical one-component saturation equation that relates the concentration of bound (B) to free ligand (F):

$$B = \frac{B_{\text{max}} F}{K_L + F}, \quad (5)$$

where $B_{\max}$ is the maximal binding capacity and K_L is the binding constant. Equation (5) was fitted to the experimental data by means of a non-linear least-squares procedure (Wilkinson 1961).

Results

Separation and assay of propofol and metabolites

Propofol was infused as a concentrated solution in dimethylsulfoxide simultaneously with [^3H]water by means of two infusion pumps. The concentration and the infusion rate of propofol were adjusted in such a way as to ensure final portal concentrations between 33 and 250 μM . Quantitative evaluation of propofol in the outflowing perfusate requires purification because transport of the drug certainly overlaps metabolic transformation, the latter leading to the appearance of metabolites in the outflowing perfusate. Extraction of the perfusate with ethyl acetate certainly eliminates most polar metabolites such as glucuronide and sulfate conjugates. Non-polar metabolites, however, are probably extracted by ethyl acetate and for this reason, the perfusate samples were submitted to purification by HPLC. Figure 1 shows three representative chromatograms. Fluorescence was used for detection with excitation at 276 nm and emission at 310 nm, reported to be an ideal combination for propofol (Court *et al.* 1999). The first chromatogram (figure 1A) corresponds to an ethyl acetate extract of the inflowing (portal) perfusion fluid, whose propofol content is known (130 μM). Propofol always appeared as a well-defined peak between 10 and 11 min after injection. The mean retention time of propofol in extracts of the portal fluid was equal to 10.54 ± 0.13 min. A small impurity appeared much earlier (retention time around 4.05 min). A component with a retention time around 10.56 ± 0.12 min appeared in all samples from the venous perfusate (figure 1B, C) and obviously corresponds to the propofol that escaped biotransformation during a single passage through the liver.

All samples of the effluent perfusate also presented a component with a mean retention time of 4.48 ± 0.04 min (figure 1B, C). This is likely to be 4-hydroxypropofol, which is known to be an important product of propofol hydroxylation in the endoplasmic reticulum (Court *et al.* 1999, 2001). Since 4-hydroxypropofol is not commercially available, microsomes were isolated and incubated under conditions where, according to Court *et al.* (1999), this compound can be expected to be the only significant product of propofol transformation (see the Materials and methods). Figure 2 shows two chromatograms of the ethyl acetate extract of such an incubation system after 5 min. The chromatograms reveal, besides propofol, a well-defined component with a mean retention time of 4.41 ± 0.01 min, which under the assumptions made above can be identified as 4-hydroxypropofol. Furthermore, figure 2A and B correspond to the same sample monitored at different wavelengths: (A) 276 and 310 nm, which are more suitable for propofol; and (B) 288 and 330 nm, which are more adequate for 4-hydroxypropofol. Fluorescence of the 4.41-min component was substantially higher at 288–330 nm, which is an expected phenomenon for 4-hydroxypropofol (Court *et al.* 1999). The same phenomenon was also observed when extracts of the venous perfusate samples were chromatographed and detection was done at 288–330 nm. It can thus be concluded that the 4.48-min component in the venous perfusate corresponds to 4-hydroxypropofol.

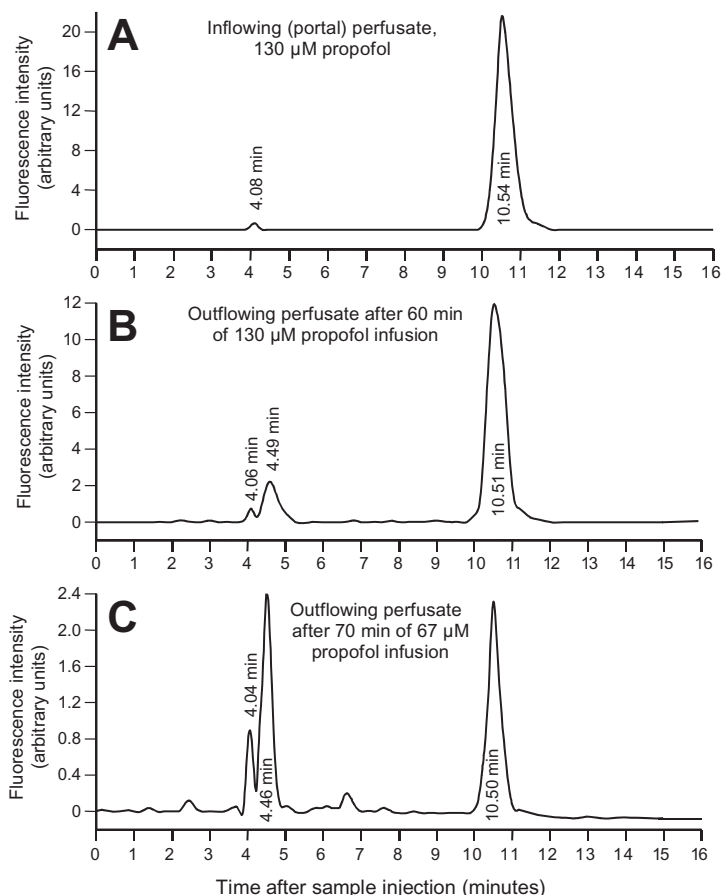


Figure 1. Typical chromatograms of perfusate from isolated rat liver. Propofol and 4-hydroxypropofol (2,6-diisopropyl-1,4-quinol) were extracted into ethyl acetate and quantified by HPLC with spectrofluorimetric detection (excitation 276 nm, emission 310 nm) as described in the Materials and methods.

The experiments with isolated microsomes were also used to calibrate the areas due to 4-hydroxypropofol fluorescence at 276–310 nm. The assumption underlying this procedure is that all decreases in propofol concentration correspond to a proportional increase in the 4-hydroxypropofol concentration in the incubation mixture. Under this assumption, the relation between increases in fluorescence due to hydroxypropofol (in arbitrary units) and decreases in propofol concentration (μM) should yield the corresponding calibration factor. Correlation analysis of this relationship produced a linear regression factor of 1.971 ± 317 ($r = 0.761$).

Outflow profiles of [^3H]water, propofol and 4-hydroxypropofol

Figure 3 shows the mean normalized outflow profiles of [^3H]water and propofol that were infused simultaneously. Normalization was accomplished by dividing the concentration of [^3H]water or propofol in the venous perfusate by the corresponding concentration in the portal perfusate. Normalization facilitates both comparison between different compounds and quantitative analysis. The outflow profile of [^3H]water was that expected for a substance that undergoes

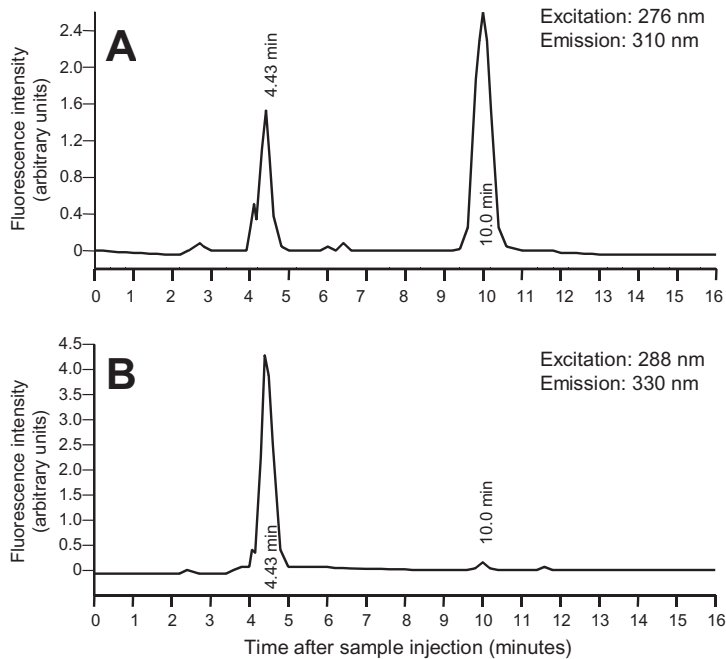


Figure 2. Typical chromatograms of microsomal incubations. Propofol was incubated with rat liver microsomes, then ethyl acetate extracts were analysed by HPLC. The chromatograms shown are from a 5-min incubation. (A, B) Optimal wavelengths for quantification of propofol and 4-hydroxypropofol, respectively.

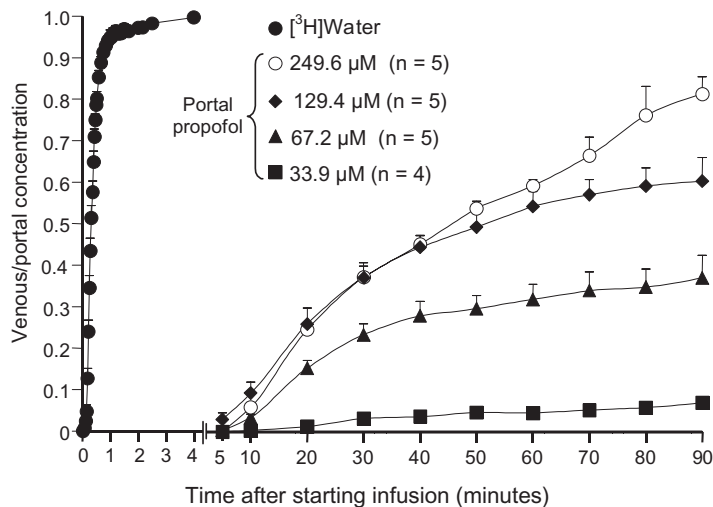


Figure 3. Normalized outflow profiles of $[^3\text{H}]\text{water}$ and propofol. Livers from fed rats were perfused with Krebs-Henseleit bicarbonate buffer, pH 7.4. Tritiated water ($2\mu\text{Ci min}^{-1}$) and propofol (at four different concentrations, as indicated) were infused simultaneously. $[^3\text{H}]\text{Water}$ was measured by liquid scintillation and propofol was assayed by HPLC. The venous/portal concentration ratios were plotted versus the time after onset of the infusion. Data are means $\pm$ mean standard errors.

flow-limited distribution into the aqueous space of the liver without undergoing substantial transformation: the normalized concentration of [^3H]water increased rapidly to values close to unity within the first 2 min (Bracht *et al.* 1980, Kelmer-Bracht *et al.* 1993). Propofol, however, was substantially delayed: significant amounts did not appear before 5 min after starting infusion. This delay predominantly reflects a much higher, real or apparent distribution space when compared with [^3H]water. At all portal concentrations, the venous/portal fraction of propofol approached asymptotically steady-state values below unity, a fact that most likely reflects metabolic transformation. For the lowest portal concentration (33.9 μM), the transformed fraction was around 95%. As the portal concentration was raised, however, the transformed fraction under steady-state conditions decreased progressively, denoting a saturable process. Note too that at the highest propofol concentrations used in the present study (249.6 μM), a secondary burst in the venous concentration was apparent during the last 20 min of infusion (70–90 min).

The kinetics of 4-hydroxypropofol appearance is shown in figure 4. The fluorescence areas of the 4-hydroxypropofol peaks at 276–310 nm were transformed into μM concentration by means of the calibration factor mentioned above. The expected error of this calibration is around 16%, but it was probably the same for all concentrations, so that the relative proportions were preserved. The metabolite 4-hydroxypropofol also began to appear relatively late in time and at concentrations that are a complex function of the portal propofol concentrations. For portal propofol concentrations of 33.9 and 67.2 μM , the hydroxypropofol production increased during the first 40 min of infusion and stabilized during the rest of the infusion time. With portal 129.4 μM propofol, however, a declining tendency at the longest infusion times was already apparent. At 249.6 μM propofol, a clear decline in hydroxypropofol production was observed from 50 to 90 min of infusion. The amount of 4-hydroxypropofol that appeared in the effluent

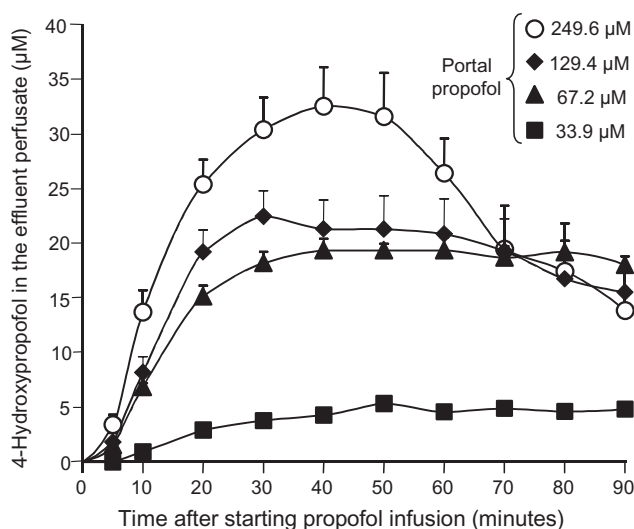


Figure 4. Outflow profiles of 4-hydroxypropofol. The concentrations of 4-hydroxypropofol measured by HPLC in the propofol infusion experiments shown by figure 2 were evaluated as described in the Results and represented against the time after the onset of propofol infusion. The mean data represent (μM) concentrations $\pm$ the corresponding mean standard errors.

perfusate at 90 min after initiation of propofol infusion corresponded to variable fractions of the total biotransformed propofol. For each of the four mean inflowing propofol concentrations shown in figure 4, namely 33.9, 67.2, 129.4 and 249.6 μM , 4-hydroxypropofol accounted for 17.8, 41.7, 39.2 and 30.2%, respectively, of the total propofol transformation.

Model analysis of the propofol outflow profiles

Full evaluation of the dilution curves in figure 4 requires model analysis. When analysed according to the model of Goresky *et al.* (1970, 1983a), the forms of the outflow profiles of propofol strongly suggest flow-limited distribution, a situation in which exchange rates between cellular and extracellular spaces tend to infinity and cannot be determined. More specifically, flow-limited distribution is suggested by the fact that the propofol curve was always delayed and entirely displaced to the right in relation to the [^3H]water curve. Dilution curves of substances such as [$1\text{-}^{14}\text{C}$]palmitate, whose distribution is not flow limited, are situated, especially in the initial up-slope, at the left of the labelled water curve (Ferraresi-Filho *et al.* 1992). If the distribution of propofol is really flow limited, the experimental curves should conform to equations (1) and (2). To verify this, these equations were fitted to all experimental curves using the numerical procedures described in the Materials and methods. In most cases, a good agreement was found between theory and experiment. Figure 5 shows an example. The experimental data shown were obtained with 67.2 μM propofol in the portal perfusate. The data points (●) were compared with the theoretical curve (—), calculated with optimized values for Φ , k_3 and t_0 , which are given in the legend to figure 5. The calculated curve describes reasonably well the experimental curve. If k_3 is set equal to zero, the curve calculated with the optimized values of Φ and t_0 represents the curve of the appropriate reference for propofol (---), as defined by

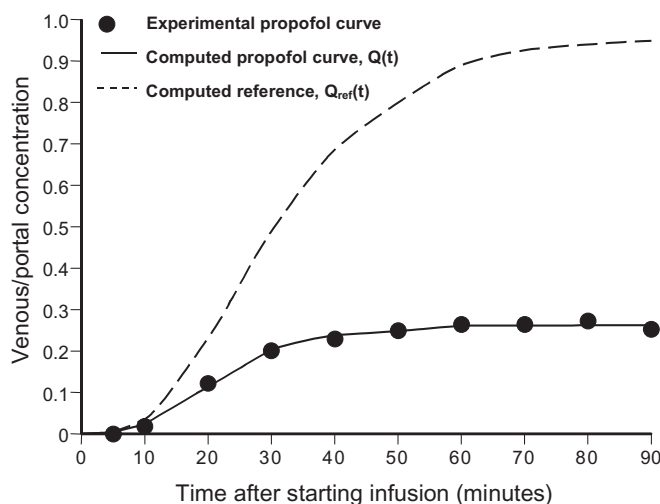


Figure 5. Fitting of equations (1) and (2) to a typical propofol outflow profile. The portal propofol concentration was 67.2 μM . Experimental data are shown (full symbols) as well as calculated curves. The continuous line ($Q(t)$) was calculated by combining equations (1) and (2) and introducing the optimized values of the parameters: $t_0 = 0.0755$ min, $k_3 = 0.0473$ $\text{ml min}^{-1} \text{ml}^{-1}$ and $\Phi = 122.1$. The standard error of the estimate was equal to 0.00788. The computed reference ($Q_{\text{ref}}(t)$) (traced line) was calculated using equation (2).

equation (2). This curve was very close to the experimental propofol curve at the initial up-slope, but it departed progressively and tended asymptotically to unity.

Equations (1) and (2) were fitted to all individual indicator dilution experiments (a total of 19 liver perfusion experiments). Similar good fits were obtained for the portal propofol concentrations in the range 33.9–129.4 μM . In the case of the highest propofol concentration used in the present study, 249.6 μM , a small but systematic deviation was found between experiment and theory for the last two measurements (80 and 90 min). This deviation reflects the fact that with 249.6 μM portal propofol, the venous concentration tended to increase again after 70 min of infusion instead of stabilizing as it occurred with all other concentrations (figure 3) and as predicted by equation (1).

Transport, distribution and transformation parameters of propofol

The two important parameters obtained by fitting equations (1) and (2) to the experimental propofol outflow profiles, the space ratio ($1 + \Phi$) and the composite rate coefficient for biotransformation (k_3), are represented in figure 6 against the portal propofol concentration. The propofol-to-water space ratio ($1 + \Phi$) was very high for all propofol concentrations. At 33.9 μM portal propofol, it was equal to 284.1 ± 39.4 . It decreased by approximately 50% when the portal propofol concentration was raised to 129.4 μM . However, when the portal propofol concentration was further increased to 249.6 μM , the space ratio ($1 + \Phi$) again increased by a small extent.

As shown in figure 6, the rate coefficient for biotransformation (k_3), which sums up all irreversible steps of propofol metabolic transformation, decreased

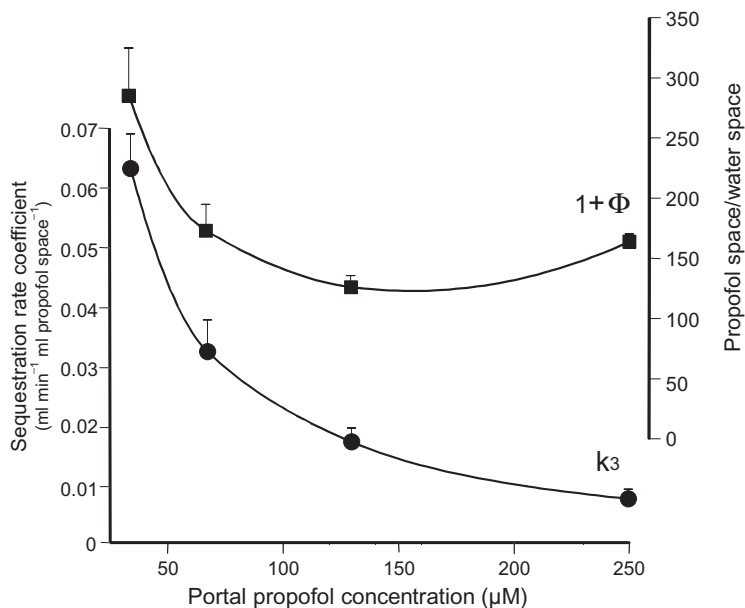


Figure 6. Derived parameters of indicator dilution experiments with variable portal propofol concentrations. The parameters Φ and k_3 were obtained by means of least-squares fits of equations (1) and (2) to the experimental data. Each datum point is the mean of four or five experiments. The vertical bars represent mean standard errors. They are not shown when smaller than the symbol sizes.

progressively with the increasing portal propofol concentrations. These decreasing values are expected as a consequence of saturation of the enzymic systems catalysing metabolic transformation (Kelmer-Bracht *et al.* 1993). This was indeed the case, as revealed by the steady-state rates of biotransformation of propofol, expressed as $\mu\text{mol min}^{-1} (\text{g liver wet weight})^{-1}$, which are shown in figure 7 against the portal concentration. The steady-state rates of propofol biotransformation were calculated as the differences between the rates of propofol infusion and of propofol outflow under steady-state conditions. Values corresponding to steady-state conditions were obtained from equation (1) by integrating between $t = t_0$ and $t \rightarrow \infty$. The portal concentration for half-maximal transformation rates obtained by graphical interpolation from figure 7 can be expected at about $25 \mu\text{M}$.

Figure 7 also shows the relation between the portal propofol concentration and the cellular propofol concentration (C_i). The latter was calculated according to equations (3) and (4). This calculation assumes flow-limited distribution of propofol and knowledge of the parameters C_e , Φ and θ , where C_e is the extracellular concentration. Rigorously, the extracellular concentration decreases along the sinusoids due to metabolic transformation so that a mean must be used for C_e . In the present work, the logarithmic mean suggested by Goresky *et al.* (1983b), which is given by equation (4), was used. Note that if a mean is used for C_e , the calculated C_i values will also be mean intracellular concentrations. The parameter θ is the ratio of intra- to extracellular water spaces in the liver, which in the haemoglobin-free perfused rat liver is equal to 1.5 (Ishii *et al.* 1987, Ferraresi-Filho *et al.* 1992, Kelmer-Bracht *et al.* 1993). It should be added that if calculated according to equation (3), C_i will refer to the whole intracellular water space. This does not imply, however, that the drug is only present as a free solute in the cellular aqueous medium. Actually, the calculated C_i values

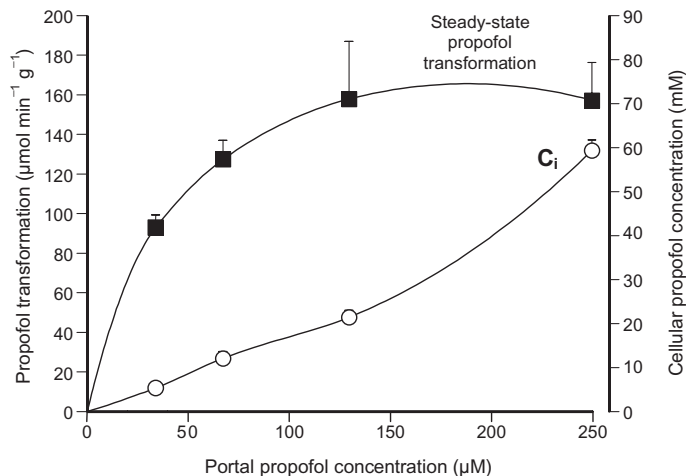


Figure 7. Steady-state rates of propofol biotransformation and cellular concentrations of propofol as a function of several portal propofol concentrations. The steady-state rates of propofol biotransformation, expressed as $\mu\text{mol min}^{-1} (\text{g liver wet weight})^{-1}$, were calculated as the differences between the rates of propofol infusion and the rates of propofol outflow under steady-state conditions. Values corresponding to steady-state conditions were obtained from equation (1) by integrating between $t = t_0$ and $t \rightarrow \infty$. The cellular propofol concentrations were calculated using equations (3) and (4). Each datum point is the mean of four or five experiments. The vertical bars represent mean standard errors. They are not shown when smaller than the symbol sizes.

represent the sum of bound plus free propofol. Owing to the high values of Φ , the cellular concentrations were much higher than the extracellular ones, as revealed by figure 7 in which C_e was expressed in μM and C_i in mM. Figure 7 shows a linear dependence between C_e and C_i for C_e values up to about $125\mu\text{M}$. For the highest extracellular concentration, however, an extra non-proportional increase in C_i was found.

Binding of propofol to isolated mitochondria

The very high cellular contents of propofol when compared with the extracellular concentrations suggest that the liver binds propofol with a high capacity and affinity. In order to get additional evidence for this phenomenon, binding of propofol to isolated rat liver mitochondria was investigated in the range up to $300\mu\text{M}$ and with a mitochondrial protein concentration of 2 mg ml^{-1} . The mean results are shown in figure 8, where bound propofol is shown versus free propofol. The data describe a saturation curve and the binding isotherm, represented by equation (5), was fitted by means of a non-linear least-squares procedure (Wilkinson 1961). The continuous line in figure 8 represents the curve calculated by means of equation (5) with the optimized B_{max} and K_L values. A maximal binding capacity (B_{max}) of $136.5 \pm 31.43\text{ nmol (mg protein)}^{-1}$ was found. Half-maximal binding (represented by K_L) can be expected at a free propofol/mitochondrial protein ratio of $89.8 \pm 41.2\text{ nmol mg}^{-1}$.

Action of propofol on liver structure

The appearance of propofol in the outflowing perfusate was visibly accelerated after 80 min of the $250\text{-}\mu\text{M}$ infusion (figure 3). In parallel with this phenomenon, the production of 4-hydroxypropofol was substantially reduced (figure 4). These

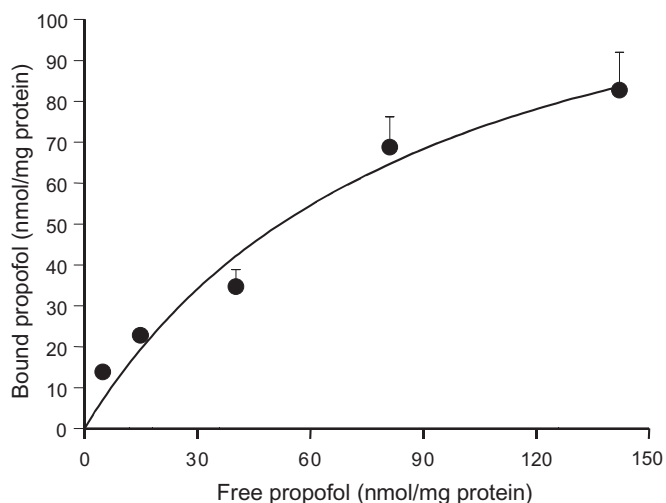


Figure 8. Binding of propofol to isolated rat liver mitochondria. Binding was measured as described in the Materials and methods. The data points represent means of five experiments. Bars represent mean standard errors; when not shown, they are smaller than the symbol sizes. The binding isotherm (equation 5) was fitted to the experimental data by means of a non-linear least-squares procedure. The traced line represents the fitted curve calculated according to equation (5) with the following optimized parameters: $B_{\text{max}} = 136.5 \pm 31.43\text{ nmol (mg protein)}^{-1}$; $K_L = 89.8 \pm 41.2\text{ nmol (mg protein)}^{-1}$. The error parameters represent standard deviations of the fitting procedure. The correlation coefficient was equal to 0.985.

phenomena could reflect liver damage induced by propofol. To verify this possibility, livers perfused solely with the vehicle (dimethylsulfoxide, $60 \mu\text{l min}^{-1}$) and vehicle plus $250 \mu\text{M}$ propofol were examined microscopically after haematoxylin and eosin staining. Figure 9A shows the control experiment (vehicle alone) and

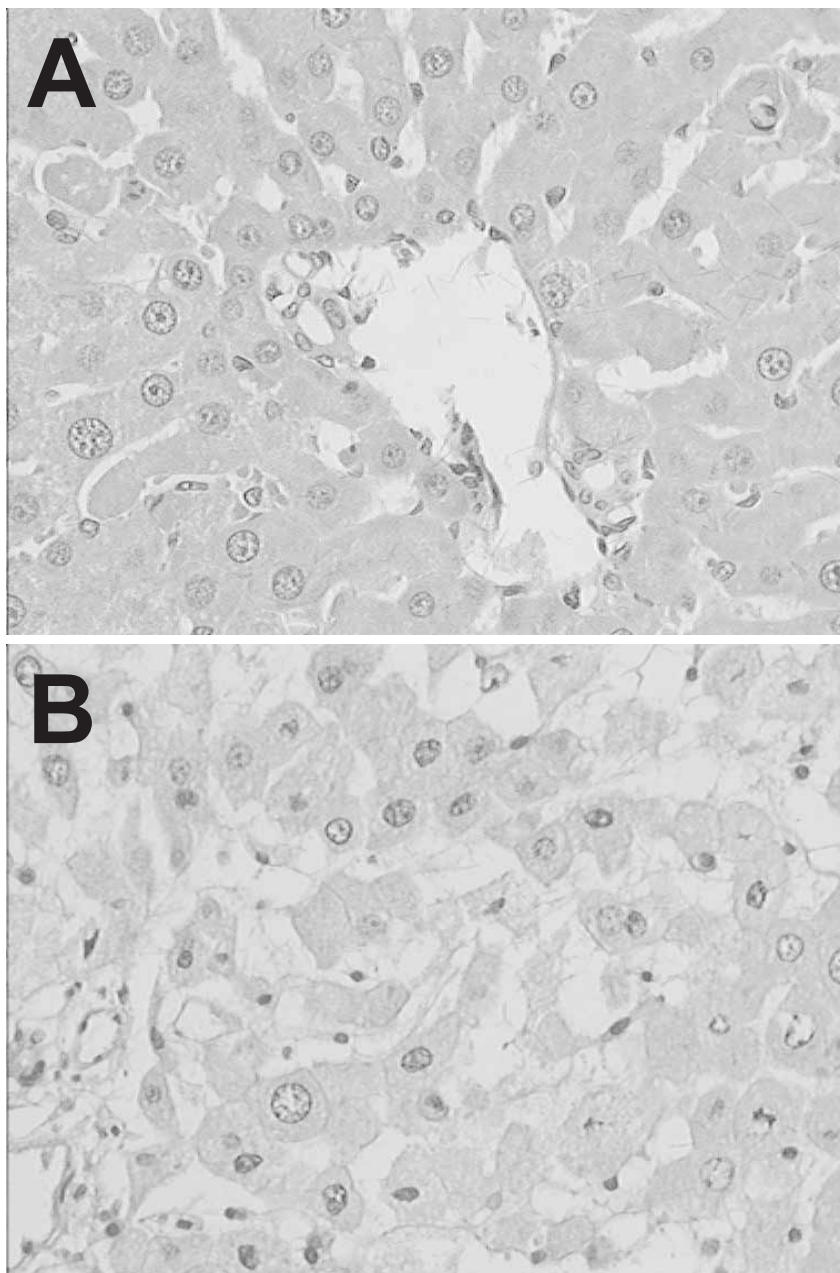


Figure 9. Action of a high propofol concentration on liver structure. (A) Typical haematoxylin and eosin stainings ($200\times$) after 90 min of vehicle infusion (dimethylsulfoxide, $60 \mu\text{l min}^{-1}$); (B) corresponding preparation ($200\times$) after 90 min of vehicle plus $250 \mu\text{M}$ propofol infusion.

figure 9B the influence of propofol after an infusion of 90 min. Livers treated solely with dimethylsulfoxide did not exhibit necrosis but variable degrees of vacuolization. Livers treated with 250 μ M propofol, however, presented severe multifocal necrosis with de-arrangement of the hepatocyte cords.

Discussion

The interactions of propofol and the rat liver present two important characteristics: (1) propofol permeates the plasma membrane at such high rates that its distribution can be considered as flow limited instead of barrier limited; and (2) the drug concentrates within the hepatic tissue to very high levels. The high rates of plasma membrane permeation are indicated by two main characteristics of the propofol outflow profiles. The first can be deduced from simple inspection of the outflow profiles: irrespective of the portal concentration, the appearance time of propofol was always considerably delayed in relation to the [^{3} H]water appearance time. This is typical for substances that undergo flow-limited distribution and subsequently distribute themselves into a space that is superior to the water space (Goresky *et al.* 1983a, Kelmer-Bracht *et al.* 1993). In opposition to this, for substances undergoing barrier-limited distribution, some material does not exchange with the cellular space during a single passage. This fraction, which is restricted to the vascular space during a single passage, appears at a time that precedes the appearance of tritiated water, which also distributes in a flow-limited manner (Goresky *et al.* 1970, Bracht *et al.* 1980). The second argument in favour of a flow-limited distribution is that the outflow-profiles could be adequately described by an equation set derived under the assumption of flow-limited distribution, namely equations (1) and (2). These equations result from the application of the flow-limited hypothesis to the assumptions underlying the space-distributed variable transit-time model of Goresky *et al.* (1970, 1983a).

The high apparent space of distribution of propofol is a reflection of the high cellular concentration and agrees with the high tissue/water and tissue/blood partition coefficients for propofol measured by Weaver *et al.* (2001) in sheep. It is also in agreement with the high distribution spaces reported in all pharmacokinetic studies in several mammalian species (Servin *et al.* 1988, Kanto and Gepts 1989, Zoran *et al.* 1993, Baroni *et al.* 2000). The high cellular concentrations most probably reflect binding to cellular structures, including membranes and proteins. Binding to proteins has already been demonstrated for at least two Ca^{2+} -ATPases in different areas of the cell, namely the plasma membrane and endoplasmic reticulum (Lopez and Kosk-Kosicka 1997). Furthermore, experiments described herein have also shown that propofol can bind to isolated mitochondria. It is likely that both phenomena, binding to mitochondria and to Ca^{2+} -ATPases, contribute to the total binding capacity detected in the whole liver, but it is highly probable that all membranes and many proteins contribute to the total binding capacity. The possibility that the high intracellular concentrations are the consequence of some type of concentrative active transport is unlikely. Since exchange is very fast, it would be very difficult for the cell to maintain such an active transport system because its activity would have to exceed by far the maximal capacity of the respiratory chain in phosphorylating ATP. Furthermore, propofol itself inhibits oxidative phosphorylation, a phenomenon that impairs all energy-dependent processes including active transport (Branca *et al.* 1991a, b).

There are two fundamental differences between the present study and the pharmacokinetic studies carried out with several species (Servin *et al.* 1988, Kanto and Gepts 1989, Zoran *et al.* 1993, Baroni *et al.* 2000). The first is rather obvious, i.e. in the present study, analysis was restricted to the liver. The second difference concerns the fact that in the present study the heterogeneity of the circulation was taken into account, whereas in all pharmacokinetic studies the whole circulatory system was taken as a homogeneous compartment. The heterogeneity of capillary transit times in the present experiments is indicated by the [^3H]water curve (Goresky *et al.* 1970, Bracht *et al.* 1980). The circulatory transit times are not homogeneous even for a single organ such as the liver. In the whole organism, such an assumption, which is inherent to compartmental pharmacokinetics, is evidently an oversimplification. This is possibly one of the reasons for which conclusions have oscillated between the two- and three-compartment models for propofol, sometimes within the same experimental series (Kanto and Gepts 1989, Zoran *et al.* 1993). On the other hand, the fact that the distribution of propofol in the liver is flow-limited also means that equilibrium exists between blood and the liver cells along the sinusoids. It is possible, though not yet demonstrated, that flow-limited distribution also occurs in other organs that can transform propofol. This would justify the assumption of most pharmacokinetic studies of a central compartment where biotransformation occurs, this central compartment being thus composed of blood, liver and eventually other propofol-transforming organs (Servin *et al.* 1988, Kanto and Gepts 1989, Zoran *et al.* 1993, Baroni *et al.* 2000).

The behaviour of propofol is very similar to that of the non-steroidal anti-inflammatory drug niflumic acid, whose distribution is also flow-limited (Kelmer-Bracht *et al.* 1993). The maximal apparent space of distribution of niflumic acid, however, exceeds the water space by a factor of 43.5, which is much less than the maximal space found for propofol, which exceeded the water space by a factor of 284.1. The behaviour of niflumic acid could also be described by the model of Goresky *et al.* (equations 1 and 2) in the range 10–1000 μM . In the case of propofol, however, deviations between theory and practice began to appear at a portal concentration of 249.6 μM after 70 min of infusion because the appearance of the drug was accelerated instead of stabilized, as predicted by theory and as occurred at lower portal concentrations. In parallel with this phenomenon, the production of 4-hydroxypropofol was substantially reduced whereas microscopic examination revealed considerable tissue damage. Probably, propofol at 249.6 μM began to exert deleterious effects on membranes at least after long infusion periods. It is known that the drug not only penetrates membranes very easily, but also can change its properties. For example, the drug has the ability to fluidize membranes (Tsuchiya 2001), a property which at high concentrations might destroy membrane structure and functions.

It has been frequently emphasized that propofol is rapidly metabolized, its transformation being, for example, 10 times faster than that of thiopental (Larijani *et al.* 1989). The data obtained in the present work provide full confirmation of this characteristic for the rat liver. At a portal concentration of 33 μM , the steady-state extraction was already 95%, making it practically impossible to obtain reliable measurements of dilution curves with lower portal concentrations. However, plasma propofol concentrations during anaesthesia are generally below 33 μM (Shafer *et al.* 1988, Engdahl *et al.* 1998). Consequently, it seems reasonable

to expect that, under most clinical situations, all the propofol that reaches the liver is almost totally transformed during a single passage. The interpolated portal concentration for half-maximal rates of biotransformation, about 25 μM , is in good agreement with several enzyme kinetic data. For example, K_m values between 20 and 41 μM have been found for several systems catalysing hydroxylation reactions (Court *et al.* 2001) and a K_m of 32.5 μM has been reported for glucuronidation of propofol by rat liver microsomes (Shimizu *et al.* 2003). In the present experiments, the total propofol transformation exceeded the rate of 4-hydroxypropofol release, an indication that glucuronic acid and sulfate derivatives were also produced by the liver. On the other hand, the fact that the liver could release significant amounts of 4-hydroxypropofol is an indication that hydroxylation is not necessarily the rate-limiting step for conjugation. The availability of glucosyl units and sulfate for conjugation in the perfused liver should not be responsible for the release of non-conjugated 4-hydroxypropofol. Sulfate is present in the perfusion fluid and livers from fed rats, such as those employed in the present study, have a glycogenolytic flux between 1.5 and 2.0 $\mu\text{mol min}^{-1} \text{g}^{-1}$ (Itinose *et al.* 1989), whereas the maximal rate of 4-hydroxypropofol release was equal to 0.11 $\mu\text{mol min}^{-1} \text{g}^{-1}$. The availability of glucosyl units in the perfused liver, for example, is high enough to ensure rapid transformation of acetaminophen, a compound which is released by the liver solely in conjugated form (Itinose *et al.* 1989, 1995). Release of 4-hydroxypropofol by the perfused liver may be an indication that the liver operates as an active source of 4-hydroxypropofol for conjugation in tissues such as the kidney, gut wall and lung when the organism is actively metabolizing propofol and excreting propofol metabolites. Conjugation activities have been demonstrated to exist in these tissues, a fact that corroborates the hypothesis (Raoof *et al.* 1996a, b). It should be added that conjugation to glucuronic acid or sulfate seems to be essential for excretion because neither free propofol nor free 4-hydroxypropofol have been found in significant amounts in the urine (Favetta *et al.* 2000).

The high solubility of propofol within the hepatic cell space is a property that can be extended to its metabolite 4-hydroxypropofol. This is indicated by the observation that 4-hydroxypropofol also appears late in time, indicating that release of the metabolite to the perfusate began only after the achievement of some degree of saturation of the intracellular binding sites. These binding sites are most probably similar to those of propofol, as can be judged by the structural similarity of both compounds. It has been reported that 4-hydroxypropofol possesses approximately one-third of the hypnotic activity of propofol (Simons *et al.* 1991). For this reason, in spite of the observation that single-pass transformation of propofol in the liver can be almost complete for clinically relevant concentrations, this phenomenon should not represent a complete inactivation of the anaesthetic activity, which probably continues to be exerted, though less efficiently, by 4-hydroxypropofol.

Acknowledgements

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Role of individual human cytochrome P450 enzymes in the *in vitro* metabolism of hydromorphone

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1. The aim was to identify the individual human cytochrome P450 (CYP) enzymes responsible for the *in vitro* *N*-demethylation of hydromorphone and to determine the potential effect of the inhibition of this metabolic pathway on the formation of other hydromorphone metabolites.

2. Hydromorphone was metabolized to norhydromorphone (apparent $K_m = 206 - 822 \mu\text{M}$, $V_{\max} = 104 - 834 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) and dihydroisomorphine (apparent $K_m = 62 - 557 \mu\text{M}$, $V_{\max} = 17 - 122 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) by human liver microsomes.

3. In pooled human liver microsomes, troleandomycin, ketoconazole and sulfaphenazole reduced norhydromorphone formation by an average of 45, 50 and 25%, respectively, whereas furafylline, quinidine and omeprazole had no effect. In an individual liver microsome sample with a high CYP3A protein content, troleandomycin and ketoconazole inhibited norhydromorphone formation by 80%.

4. The reduction in norhydromorphone formation by troleandomycin and ketoconazole was accompanied by a stimulation in dihydroisomorphine production.

5. Recombinant CYP3A4, CYP3A5, CYP2C9 and CYP2D6, but not CYP1A2, catalysed norhydromorphone formation, whereas none of these enzymes was active in dihydroisomorphine formation.

6. In summary, CYP3A and, to a lesser extent, CYP2C9 catalysed hydromorphone *N*-demethylation in human liver microsomes. The inhibition of norhydromorphone formation by troleandomycin and ketoconazole resulted in a stimulation of microsomal dihydroisomorphine formation.

Introduction

Hydromorphone is a semisynthetic μ -opioid analgesic prepared by electrolytic reduction of morphine or by oxidation of dihydromorphone (Eddy and Reid 1934, Rapoport *et al.* 1950). It is used for the relief of moderate to severe pain and is a useful alternative to morphine where patients are not responsive to this drug or when the adverse effects of morphine, such as nausea and sedation, are severe (Lawlor *et al.* 1997).

In addition to the free drug, hydromorphone has been reported to be excreted in human urine as: norhydromorphone, dihydromorphone, dihydroisomorphine, hydromorphone-3-glucuronide, dihydromorphone-3-glucuronide, dihydroisomorphine-3-glucuronide and hydromorphone-3-sulfate (Cone *et al.* 1977, Zheng

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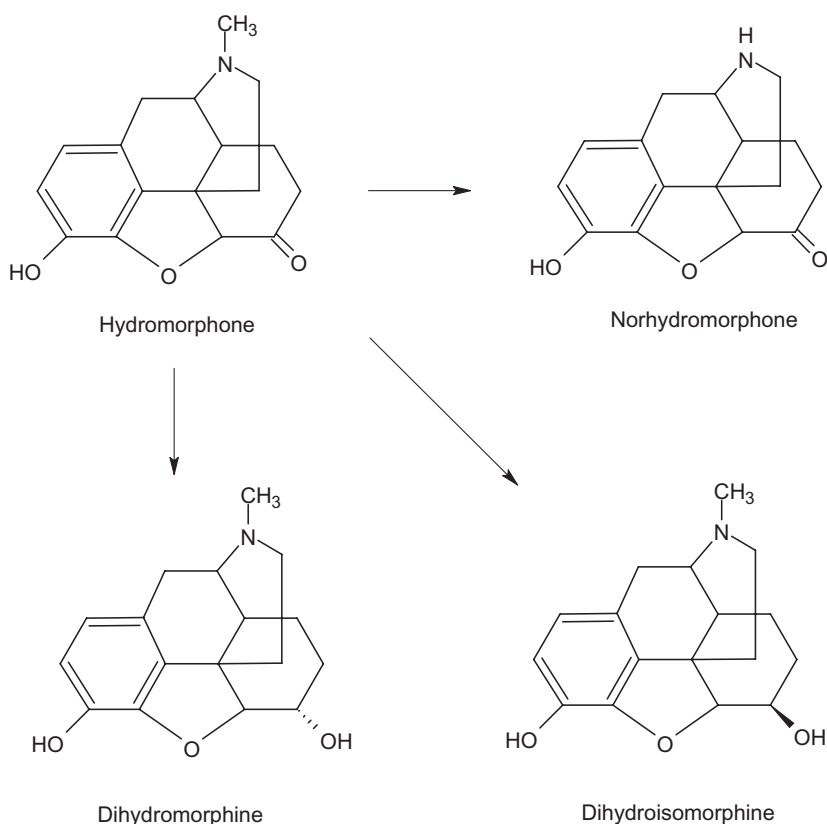


Figure 1. Chemical structures of hydromorphone, norhydromorphone, dihydromorphone and dihydroisomorphine.

1997). Despite the fact that the *N*-demethylated (norhydromorphone) metabolite of hydromorphone (figure 1) is known to be formed, the specific cytochrome P450 (CYP) enzymes involved in catalysing this metabolic pathway of hydromorphone have not been identified. Such information is important in predicting potential drug interactions with hydromorphone because patients taking an opioid analgesic are often on multiple drug therapy.

The study was conducted to identify the specific human CYP catalysts of *N*-demethylation of hydromorphone by employing an experimental strategy that involved the use of human liver microsomes, enzyme-selective chemical inhibitors and human recombinant CYP enzymes. The effect of the inhibition of the formation of norhydromorphone on the formation of dihydromorphone (6 α -hydroxyl metabolite, formed by the 6-ketone reduction of hydromorphone; figure 1) and dihydroisomorphine (6 β -hydroxy metabolite) was also investigated.

Materials and methods

Chemicals

Hydromorphone hydrochloride (99.5% pure; liquid chromatography coupled with tandem mass spectrometry detection, i.e. LC/MS/MS) was kindly provided by Knoll Pharma, Inc. (Mississauga, Ont., Canada). Nalorphine hydrochloride was obtained from Health Canada (Ottawa, Ont., Canada). Norhydromorphone, dihydroisomorphine and dihydromorphone were synthesized in the authors'

laboratory by Dr Ming Zheng (1997). Elemental and spectral analyses indicated that the purities of the synthetic materials were greater than 99.5%. Troleandomycin (TAO), sulfaphenazole, omeprazole, quinidine sulfate, furafylline, ketoconazole and β -nicotinamide adenine dinucleotide phosphate (NADPH) were from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of high purity and obtained from commercial sources.

Human liver microsomes and recombinant CYP enzymes

Pooled human liver microsomes (cat. no. H161), individual human liver microsomes (cat. nos HG23, HG42, HG66, HG112), microsomes from baculovirus-infected insect cells co-expressing NADPH-cytochrome P450 reductase and human CYP1A2 (cat. no. P203), CYP2C9 (cat. no. P218), CYP2D6 (cat. no. P217), CYP3A4 (cat. no. P207) or CYP3A5 (cat. no. P235), and the corresponding control insect cell microsomes (cat. no. P201) were from GENTEST Corp. (Woburn, MA, USA). The CYP and protein concentration of each enzyme preparation were provided by the supplier.

Incubation conditions

Each standard 200- μ l incubation contained 50 mM hydroxyethylpiperazine ethane sulfonate (HEPES) buffer, pH 7.4, 0.1 mM ethylenediamine tetra-acetic acid, 3 mM $MgCl_2$, human liver microsomes (100 μ g protein), hydromorphone (at the concentrations indicated in each figure legend) and 1 mM NADPH. Each incubation was performed at 37°C for 20 min and terminated by the addition of 500 μ l 0.2 M sodium bicarbonate, pH 10. The internal standard (nalorphine) was added (5 μ g ml⁻¹) and the mixture was kept on ice. The metabolites were extracted twice with 4 ml 1-propanol:dichloromethane (1:9) using Chem Elut liquid-liquid extraction cartridges (Varian, Inc., Harbor City, CA). The organic layer was evaporated to dryness under a stream of nitrogen and the residue reconstituted with 200 μ l 4% methanol in water containing 0.1% formic acid. Initial experiments showed that the assay was linear with respect to incubation time and the amount of enzymes, and that 50 mM HEPES, pH 7.4, buffer yielded the greatest amount of the dihydroisomorphine metabolite when compared with other buffers such as 50 mM Tris and 50 or 100 mM potassium phosphate (data not shown). The formation of norhydromorphone was not influenced by the buffer solution used. Incubations were also performed in the absence of NADPH to assess if metabolite formation was NADPH dependent.

To investigate the metabolism of hydromorphone by individual human CYP enzymes, microsomes containing 10 pmol human recombinant CYP1A2, CYP2C9, CYP2D6, CYP3A4 or CYP3A5 and the corresponding insect cell control microsomes were incubated with 1 mM hydromorphone for 2 h as described above. The reaction was terminated by the addition of 25 μ l perchloric acid, internal standard, and frozen until analysis. For analysis, 20 μ l of the samples were injected directly onto the liquid chromatography (extraction was not performed to improve sensitivity).

Metabolite analysis

Incubations were analysed for the parent drug and its metabolites by LC/MS/MS using a Hewlett Packard series II HPLC 1090 system (Hewlett Packard, Avondale, CA) with a C18 (150 $\times$ 2.0 mm, 5 μ m) column. The mobile phase started as a solution of 4% methanol in water containing 0.1% formic acid for 6 min, increased to 20% methanol within the next 12 min and then sharply (0.1 min) increased to 57% methanol and kept at this concentration for 3 min before decreasing sharply (0.1 min) to 4% methanol and kept at this initial methanol concentration for column equilibration for the next 8 min. The flow rate and injection volume were 0.12 ml min⁻¹ and 20 μ l, respectively. A Fisons VG Quattro triple quadrupole mass spectrometer (Fisons, Inc., Pointe Claire, Quebec) equipped with an electrospray interface was used for detection. The operation of the LC/MS/MS system was controlled by the MassLynx 3.1 computer software program (Fisons, Inc., Pointe Claire, Quebec). Tandem mass spectrometry (MS/MS) analysis was performed in the positive ion mode using multiple reaction monitoring. The transitions monitored were: hydromorphone (286 > 185), dihydromorphine and dihydroisomorphine (288 > 185), norhydromorphone (272 > 185), and the internal standard nalorphine (312 > 152). The amount of each metabolite was calculated from the area ratio of the metabolite and the internal standard. The LC/MS/MS assay validated with respect to linearity ($r^2 > 0.99$), accuracy (< 10%), intraday precision (< 10%), interday precision (< 10%), and extraction efficiency for norhydromorphone (83 $\pm$ 3%), dihydroisomorphine (90 $\pm$ 2%) and dihydromorphine (90 $\pm$ 4%). Owing to the fact that the calibration curves allow for these recoveries, these data are presented for information only.

Enzyme kinetic analysis

To determine the apparent K_m and V_{max} for the metabolism of hydromorphone in human liver microsomes, enzyme kinetic experiments were performed (in duplicate incubations on the same day) with eight to 10 concentrations of hydromorphone ranging from 5 to 1500 μ M. The metabolite formation-substrate concentration data were fitted to the Michaelis-Menten equation and the apparent K_m and V_{max} were determined by non-linear regression analysis (SigmaPlot 2000, v.6.0). The *in vitro* intrinsic clearance was calculated as the ratio of $V_{max}/\text{apparent } K_m$ and expressed as μ l min⁻¹ mg⁻¹ microsomal protein.

Chemical inhibition experiments

The effect of CYP enzyme-selective chemical inhibitors such as furafylline (CYP1A2), sulfaphenazole (CYP2C9) (Newton *et al.* 1995), omeprazole (CYP2C19) (Ko *et al.* 1997), quinidine (CYP2D6) (Newton *et al.* 1995), TAO (CYP3A) (Chang *et al.* 1994, Newton *et al.* 1995), and ketoconazole (CYP3A) (Newton *et al.* 1995) on norhydromorphone formation was examined by conducting the hydromorphone metabolism assay in the presence of one of these chemicals. The inhibitor concentrations used were similar to those previously shown to inhibit the respective enzymes (Chang *et al.* 1994, Newton *et al.* 1995, Ko *et al.* 1997); furafylline, sulfaphenazole and TAO at 5, 10 and 20 μM ; omeprazole, quinidine and ketoconazole at 1, 5 and 10 μM . Quinidine sulfate was dissolved in water, whereas the other chemical inhibitors were dissolved in methanol (final concentration of the vehicle was $\leq 0.5\%$ v/v). In the experiments with TAO and furafylline, each compound was pre-incubated with microsomes and NADPH for 30 min before the addition of the substrate (Chang *et al.* 1994, Newton *et al.* 1995). Experiments with the other inhibitors were conducted in the absence of a pre-incubation step.

Statistical analysis

Statistical analysis to investigate the effects of inhibitor was carried out in GraphPad Prism (San Diego, CA) (v.3.02) using one-way analysis of variance with Dunnett's multiple comparison test.

Results

Metabolite identification by LC/MS/MS

Hydromorphone was metabolized to norhydromorphone and dihydroisomorphine by human liver microsomes, but this required the presence of NADPH. The identity of the metabolites was confirmed by comparison with the retention times of the authentic reference standards. Dihydromorphone was an impurity ($\leq 0.5\%$) present in the hydromorphone, but it was well separated from hydromorphone, norhydromorphone and dihydroisomorphine by the LC/MS/MS method. Lengthening the incubation time or increasing the concentration of microsomal protein did not increase the amount of dihydromorphone, indicating that this metabolite was not formed enzymatically by human liver microsomal enzymes (data not shown).

Enzyme kinetic analysis of hydromorphone metabolism by human liver microsomes

Initial experiments to characterize the enzyme kinetics of hydromorphone metabolism were performed with pooled human liver microsomes and the apparent K_m and $V_{\max}$ are shown in table 1. Enzyme kinetic analysis with individual

Table 1. Enzyme kinetic analysis of hydromorphone metabolism by human liver microsomes.

HLM sample	Microsomal formation of norhydromorphone			Microsomal formation of dihydroisomorphine		
	K_m^a	$V_{\max}$	Cl_{int}	K_m^a	$V_{\max}$	Cl_{int}
Pooled	245	156	0.65	223	44	0.20
HL 112	822	834	1.04	296	31	0.12
HL 42	206	305	1.54	62	17	0.32
HL 66	270	141	0.55	258	48	0.19
HL 23	352	104	0.30	557	122	0.22

^aApparent K_m (μM); $V_{\max}$ ($\text{pmol min}^{-1} \text{mg}^{-1}$ microsomal protein); Cl_{int} ($\mu\text{l min}^{-1} \text{mg}^{-1}$ microsomal protein) = *in vitro* clearance intrinsic ($V_{\max}/K_m$).

Pooled human liver microsome (HLM) sample was comprised of 10% of the microsomal protein from each of the 10 individual human liver microsome preparations.

Results are the average of two independent experiments performed in duplicate.

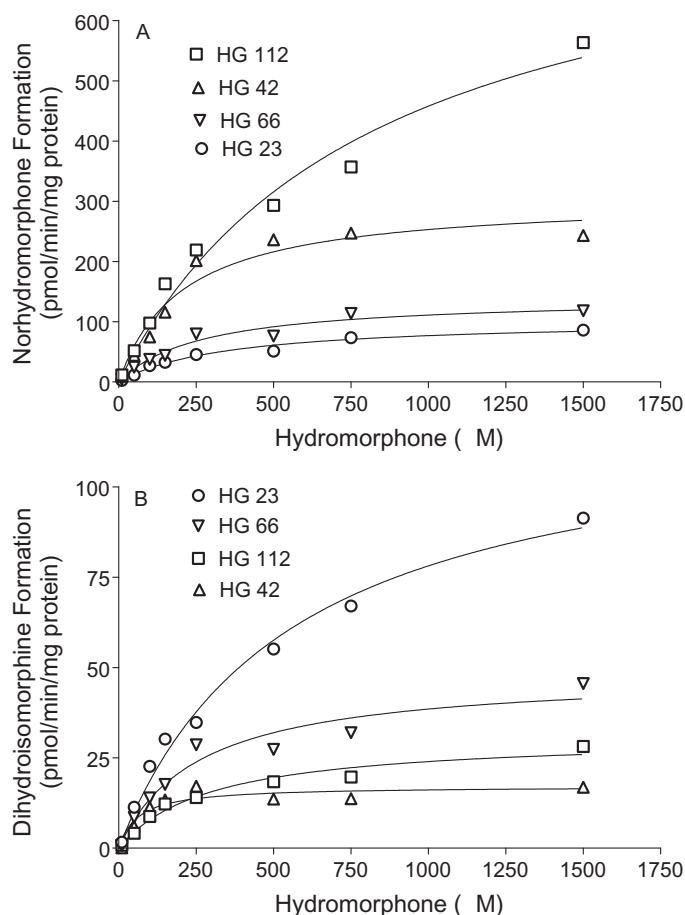


Figure 2. Enzyme kinetic analysis of the metabolism of hydromorphone to norhydromorphone (A) and dihydroisomorphine (B) by human liver microsomes. Hydromorphone (10–1500 μ M) was incubated with individual human liver microsomal preparations (HG23, HG42, HG66, HG112, 100 μ g microsomal protein) and NADPH (1 mM) at 37°C for 20 min. Each point is the average of two determinations.

human liver microsome samples indicated considerable interindividual differences in the enzymatic conversion of hydromorphone to norhydromorphone (figure 2A) and dihydroisomorphine (figure 2B). The apparent K_m for the metabolism of hydromorphone to norhydromorphone ranged from 206 to 822 μ M, whereas an eightfold difference was obtained for the V_{max} of this reaction (table 1). Similar interindividual differences in microsomal formation of dihydroisomorphine were also obtained. In three of the four individual samples tested, human liver microsomes were more efficient (three- to 10-fold) in catalysing norhydromorphone formation when compared with dihydroisomorphine formation, as assessed by the ratio of V_{max} /apparent K_m (*in vitro* intrinsic clearance) (table 1).

Inhibition of human liver microsomal hydromorphone metabolism

To determine the role of specific CYP enzymes in the *N*-demethylation of hydromorphone in human liver microsomes, the hydromorphone metabolism assay was conducted in the presence of a CYP enzyme-selective chemical

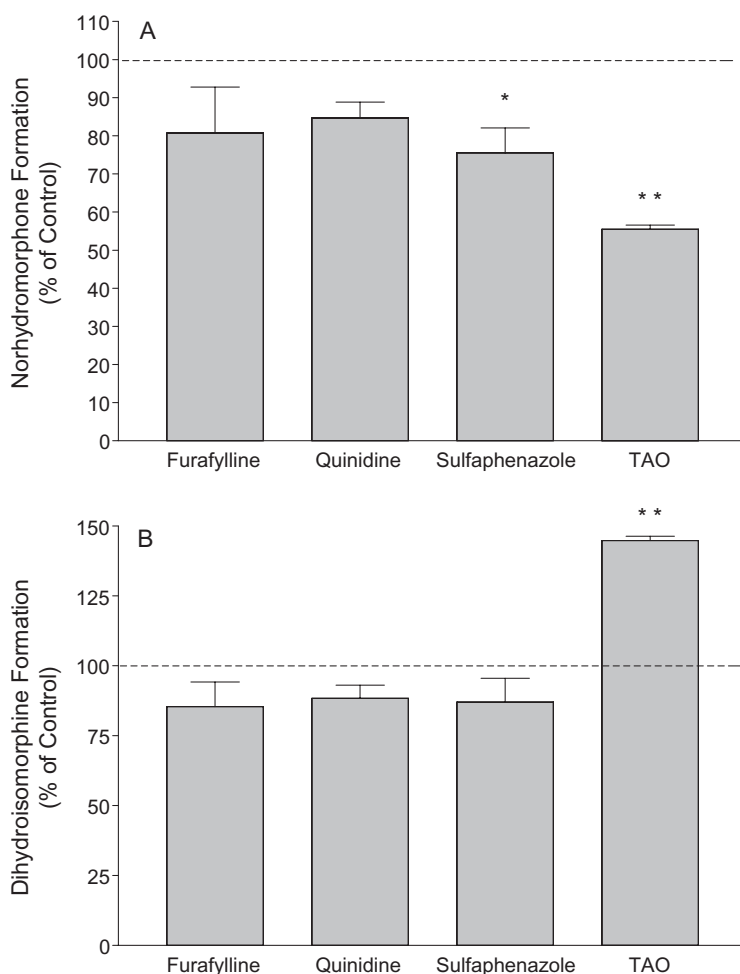


Figure 3. Effect of CYP enzyme-selective inhibitors on the rate of hydromorphone metabolism to norhydromorphone (A) and dihydroisomorphine (B) in pooled human liver microsomes (pool of 10 livers). The hydromorphone (500 μ M) metabolism assay was conducted as in figure 2, but 1 with the inclusion of furafllyline (5 μ M), quinidine (1 μ M), sulfaphenazole (5 μ M) or TAO (5 μ M), as described in the Materials and methods. Values are the mean $\pm$ SD ($n=4$, $n=3$ for quinidine) shown as the percentage of control activity (no inhibitor). The control activities for the formation of norhydromorphone and dihydroisomorphine were 293 and 20 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein, respectively. * $p < 0.05$, ** $p < 0.001$ compared with controls.

inhibitors, such as furafllyline (CYP1A2), sulfaphenazole (CYP2C9), omeprazole (CYP2C19), quinidine (CYP2D6) and TAO or ketoconazole (CYP3A) (Chang *et al.* 1994, Newton *et al.* 1995, Ko *et al.* 1997). As shown in figure 3, TAO (5 μ M) and sulfaphenazole (5 μ M) reduced the enzymatic formation of norhydromorphone by 45 and 25%, respectively, in a sample of pooled human liver microsomes. In contrast, furafllyline (5 μ M), quinidine (1 μ M) and omeprazole (1 μ M) did not result in a statistically significant effect on norhydromorphone formation (figure 3 and data not shown). Increasing the concentrations of furafllyline, quinidine and omeprazole also did not reduce the formation of norhydromorphone. To confirm the results obtained with TAO, chemical inhibition experiments were performed with ketoconazole, which is another CYP3A-selective inhibitor (Newton *et al.* 1995).

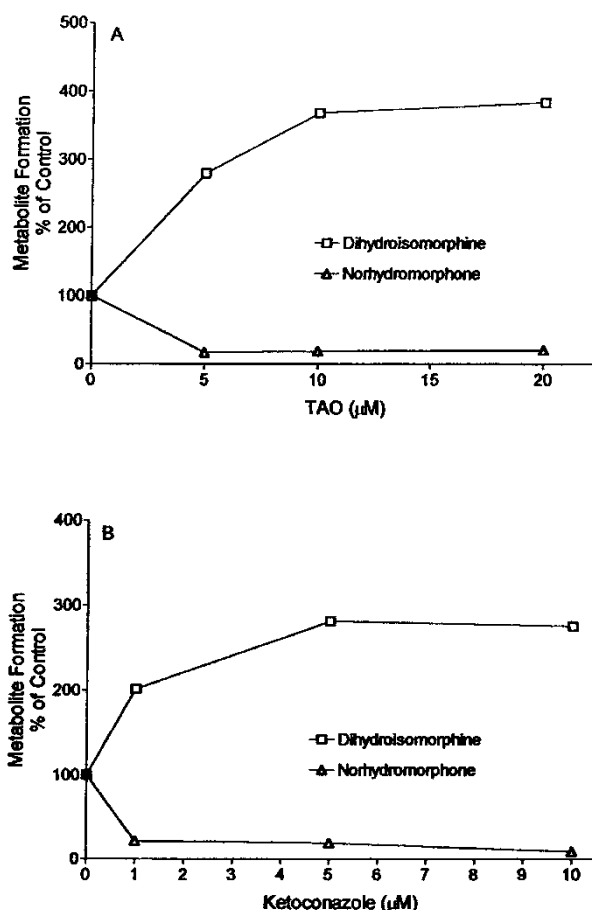


Figure 4. Effect of TAO (A) and ketoconazole (B) on the rate of hydromorphone metabolism to norhydromorphone and dihydroisomorphine in an individual human liver microsomal preparation (HG 112) containing a high CYP3A4 content. Hydromorphone ($500\text{ }\mu\text{M}$) metabolism assay was conducted as described in figure 2, but with the inclusion of TAO (5, 10 or $20\text{ }\mu\text{M}$) or ketoconazole (1, 5 or $10\text{ }\mu\text{M}$) as described in the Materials and methods. Each point is the average of two determinations. The control activities for the formation of norhydromorphone and dihydroisomorphine were 102 and $96\text{ pmol min}^{-1}\text{ mg}^{-1}$ protein, respectively.

Similar to the effect of TAO, ketoconazole reduced the enzymatic formation of norhydromorphone by about 50% (data not shown). In an individual human liver microsome sample with a high CYP3A protein content, TAO and ketoconazole reduced norhydromorphone formation by 80%. Maximal inhibition was achieved at inhibitor concentrations ($5\text{ }\mu\text{M}$ for TAO and $1\text{ }\mu\text{M}$ for ketoconazole) that reflect selective inhibition of CYP3A (Chang *et al.* 1994, Newton *et al.* 1995). Interestingly, the inhibition of norhydromorphone formation by TAO (figure 4A) and ketoconazole (figure 4B) was accompanied by an increase in dihydroisomorphine formation. Similar effects by TAO (figure 3A, B) and ketoconazole (data not shown) were obtained when pooled human liver microsomes were used in the incubations. In contrast, the inhibition of norhydromorphone formation by sulfaphenazole (figure 3A) did not result in a stimulation of dihydroisomorphine formation (figure 3B).

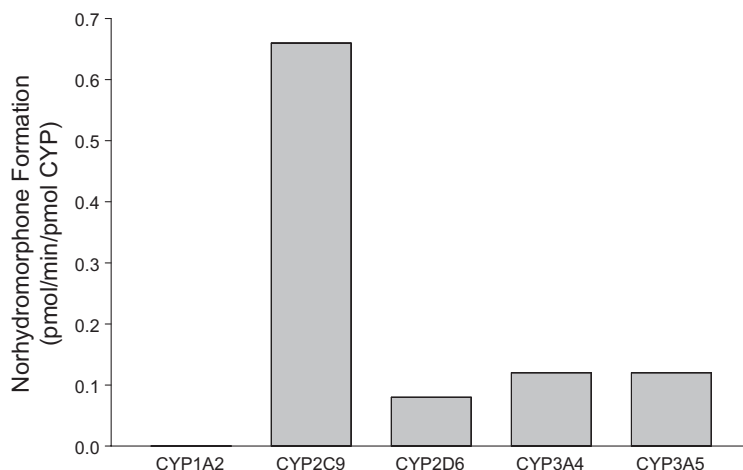


Figure 5. Metabolism of hydromorphone to norhydromorphone by human recombinant CYP enzymes. Hydromorphone (1 mM) was incubated with 10 pmol CYP1A2, CYP2C9, CYP2D6, CYP3A4 or CYP3A5, and NADPH (1 mM) at 37°C for 2 h. The results are the average of two experiments.

Metabolism of hydromorphone by human recombinant CYP enzymes

To corroborate the findings obtained with human liver microsomes and CYP enzyme-selective inhibitors, experiments were performed with human recombinant CYP enzymes. As shown in figure 5, CYP2C9, CYP2D6, CYP3A4 and CYP3A5 were active in catalysing the formation of norhydromorphone, whereas CYP1A2 was inactive. By comparison, none of these enzymes catalysed the metabolism of hydromorphone to dihydroisomorphine (data not shown).

Discussion

The present study provides the first direct demonstration for the role of specific CYP enzymes in the *N*-demethylation of hydromorphone. The novel finding also indicates that the inhibition of this metabolic pathway is associated with alteration in the 6-ketone reduction pathway that forms dihydroisomorphine.

CYP3A enzymes contributed substantively to the *N*-demethylation of hydromorphone to form norhydromorphone in human liver microsomes. This conclusion is based on the observation that TAO and ketoconazole, which are selective inhibitors of CYP3A (Chang *et al.* 1994, Newton *et al.* 1995), reduced the rate of this metabolic reaction. In agreement with these findings, human recombinant CYP3A4 and CYP3A5 were catalytically active in the metabolic conversion of hydromorphone to norhydromorphone. CYP2C9 is another catalyst of norhydromorphone formation, as judged by experiments with recombinant CYP2C9 and in human liver microsomal experiments with a CYP2C9-selective inhibitor sulfaphenazole. In addition to these enzymes, recombinant CYP2D6 also catalysed this reaction. However, quinidine, which is a CYP2D6-selective inhibitor (Newton *et al.* 1995), did not significantly reduce norhydromorphone formation in our pooled sample of human liver microsomes, suggesting a lack of contribution by the human liver microsomal CYP2D6 enzyme.

The involvement of more than one CYP enzyme catalysing the metabolism of hydromorphone to form norhydromorphone is consistent with the present enzyme kinetic analysis indicating differences in the apparent K_m in norhydromorphone formation in the panel of individual human liver microsomes. However, the relative contribution of CYP3A and CYP2C9 to hepatic norhydromorphone formation is likely to differ between patients because of the interindividual differences in the hepatic expression of these enzymes (Shimada *et al.* 1994). Whereas CYP3A is subject to induction and inhibition (Guengerich 1999), CYP2C9 is subject to genetic polymorphism (Lee *et al.* 2002). In a microsome sample known to express high levels of CYP3A protein, TAO inhibited norhydromorphone formation to a major extent (80%), suggesting that CYP3A was the major catalyst for this reaction in that particular individual. Interestingly, CYP3A is also a major catalyst in the *N*-demethylation of dihydrocodeine (Kirkwood *et al.* 1997), codeine (Caraco *et al.* 1996), dextromethorphan (Schmider *et al.* 1997) and ethylmorphine (Liu *et al.* 1995), which are structurally similar to hydromorphone. In the case of dextromethorphan and ethylmorphine, an enzyme(s) other than CYP3A may also contribute to the *N*-demethylation reaction (Liu *et al.* 1995, Schmider *et al.* 1997).

It is known that a compound can inhibit the enzymatic formation of one metabolite while simultaneously stimulating the production of another metabolite of the same parent drug (Wang *et al.* 2000). In the present study, the inhibition of norhydromorphone formation by TAO and ketoconazole was accompanied by an increase in the formation of dihydroisomorphine in the same human liver microsome samples. The mechanism for these effects by TAO and ketoconazole is not known. However, it is not likely that the effect was due to stimulation of CYP3A by these two compounds because recombinant CYP3A4 and CYP3A5 were catalytically inactive in the formation of dihydroisomorphine. In contrast to the stimulation of dihydroisomorphine (6 β -hydroxyl metabolite) formation by TAO and ketoconazole, these compounds did not have any effects on the production of dihydromorphine (6 α -hydroxyl metabolite), which is consistent with the well-known phenomenon of product stereoselectivity in enzymatic ketone reduction (Prelusky *et al.* 1982). Interestingly, the inhibition of norhydromorphone formation by sulfaphenazole did not result in stimulation of dihydroisomorphine formation.

In summary, based on the use of recombinant CYP enzymes, CYP enzyme-selective chemical inhibitors/substrates and human liver microsomes, the present study shows for the first time that human liver microsomal CYP3A and CYP2C9 are the primary catalysts of hydromorphone *N*-demethylation to form norhydromorphone. The TAO and ketoconazole inhibition of norhydromorphone formation was accompanied by an increase in dihydroisomorphine. Finally, dihydromorphine was an impurity of the hydromorphone used in the present study and this metabolite was not formed enzymatically by human liver microsomes.

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Identification of 2,3-diaminophenazine and of *o*-benzoquinone dioxime as the major *in vitro* metabolites of benzofuroxan

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1. The results of an *in vitro* study of the metabolism of benzofuroxan using either cytosolic or microsomal fractions obtained from rat liver are reported.

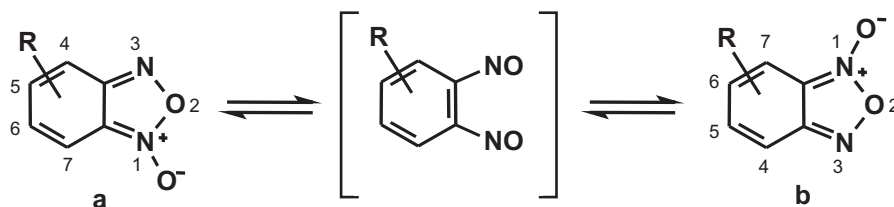
2. Benzofuroxan was incubated with an appropriate volume of cytosol or microsomal suspension; control incubations were performed without the β -nicotinamide adenine dinucleotide phosphate-generating system or, alternatively, by using the subcellular fractions inactivated by heating. Incubation mixtures were analysed by high-performance liquid chromatography. Two principal metabolites (**M1**, **M2**) were identified in the cytosolic fraction only. The dependence of **M2** formation on thiol cofactors, incubation time and protein concentration was examined.

3. The two metabolites were isolated and characterized by their ¹H-, ¹³C-nuclear magnetic resonance, infrared and mass spectra. The structures of *o*-benzoquinonedioxime (2) and 2,3-diaminopleuozuc (3), were arranged to M1 and M2 respectively. The proposed structures were confirmed by the identity of the metabolites with authentic samples obtained by synthesis. X-ray analysis showed that the dioxime metabolite had an *amphy* configuration.

4. A metabolic scheme for the formation of the two products is proposed.

Introduction

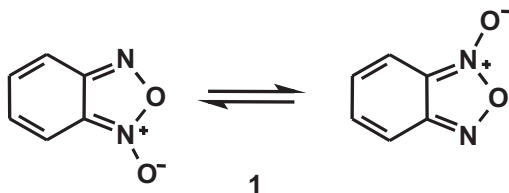
Benzofuroxans are stable compounds that exhibit rapid tautomeric equilibrium $a \rightleftharpoons b$ at room temperature:



The isomerization probably occurs via the corresponding intermediate *o*-dinitrosobenzene derivatives. Thermodynamic equilibrium concentrations are dependent on a number of factors, in particular the solvent, nature and position of

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the substituents on the ring. The chemistry and physicochemical properties of the benzofuroxans have been reviewed (Gasco and Boulton 1981, Khmel'nitskii *et al.* 1996). Noteworthy is the easy reduction of these substances that can lead to a variety of products, depending on the conditions. In particular, Gasco *et al.* (1994) studied the reduction of benzofuroxan (**1**) by ferrous salts and found that the *o*-nitroaniline was the principal reaction product. This same aniline was obtained by reacting **1** with oxyhaemoglobin (HbO₂⁺) (Medana *et al.* 2001).



Recently, **1** was used to design new Ca²⁺-channel blockers and Ca²⁺-channel activators (Gasco *et al.* 1996, Visentin *et al.* 1999).

Surprisingly, no data are reported in the literature on the metabolism of benzofuroxans. The present paper reports the results of an *in vitro* study of the metabolism of **1** using cytosolic and microsomal fractions obtained from rat liver.

Materials and methods

Chemical and analytical studies

o-Benzoquinone dioxime (**2**) and 2,3-diaminophenazine (**3**) were synthesized according to the procedures reported in Alexanian *et al.* (1981) and Premasiri and Euler (1995).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 spectrometer using suitable solvents and tetramethylsilane as the internal standard. Infrared and mass spectra were recorded on a Shimadzu FT-IR 8101M and on a Finnigan Mat TSQ-700 spectrometer, respectively. Thin-layer chromatography was performed on aluminium plates precoated with silica-gel containing a fluorescent indicator (D. C. Fertigplatten F₂₅₄, E. Merck, Darmstadt, Germany); spots were visualized at 254 nm.

o-Benzoquinone dioxime (**2**): m.p. 146–147°C; ¹H-NMR (CDCl₃/DMSO-*d*₆) δ: 6.53 (1H), 6.67 (1H), 6.80 (1H), 7.20 (1H), 11.07 (2H, br, -NH₂); ¹³C-NMR (CDCl₃/DMSO-*d*₆) δ: 115.8, 124.4, 127.7, 129.8 (CH), 145.3, 148.7 (C); MS *m/z* 138; IR (KBr) 2250–3250 cm⁻¹ (br) ν(O–H), 1550–1650 cm⁻¹ (m) ν(C=C, C=N), 1050 cm⁻¹ (s) ν(N–O).

2,3-Diaminophenazine (**3**): m.p. > 300°C; ¹H-NMR (DMSO-*d*₆) δ: 6.26 (4H, br, -NH₂), 6.93 (2H, s, 1,4-H), 7.54–7.62 (2H, m, 6,7-H), 7.88–7.92 (2H, m, 5,8-H); ¹³C-NMR (DMSO-*d*₆) δ: 102.3 (1,4-CH), 126.5 (5,6-CH), 128.0 (7,8-CH), 140.4 (2,3-C), 142.1 (9,10-C), 144.1 (11,12-C); MS *m/z* 210; λ_{max} = 451 nm (protonated form), λ_{max} = 425 nm (deprotonated form); IR (KBr) 3400–3170 cm⁻¹ (s) ν(N–H), 1480–1650 cm⁻¹ (m, s) ν(C=C, C=N, NH), 840, 760 cm⁻¹ (m, s) γ(C–H), 582 cm⁻¹ (m) γ(N–H).

High-performance liquid chromatography (HPLC) analyses were performed with an HP1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A) and a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data analysis was done using an HP ChemStation system (Agilent Technologies). The analytical column was a LiChrospher (Merck) C18 endcapped column (250 × 4 mm, 5 μm particle size). The mobile phase used was methanol with 0.05% trifluoroacetic acid (eluent A) and water with 0.05% trifluoroacetic acid (eluent B) with a gradient composition starting from 30% A for 2 min followed by a linear increase to 50% A over 4 min, then 50% A between 4 and 12 min, and finally a decrease to 30% A over 15 min. The injection volume was 50 μl (Rheodyne, Cotati, CA, USA). The peaks were recorded at 420 nm referenced against a 600 nm wavelength.

Titriplex (diethylenetriaminopentacetic acid) and magnesium chloride were purchased from Merck. Methanol, acetonitrile (HPLC grade), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP⁺, NAD⁺, benzofuroxan, *o*-phenylenediamine, *p*-benzoquinone, L-cysteine, glutathione and trifluoroacetic acid were from Sigma-Aldrich (Milan, Italy). All other chemicals and solvents were used as received without further purification. All solvent mixtures were expressed as v/v.

Preparation of liver cytosol and microsomes

Male Wistar rats weighing 250–300 g (Morini, S. Polo d'Enza, Italy) were allowed food and water *ad libitum*. The animals were killed by decapitation, and the livers were perfused *in situ* with an ice-cold 1.15% (v/v) KCl solution and homogenized in a Potter–Elvehjem glass–Teflon homogenizer. The homogenates were centrifuged for 20 min at 10 000g and the supernatant fractions for 60 min at 100 000g in a Beckman L5-50 ultracentrifuge. The cytosolic and microsomal fractions were recovered. Microsomes were washed twice by resuspension in the above KCl solution, resedimented by centrifugation for 35 min at 100 000g and finally resuspended in 100 mM Titriplex/pH 7.4 0.1 M phosphate buffer mixture. All operations were performed at 4°C. Metabolic assays were carried out with microsomes and cytosol either fresh or frozen in phosphate buffer and stored at –80°C. Microsomal and cytosolic proteins were assayed by the method of Lowry as modified by Schacterle and Pollack (1973).

Incubation conditions

The standard incubation mixture, in 10 ml polyethylene tubes, contained 1.3 mM MgCl₂, 0.4 mM NADP⁺, 3.5 mM glucose 6-phosphate, 0.5 U ml^{–1} glucose 6-phosphate dehydrogenase in a 0.1 M phosphate buffer, pH 7.4, containing 1.5 mM Titriplex (an ethylenediamine tetra-acetic acid analogue) and 1.5 mM benzofuroxan, brought to a final volume of 3 ml. After pre-equilibration of the mixture at 37°C, an appropriate volume of cytosol or microsomal suspension was added to give a final protein concentration of 1 mg ml^{–1}; the mixture was shaken under atmosphere for 30 min at 37°C. Control incubations were done without the β -nicotinamide adenine dinucleotide phosphate (NADPH)-generating system or, alternatively, by using the subcellular fractions inactivated by heating for 5 min at 80°C. When reduced glutathione and L-cysteine were used, the final concentrations were 3 and 1 mM, respectively. An aliquot (400 μ l) of the incubation mixture was treated with 400 μ l acetonitrile. The precipitated proteins were separated by centrifugation, the supernatant was filtered through polytetrafluoroethylene (PTFE) membrane filters 0.45 μ m pore size (Alltech) and analysed by reverse-phase HPLC.

The concentration of **M2** was calculated from the standard curve obtained with a synthetic sample of the metabolite. Linear response was obtained in the concentration range 0.8–8.6 μ g ml^{–1}. In order to check the dependence of **M2** formation on time, the incubations were done using the conditions reported above, except that the final volume of the incubation was 6 ml. At fixed times (5, 10, 15, 30, 40, 60 min), aliquots (100 μ l) of the incubation mixture were treated with an equal volume of acetonitrile, and after centrifugation and PTFE filtration were analysed by HPLC. Similarly, to check the dependence of metabolite formation on protein concentration, the incubations were done in the presence of different cytosolic protein concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg ml^{–1}).

Isolation of metabolites

To isolate sufficient amounts of metabolites for their characterization, 20 incubations of 25 ml were done in 100 ml flasks using the conditions described above for cytosolic analytical incubations, except that the final protein concentration and the time of incubation were 1.5 mg ml^{–1} and 1 h, respectively. The pooled incubation media were extracted three times with an equal volume of chloroform/ethylacetate/2-propanol mixture, 45/45/10; the organic phase was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude extract was dissolved in chloroform and separated into components by column chromatography using chloroform/methanol 95/5.

Results and discussion

Benzofuroxan was incubated in the cytosolic fraction obtained from rat liver according to the procedure described above. After 1 h of incubation, HPLC analysis showed the presence of two major metabolites, **M1** and **M2** (figure 1).

The influence of a number of incubation conditions on the metabolism of **1** was studied (table 1). Neither of the two metabolites was formed following the incubation of **1** in the microsomal fraction, nor were either metabolites formed in the absence of an NADPH-generating system or with heat-inactivated cytosol.

The formation of **M2** increased linearly with time for 30 min (figure 2A) as well as with cytosolic protein concentrations in the range 0.2–1.0 mg ml^{–1} (figure 2B).

When an additional amount of cytosolic protein was used, a decrease in the more polar metabolite **M1** was observed, until it completely disappeared. The presence of thiol cofactors such as L-cysteine and glutathione greatly influenced

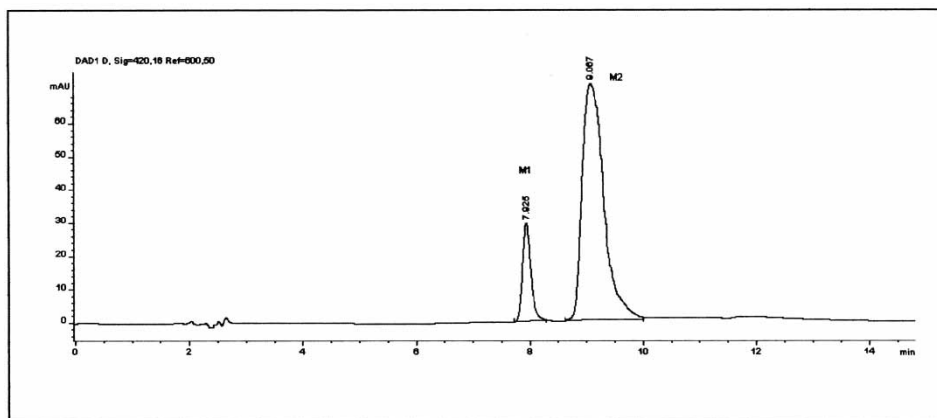


Figure 1. Reverse-phase HPLC analysis of the cytosolic incubation mixture.

Table 1. Influence of incubation conditions and thiol cofactors on formation of **M2**.

	Metabolite M2 formed as a percentage of standard incubation*
Standard	100
Minus NADPH-generating system	n.d.
With boiled cytosol (5 min at 80°C)	n.d.
With microsomes instead of cytosol	n.d.
Plus glutathione (3 mM)	15.6 ± 0.6
Plus glutathione (1 mM)	47.7 ± 0.2
Plus L-cysteine (3 mM)	41.5 ± 0.5

*Values are means ± standard deviation of duplicate assays of three different experiments. The average rate of **M2** formation in a standard incubation was 1.3 nmol min⁻¹ mg⁻¹ protein.

n.d.: Not detected.

the formation of **M2**; in particular, glutathione was more efficient than L-cysteine in decreasing its formation.

Extraction of the incubation mixture with chloroform/ethylacetate/2-propanol 45/45/10 and subsequent solvent evaporation gave a mixture of the two metabolites, which was separated by column chromatography. The first eluted metabolite was a yellow compound (m.p. 146–147°C) while the second an orange product (m.p. > 300°C). HPLC analysis of the two products, under the same conditions used to follow the progress of metabolism, revealed that the first eluted corresponded to **M1**, the second one corresponding to **M2**. The former showed in the mass spectrum a molecular ion at 138 *m/e* (base peak). The IR spectrum (KBr) showed absorption bands in the 1550–1650 cm⁻¹ region, which could be due to the presence of C=C and C=N groups, and a very broad absorption between 2250 and 3250 cm⁻¹ characteristic of strong hydrogen bonding of OH groups. An ¹H-NMR spectrum recorded in CHCl₃ with a drop of DMSO for increased solubility gave four somewhat broad signals with the same integration ratio 1:1, in the region 7.2–6.5 ppm typical of conjugated double-bond protons (Gueuther 1995), and a very broad signal at 11.07 ppm (2H), which disappeared when the sample was treated with a drop of D₂O. The spectrum recorded in DMSO appeared as an envelope of very broad bands in the region 5.8–7.5 ppm. The ¹³C-NMR spectrum (CDCl₃/

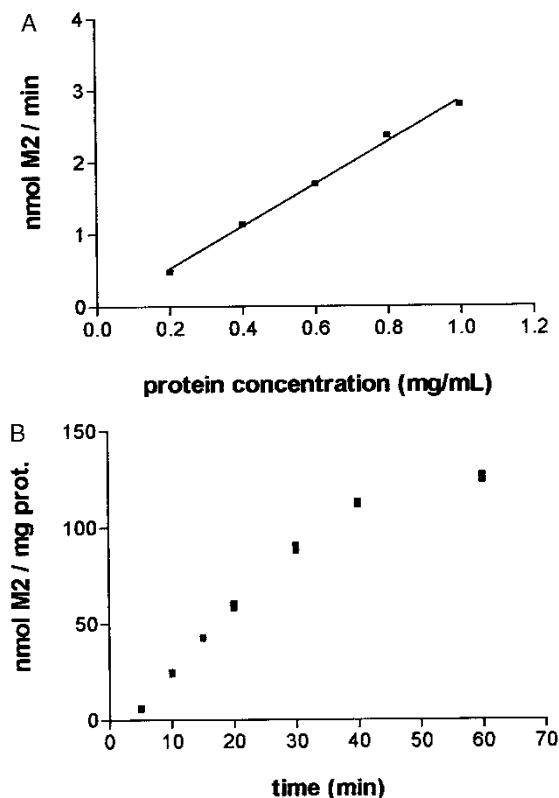


Figure 2. (A) Dependence of **M2** formation on cytosolic protein concentration. Values are averages for three separate experiments. (B) Dependence of **M2** formation on incubation time. Values are averages for three separate experiments.

DMSO) showed six absorptions, again rather broad. Two of them, at 148.7 and 145.3 ppm, respectively, were very weak, whereas the other four peaks, in the range 130–115 ppm, appeared as positive signals in the DEPT spectrum. Based on these analyses, the *o*-benzoquinone dioxime (**2**) was proposed for the compound under study. This assignment was confirmed by the identity of the mass, NMR and IR spectra of the product with those of a true sample of *o*-benzoquinone dioxime obtained by synthesis. An *amphy* configuration to the dioxime substructure was strongly suggested by the number of ^{13}C signals and by the ^1H -NMR pattern, and was confirmed by X-ray analysis. Figure 3 shows the Oak Ridge Thermal Ellipsoid Plot (ORTEP) diagram (Johnson 1975) of **2** with thermal ellipsoids and an atom numbering scheme. Distances and bond angles were in keeping with localized double bonds between C(2)-N(2), C(1)-N(1), C(3)-C(4), C(5)-C(6) (Gervasio and Marabello personal communication). Note the strong intramolecular hydrogen bonding H(2)⋯N(1) (1.45(6) Å) responsible for the conformational stability.

Heating of **2** in the NMR tube revealed the disappearance of the original proton pattern and the appearance of a $\text{A}_2'\text{X}_2'$ pattern in the region 7.5–8.2 ppm, and of only three peaks in the ^{13}C -NMR spectrum (116.5, 132.0, 149.1 ppm). This behaviour is in keeping with the formation of benzofurazan (Zincke and Schwarz 1899, Kamienski *et al.* 1989).

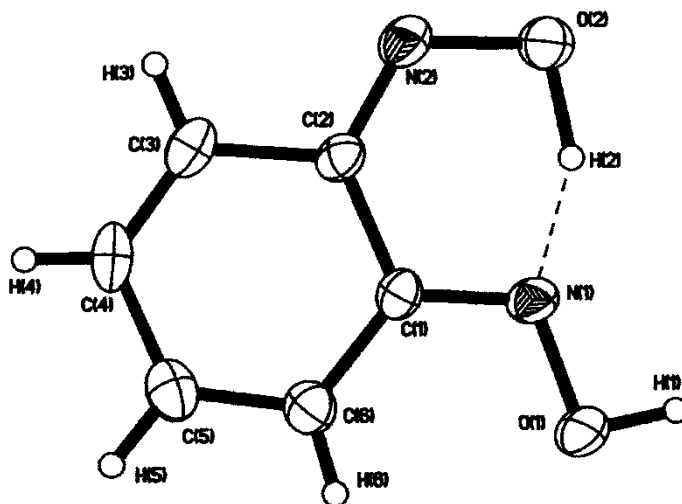


Figure 3. ORTEP diagram of **2** with thermal ellipsoids and the atom numbering scheme.

The second metabolite isolated by column chromatography (**M2**) showed a very intense molecular ion (base peak) at 210 m/e and low-intensity fragment ions. This strongly suggested an aromatic structure for the product. A typical $A_2'B_2'$ system and two singlets at 6.93 (2H) and 6.27 (4H) ppm, respectively, were present in the ^1H -NMR spectra recorded in DMSO (Premasiri and Euler 1995). The last signal was completely removed by exchange with D_2O . The ^{13}C -NMR spectrum showed six signals in the aromatic region, three of which (128.0, 126.5, 102.2 ppm) appeared as positive signals under the DEPT sequence. The IR spectrum (KBr) indicated the presence of a doublet in the region of N-H stretching and at lower frequencies other absorptions typical of a -NH hydrogen bonded group. Medium and strong absorption bands in the $1480\text{--}1650\text{ cm}^{-1}$ region were in keeping with the presence of C=C, C=N and NH groups. Based on these analyses, the 2,3-diaminophenazine structure (**3**) was proposed for the compound. This assignment was confirmed by the identity of the mass, NMR and IR spectra of the product with those of a true sample of 2,3-diaminophenazine obtained by synthesis.

A possible mechanism that justifies the production of **2** and **3** from **1** is shown in the figure 4.

The first step implies the production of **2** from **1** under the action of reducing cytosolic enzymes. This transformation is a well-known reaction undergone by benzofuroxan when treated with a number of chemical reducing agents (Gasco and Boulton 1981). It is known that oximes can be enzymatically reduced to amines through the intermediate formation of imino derivatives (Testa and Soine 2003). It has also been reported that chemical interaction of *o*-phenylenediamine (**5**) with *o*-benzoquinone diimine (**4**) can produce **3** (Nogami *et al* 1975, Loveless *et al.* 1981, Iseminger *et al.* 1997). Therefore, these transformations could rationalize the formation of **3** following the incubation of **1** with the rat liver cytosolic fraction. The formation of **3** by gentle heating of an acetic acid solution of **1**, with an excess of **5**, through the formation of dioxime **2**, has also been described by Zincke and Schwarz (1899). However, the chemical formation of **3** through this pathway during the metabolism of **1** is ruled out since it was ascertained that such transformation ($2 + 5 \rightarrow 3$) does not occur in physiological conditions (pH 7.4, 37°C). By contrast,

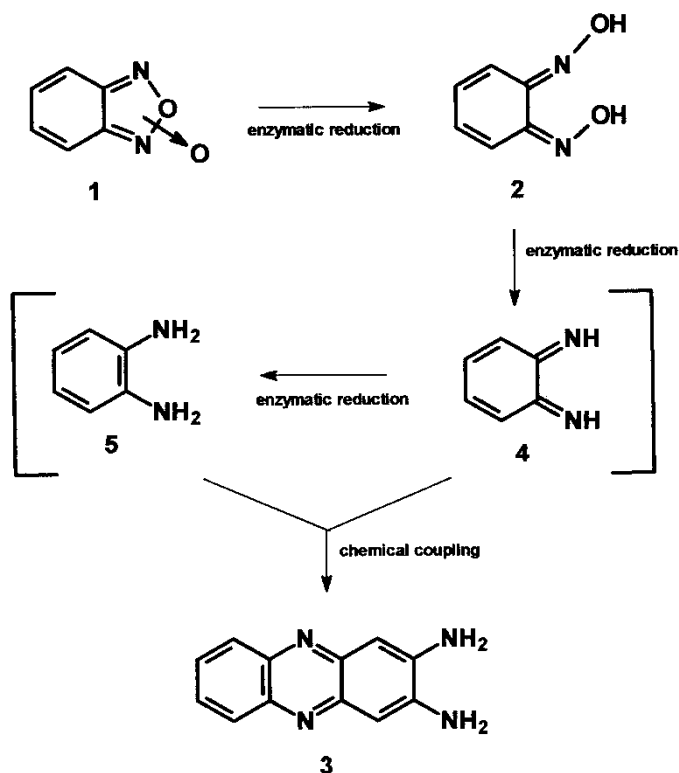


Figure 4. Hypothetical mechanism of production of 2 and 3 from 1.

coupling between 2 and benzoquinone diimine (4) and subsequent reduction to give 3 cannot be excluded.

The ability of thiol cofactors to inhibit the formation of the final 2,3-diaminophenazine (3) (table 1) is in keeping with the proposed metabolic scheme since these products can trap benzoquinone diimine through both nucleophilic addition and reduction reactions (Testa and Soine 2003). It is known that low molecular thiols can react with benzofuroxan (1) leading to the formation of benzoquinone dioxime and the corresponding disulphide (Shipton *et al.* 1977). In effect, it was found that this reaction occurs in physiological solution; however, it does not contribute to inhibit the formation of 3 because the dioxime is the first intermediate in the metabolic pathway of 1. Finally, the observed decrease in the formation of 3, when metabolism is conducted in the presence of thiols, cannot be attributed to the reaction of these products with either dioxime (2) or diaminophenazine (3), since it was ascertained that these reactions do not occur under physiological conditions.

In conclusion, the metabolic fate of benzofuroxan in a rat liver cytosolic fraction was studied and a new metabolic pathway was proposed for the formation of the two major metabolites, 2 and 3.

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Apparent absolute oral bioavailability in excess of 100% for a vitronectin receptor antagonist (SB-265123) in rat. I. Investigation of potential experimental and mechanistic explanations

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1. SB-265123 is a novel $\alpha v\beta 3$ (the vitronectin receptor) antagonist. Previous rat studies with it revealed an apparent absolute oral bioavailability (F_{app}) of greater than 100%. The present studies were conducted to investigate the potential causes for this observation.

2. Of 49 SB-265123 analogues evaluated in rat using an identical experimental design, $F_{app} > 100\%$ was observed for 22 of them, suggesting that the observed $F_{app} > 100\%$ with SB-265123 was not anomalous. All 22 compounds had clearances $< 15 \text{ ml min}^{-1} \text{ kg}^{-1}$. However, $F_{app} > 100\%$ were not recorded for all low-clearance analogues.

3. Using SB-265123 as a model to investigate potential artefacts, it was demonstrated that using a chiral assay did not decrease F_{app} . Additionally, qualitative sample analysis demonstrated that no metabolites were present in the plasma that could interfere with the liquid chromatography coupled with tandem mass spectrometry detection assay. The high F_{app} was also dose-order-, delivery system- and vehicle-independent, and was not affected by the feeding status of the animals. Furthermore, a linearity experiment and an absorption study indicated that oral administration of SB-265123 does not result in hepatic portal vein concentrations that exceed the pharmacokinetic linearity of SB-265123.

4. These observations suggest that the observed $F_{app} > 100\%$ for SB-265123 is not due to an experimental artefact or an obvious pharmacokinetic non-linearity. The mechanism(s) for this phenomenon is explored further in the second part of the present paper.

Introduction

SB-265123 [(S)-10,11-dihydro-3-[3-(pyridin-2-ylamino)-1-propyloxy]-5H-di-benzo[a,d] cycloheptene-10-acetic acid; figure 1] is a potent and selective $\alpha v\beta 3$ integrin (the 'vitronectin receptor') antagonist that might be useful for the treatment of a variety of diseases, including cancer, restenosis and osteoporosis (Miller *et al.* 1999, 2000). SB-265123 demonstrates favourable preclinical pharmacokinetic properties with excellent bioavailability in the mouse, rat, dog and monkey (Ward *et al.* 1999a). Furthermore, it demonstrates an apparent absolute oral bioavailability (F_{app}) of approximately 118% when administered as a solution formulation in the rat. In a preliminary experiment in bile duct-exteriorized rats, SB-265123 was extensively excreted in bile both as parent and as a glucuronide conjugate, and although bile duct exteriorization resulted in a twofold increase in plasma clearance, F_{app} remained $> 100\%$ ($F_{app} = 122\text{--}140\%$).

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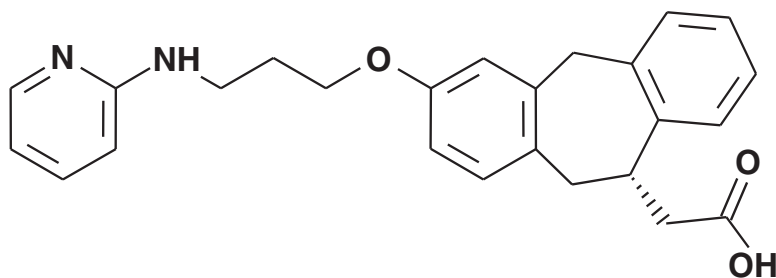


Figure 1. Chemical structure of SB-265123 [(*S*)-10,11-dihydro-3-[3-(pyridin-2-ylamino)-1-propyloxy]-5H-dibenzo[*a,d*]cycloheptene-10-acetic acid].

The present investigation was conducted to investigate potential mechanism(s) underlying the observation of $F_{app} > 100\%$ in the rat for SB-265123. In a series of studies, interfering circulating metabolites, vehicle interference with the assay, recovery from the intravenous (i.v.) infusion apparatus, dose-order, feeding status, dose vehicle, non-linear systemic elimination kinetics, and non-linear hepatic extraction (resulting from excessively high portal concentrations following oral administration) were all explored as potential mechanistic or artefactual explanations for the observed high F_{app} for SB-265123 in the rat. These experiments suggest a more complex explanation for the observed $F_{app} > 100\%$, which is further explored in a companion manuscript (Ward *et al.* 2004).

Materials and methods

Materials

SB-265123 (molecular weight = 402.49, purity $\geq 98\%$) and other proprietary structural analogues were prepared by the Department of Medicinal Chemistry, GlaxoSmithKline. All other reagents were purchased from standard vendors and were of the highest available purity.

Animals

Male outbred Sprague–Dawley rats (CrI:CD(SD)IGS BR; Charles River, Raleigh, NC, USA), weighing 250–400 g, were used. Rats were housed according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) in individual cages in unidirectional airflow rooms with controlled temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($50 \pm 10\%$) and 12-h light/dark cycles (06.00–18.00 hours). Filtered tap water was available *ad libitum* and was periodically analysed for impurities by the Philadelphia Suburban Water Company, PA, USA. Animals were fed a standard animal diet (Purina Mills, St Louis, MO, USA); food was available *ad libitum* except for overnight periods before dosing, as described below. Whenever overnight fasting was employed, food was provided after the 240-min blood sample was obtained on the following study day. All animal use was conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee before the study, and all surgical procedures were conducted using aseptic techniques in special-purpose operating suites.

Intravenous/oral crossover studies

Various permutations of a standard i.v. $\times$ oral crossover experiment (Ward *et al.* 1999a) were performed as detailed in table 1. Briefly, for each experiment, rats ($n=3$ per experiment unless otherwise specified) received surgically implanted femoral vein catheters for drug administration. Surgery was performed under ketamine/xylazine ($87/13 \text{ mg kg}^{-1}$, intramuscular) anaesthesia 4–5 days before the first day of the experiment; the catheters were filled with heparinized saline to maintain patency when not in use. On each study day, the rats were held in restrainers to facilitate drug administration and blood sampling (from a lateral tail vein) for up to 2 h after dosing before being returned to their cages. For the standard test procedure, on study day 1, the rats received $2 \mu\text{mol kg}^{-1}$ SB-265123 as a 30-min i.v. infusion (4.0 ml kg^{-1}). Two days later, the same rats received a bolus of $2 \mu\text{mol kg}^{-1}$ via oral gavage (16 ml kg^{-1}). These doses were administered in saline or water, respectively, each containing 5% polyethylene glycol (PEG)-300 and 0.5% dimethyl sulfoxide (DMSO). Analytical

Table 1. Study design and bioavailability data from various intravenous and oral crossover experiments with SB-265123 in rat. Dosages are all $2 \mu\text{mol kg}^{-1}$ unless otherwise specified.

Study	Design distinctive(s)	%F
Control	previous data from Ward <i>et al.</i> (1999a)	118 ± 36
Stabilization	plasma samples acid-stabilized to prevent hydrolysis of circulating glucuronides	128 ± 22
Chiral assay	chiral analytical method used to distinguish between <i>R</i> - and <i>S</i> -SB-265123	125 ± 34
BDC	bile duct-exteriorized rats, with bile salt replacement solution	136 ± 6
Dose order	dose order switched between intravenous and oral doses	145 ± 18
Low oral dose	10-fold lower oral dosage employed	123 ± 14
Food effect	rats fasted before both intravenous and oral study days	163 ± 46
Saline	PEG-300 replaced with saline as a dose vehicle	126 ± 52
Cyclodextrin	PEG-300 replaced with cyclodextrin as a dose vehicle	216 ± 48

Standard study conditions included 30-min intravenous infusion (4 ml kg^{-1}) to fed rats formulated in saline with 5% PEG-300 and 0.5% DMSO, followed 2 calendar days later by an oral bolus gavage (16 ml kg^{-1}) to fasted rats formulated in water with 5% PEG-300 and 0.5% DMSO.

verification of the concentration of test compound in each dose solution was performed using methods described below. Blood samples of approximately 0.1 ml were collected from each study animal from a lateral tail vein at various times during and up to 1440 min after drug administration; plasma (50 μl) was isolated by centrifugation and stored at or about -70°C until analysis. The maximum volume of blood collected from any one rat was 1.8 ml per study day.

Bile duct exteriorization study

To evaluate further the role of biliary excretion in the disposition of SB-265123, an experiment was conducted in bile duct-exteriorized rats ($n=3$). The study was conducted as described (Ward *et al.* 1999a), except that a bile replacement solution was infused into the distal bile duct during the time the bile duct catheter loop was open (18.15 mM cholic acid and 18.15 mM taurocholic acid, pH 8.0, in saline; $85 \mu\text{l min}^{-1} \text{ kg}^{-1}$) to replenish fluids lost during bile exteriorization.

Accelerated infusion study

To evaluate the range of pharmacokinetic linearity for SB-265123, an accelerated infusion protocol was employed (Ward *et al.* 2000). Briefly, rats ($n=3$) received surgically implanted hepatic portal vein, femoral vein and femoral artery catheters as described above. On study day 1, the rats each received a 6-h accelerated intraportal vein infusion, beginning at an initial infusion rate of $0.6 \mu\text{g kg}^{-1} \text{ min}^{-1}$ and linearly accelerating at $0.1 \mu\text{g kg}^{-1} \text{ min}^{-1}$ (total dose volume 9 ml kg^{-1}). On study day 2 (7 days later), the same rats received an identical accelerated infusion into the femoral vein. On both study days, blood samples were withdrawn from the indwelling femoral artery catheter and treated as described above.

Absorption study

To determine SB-265123 concentrations achieved in the portal circulation following oral administration, an experiment was performed in anaesthetized rats employing portal and systemic sampling. Briefly, rats ($n=3$) were anaesthetized as described above, and anaesthesia was maintained for the duration of the experiment. Immediately before drug administration, rats received surgically implanted catheters in the femoral and hepatic portal veins, and the duodenum was ligated at the pylorus to prevent back-flow of the dose solution from the duodenum into the stomach. Each rat then received SB-265123 ($2 \mu\text{mol kg}^{-1}$) as a bolus injection directly into the duodenum using the same vehicle and dose volume as described above for oral dosing. Blood samples were withdrawn from the femoral and hepatic portal vein catheters at various points for 2 h and treated as described above. At the end of the experiment, rats were euthanized with an overdose of the ketamine/xylazine anaesthetic.

Analytical procedures

Quantitative analysis of plasma samples for SB-265123 and other structural analogues was performed with liquid chromatography coupled with a tandem mass spectrometry detection assay with demonstrated specificity and acceptable within-run bias and error, as described (Ward *et al.* 1999a). Analytes were isolated from 50 μl plasma by protein precipitation and quantified using positive-ion multiple reaction monitoring with a Sciex API 3000 instrument (Applied Biosystems, Foster City,

CA, USA) with a turbo-ion spray interface. The lower limit of quantitation (based on the linearity of the assay) was $2.0\text{--}10.0\text{ ng ml}^{-1}$ for each experiment. Qualitative analysis of SB-265123 and its potential metabolites was conducted using an Agilent series 1100 liquid chromatography system (Agilent, Palo Alto, CA) coupled to a Micromass Q-TOF API mass spectrometer (Waters Corp., Milford, MA, USA). Acid-stabilized *in vivo* plasma samples were precipitated and separated on a Genesis C18 ($3 \times 150\text{ mm}$, $3\text{-}\mu\text{m}$ particle packing size) column (Jones Chromatography, Lakewood, CO, USA) operated under gradient conditions. The gradient profile increased the percent acetonitrile from 7 to 90% over 16 min with 10 mM ammonium formate (pH 5.5). Accurate mass information was obtained under positive-ion electrospray conditions using a reference lock mass. Finally, qualitative metabolite analysis was performed by monitoring for likely Phase I reactions (including oxidation, reduction, hydrolysis and hydration reactions) as well as numerous Phase II conjugative reactions (including, but not limited to, glucuronidation, glutathione, cysteine and sulfate conjugation reactions).

Data analysis

Concentration–time profiles were obtained for each animal in each study. Standard non-compartmental analysis was performed on the data from individual animals using WinNonlin Professional Version 3.3 (Pharsight, Mountain View, CA, USA) to recover area under the curve (AUC) and other non-compartmental parameters. Oral bioavailability was estimated by dividing the dose-normalized AUC (DNAUC) resulting from oral administration by the DNAUC resulting from i.v. administration. All analyses were conducted using both AUC(0– t), where t is the last time point with measurable drug concentrations in the study, and AUC(0– ∞). The conclusions from each study were identical regardless of the AUC method employed; all data shown here are AUC(0– t) to avoid any bias due to study-to-study differences in the extrapolated AUC. Either a two-tailed Student's t -test or a one-way analysis of variance was used for statistical evaluation of the observed data, as appropriate. In all cases, a probability level of $p \leq 0.05$ was predetermined as the criterion of statistical significance.

Results

Structural analogues of SB-265123

During lead optimization for this chemical series, the pharmacokinetic properties of several hundred proprietary molecules within this structural series were evaluated using identical experimental designs. Of these molecules, 49 were closely related in structure to SB-265123. The clearance and apparent oral bioavailability properties of these molecules are shown in figure 2. Of the compounds demonstrating a plasma clearance $\geq 10\text{ ml min}^{-1}\text{ kg}^{-1}$, most demonstrated poor oral bioavailability, with only four compounds demonstrating $F_{\text{app}} > 100\%$, all of which had clearances between 10 and $15\text{ ml min}^{-1}\text{ kg}^{-1}$. However, for the 30 molecules with a plasma clearance $< 10\text{ ml min}^{-1}\text{ kg}^{-1}$, $F_{\text{app}} > 100\%$ was observed for 22 compounds, with the remainder showing an F_{app} between 70 and 100%. These data suggest that the observations with SB-265123 were not anomalous. To explore this phenomenon further, additional investigations of potential artefactual and mechanistic causes for the $F_{\text{app}} > 100\%$ in this structural series were subsequently conducted using SB-265123 as a representative compound. Various experiments were conducted to evaluate the influence of different components of the study design on the i.v. and oral AUC (and therefore F_{app}) of SB-265123. The basic design and results of these experiments are summarized in table 1 and figure 3, and described in detail below.

Potential experimental artefacts

Attention was initially focussed on possible experimental artefacts for the observed $F_{\text{app}} > 100\%$. First, because the delivery systems for the i.v. and oral dosage differed (syringe, needle and tubing for i.v. infusion; syringe and gavage needle for oral gavage), recovery of SB-265123 from each apparatus was investigated. However, recovery of SB-265123 from both delivery systems was essentially 100% and did not differ from each other (data not shown.) Another possible

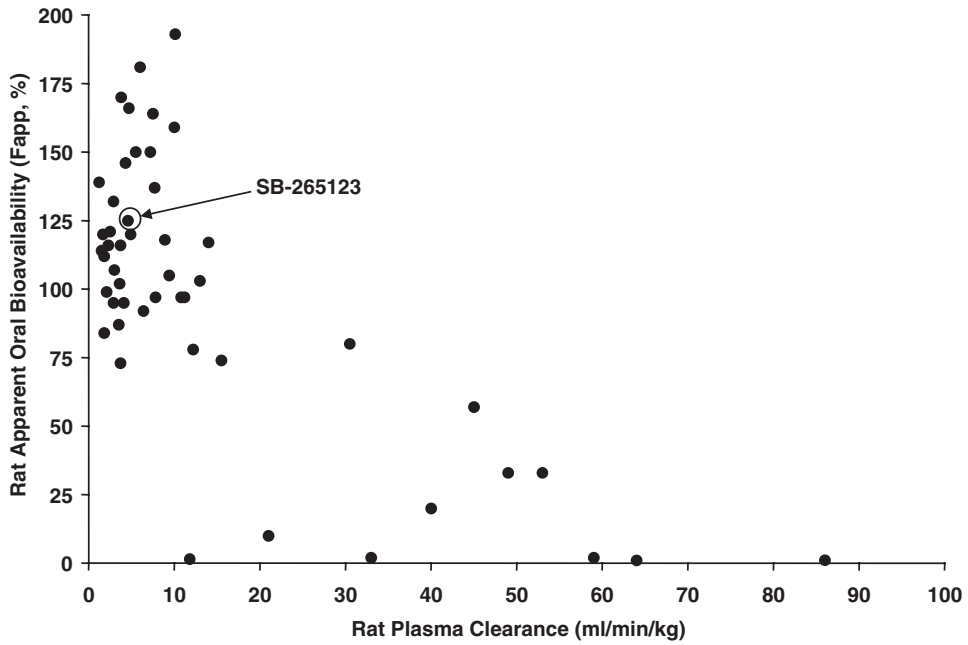


Figure 2. Clearance and apparent oral bioavailability estimates for 49 structural analogues of SB-265123 in the rat. Symbols represent average crossover data from $n=3$ rats per compound.

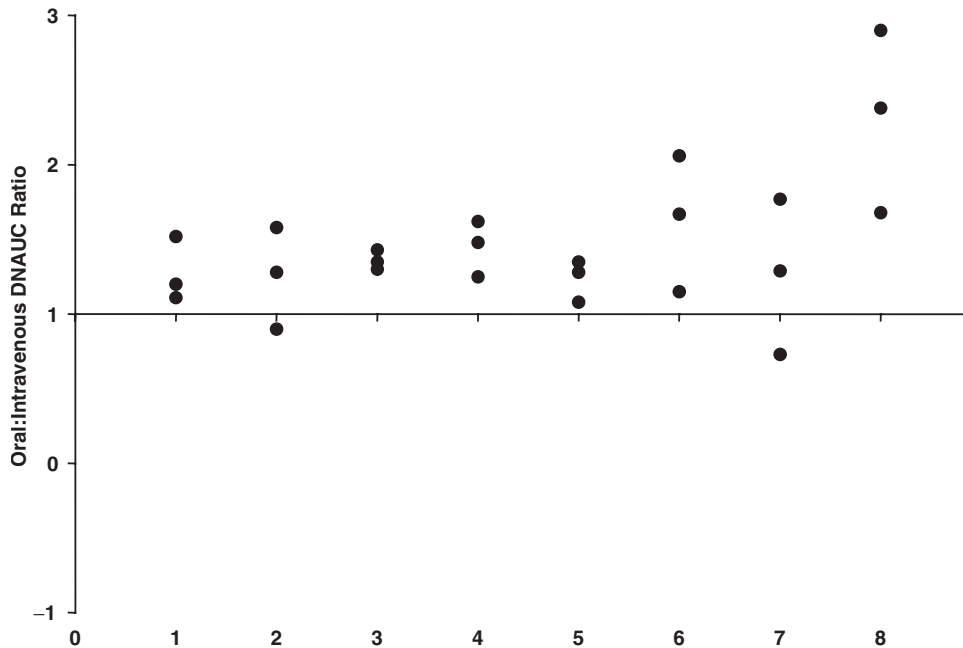


Figure 3. Ratio of oral-to-intravenous dose-normalized AUCs for SB-265123 in individual animals using different experimental designs. The study order was identical to that given in table 1: 1, acid stabilization; 2, chiral assay; 3, bile duct cannulated with bile salt replacement; 4, reversed dose-order; 5, low oral dose; 6, food effect; 7, saline vehicle; and 8, cyclodextrin vehicle.

experimental factor investigated was the dose vehicle used (0.5% DMSO and 5% PEG-300 in saline or in water for i.v. and oral dosages, respectively). Preliminary assay development work had optimized the analytical assay to prevent any effect of circulating PEG-300 on the measured plasma concentrations of SB-265123. To demonstrate further that the vehicle had no effect on the plasma assay, various concentrations of PEG-300 were added to rat plasma samples at concentrations up to threefold those likely to be achieved in plasma at $C_{\max}$, based on the pharmacokinetics of PEG-300 in the rodent (Berglund *et al.* 1969, Yamaoka *et al.* 1994). No significant signal suppression was observed in the samples with PEG-300 at any of the concentrations investigated (data not shown). To exclude further the possibility of a dose vehicle effect, an experiment was conducted using 0.5% DMSO with 20% (i.v. dose) or 6% (oral dose) hydroxypropyl- β -cyclodextrin (CavitronTM; Cerestar, Hammond, IN, USA), or with 3% DMSO and no additional excipients. The use of the alternate dose vehicles did not decrease the observed F_{app} for SB-265123 in the rat (table 1). Finally, the effect of food on the pharmacokinetics of SB-265123 was investigated. In previous studies, rats were allowed food *ad libitum* before the i.v. study (for humane considerations), with overnight fasting employed only for the oral portion of the experiment. To investigate the possibility that feeding status altered the exposure of SB-265123 following i.v. administration, an experiment was conducted in which animals were fasted overnight before both study days. This altered feeding regimen did not decrease the observed F_{app} in the rat (table 1).

Potential interfering metabolites

Previously, SB-265123 was shown to be metabolized at least in part by acyl glucuronidation (Ward *et al.* 1999a). To minimize any post-sampling acyl glucuronide hydrolysis or rearrangement in plasma, a study was performed employing acid stabilization of samples immediately after collection (addition of 3 μ l 3% phosphoric acid to each 50- μ l plasma aliquot). Oral bioavailability of the SB-265123 determined using acid stabilization of plasma samples was not different from that determined previously ($128 \pm 22\%$). However, as a precaution, acid stabilization was employed in all subsequent experiments with SB-265123. Furthermore, a repeat of the previous preliminary ($n=2$ rats; no bile acid replacement) bile duct-exteriorized rat study was also conducted using acid stabilization of plasma samples. Consistent with the previous data, bile duct exteriorization resulted in a significant decrease in the AUC generated following both i.v. and oral administration compared with similar experiments in non-bile duct-exteriorized animals due to increased plasma clearance of SB-265123 (8.61 ± 1.48 versus $4.05 \pm 2.00 \text{ ml min}^{-1} \text{ kg}^{-1}$ in exteriorized and non-exteriorized rats, respectively) (figure 4). However, despite these changes in the plasma concentration-time profiles for SB-265123 in the bile duct-exteriorized rat, F_{app} was still in excess of 100% (table 1).

Another potential metabolism/analytical issue addressed was chiral inversion. The tandem mass spectrometry method developed for SB-265123 was optimized to eliminate interference from possible metabolites with different molecular weights and/or different daughter ions produced in the mass spectral analysis. However, SB-265123 is a chiral compound, and the initial method would not have discriminated between the *S*-enantiomer (SB-265123) and its corresponding *R*-isomer, if the latter was formed as a metabolite. Therefore, a chiral analytical

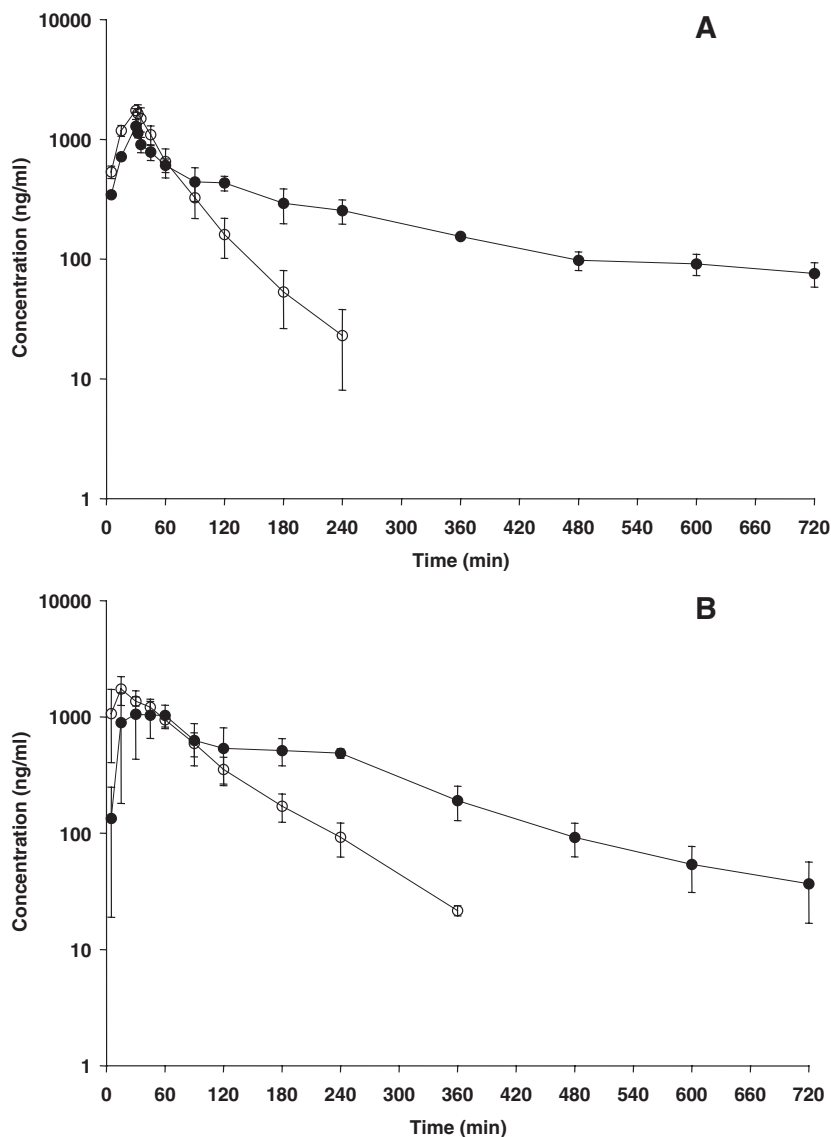


Figure 4. Comparison of plasma concentration-time profiles for SB-265123 in the intact (closed circles) and bile duct-exteriorized (open circles) rat following a $2\text{-}\mu\text{mol kg}^{-1}$ 30-min intravenous infusion (A) or oral bolus gavage (B). Symbols are the average $\pm$ SD at each time point for $n = 3$ rats per dose group; lines represent the linear interpolation between data points.

method was developed to distinguish between these two enantiomers. No significant *in vivo* chiral inversion of SB-265123 occurred in the rat, and the oral bioavailability of SB-265123 determined using the chiral assay was not different from that determined previously (table 1).

Finally, to verify that a circulating metabolite of SB-265123 was not present in sufficient quantity to interfere with the quantitative assay, qualitative liquid chromatography-mass spectrometry sample analysis was employed. Qualitative analysis of plasma samples collected at various time points following oral

administration of SB-265123 resulted in the observation of a single major peak corresponding to the parent drug. Greater than 95% of all the integrated peak area was represented by the parent drug, with the residual (< 5%) shared among a Phase I methylation reaction and two Phase II conjugation reactions, glycine and cysteine conjugation (data not shown). None of these peaks, or daughter ions thereof, would have co-eluted with the parent drug in a manner that would interfere with the quantitative analysis of SB-265123 using liquid chromatography coupled with tandem mass spectrometry detection.

Potential mechanistic explanations

To exclude the possibility of a dose-order effect (i.e. non-stationary pharmacokinetics), an experiment was conducted in which the oral dosage was administered first, followed by the i.v. dosage. No decrease in the observed F_{app} was observed in this study. Next, the possibility of pharmacokinetic non-linearity in systemic clearance and/or first-pass hepatic extraction was evaluated. An experiment was performed in which an accelerated infusion was administered either to the systemic circulation or via the hepatic portal vein, to evaluate possible non-linearities in hepatic and non-hepatic elimination processes. The concentration-time profiles for these experiments are shown in figure 5. No clear evidence of non-linearity was observed in this experiment over a concentration range of approximately 0 to 4000–5000 ng ml⁻¹. Estimation of first-pass hepatic extraction according to the method of Li *et al.* (1997) indicated a negligible first-pass for SB-265123, an observation consistent with the observed low clearance and high oral bioavailability.

Although the accelerated infusion experiment demonstrated linear pharmacokinetics at concentrations up to at least 4000 ng ml⁻¹, the possibility still existed that oral administration of SB-265123 could result in rapid and extensive absorption of a large fraction of the administered dose, generating even higher portal concentrations of SB-265123, possibly exceeding the previously established linear range. Therefore, an experiment was conducted to determine hepatic portal plasma concentrations of SB-265123 following the oral dosage employed in the previous studies (0.8 mg kg⁻¹). As shown in figure 6 and table 2, absorption of SB-265123 was indeed rapid and extensive, with maximum concentrations achieved at <30 min in both the portal and systemic circulation. Based on the method of Ward *et al.* (2001a), absorption of SB-265123 over 2 h in the anaesthetized rat was at least 60%, and maximal hepatic portal concentrations of SB-265123 in this study were <2000 ng ml⁻¹, well within the range of linearity established in the accelerated infusion experiments. To explore further the possibility of non-linearities induced by high hepatic portal concentrations following oral administration, an experiment was conducted in conscious animals in which a 10-fold lower oral dose was administered. Again, F_{app} was >100% in this study (table 1), further indicating that saturation of hepatic elimination processes following oral administration is not responsible for this observation.

Discussion

The term 'oral bioavailability' generally refers to the fraction of the administered oral dosage that reaches the systemic circulation. Furthermore, since the systemic availability of an i.v. dosage is usually considered to be unity, oral

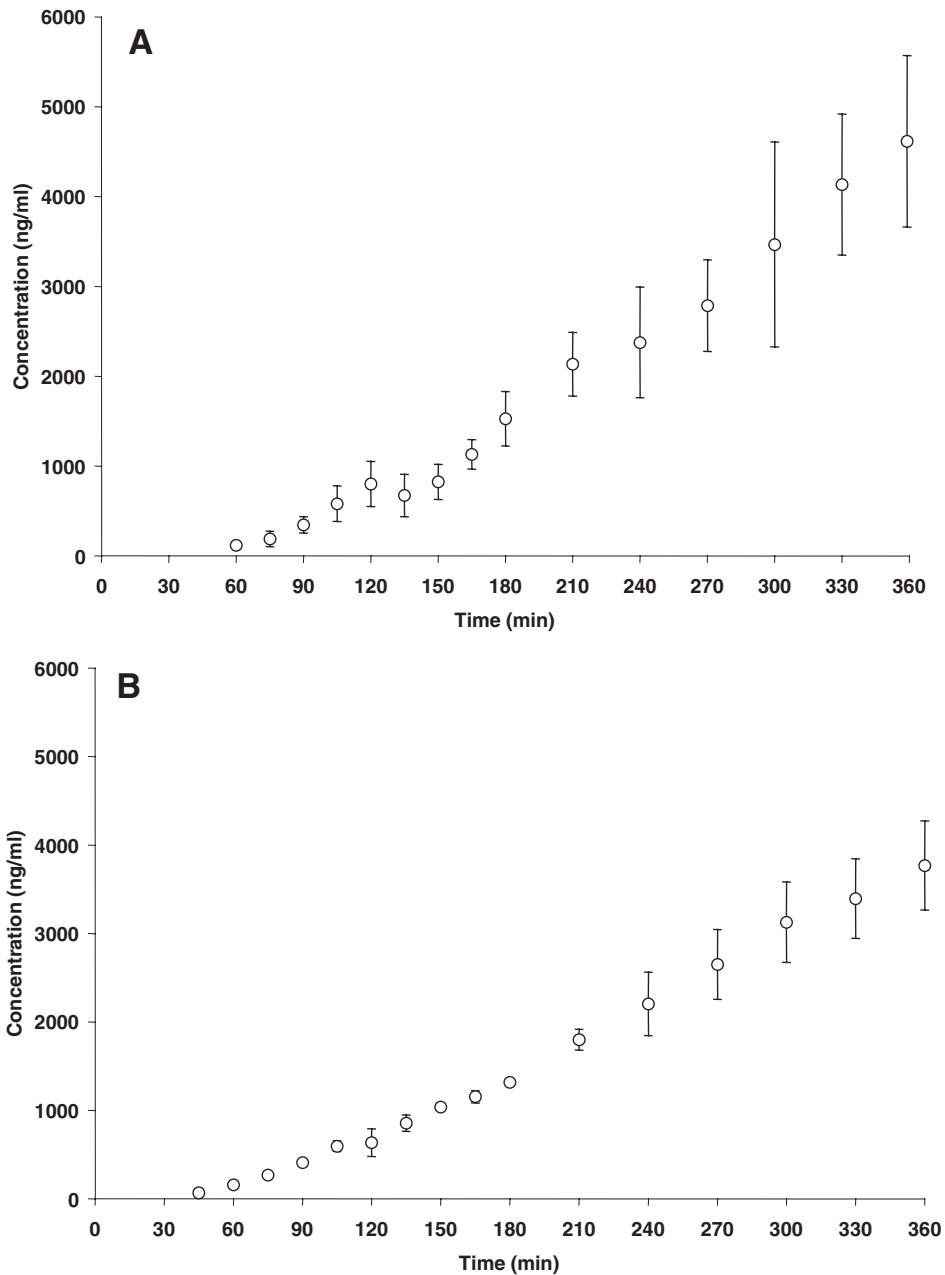


Figure 5. Plasma concentration–time profiles generated during an accelerated infusion of SB-265123 (approximately 7 mg kg^{-1} total dose) into either the hepatic portal vein (A) or the femoral vein (B). Symbols are the average $\pm$ SD at each time point for $n=3$ rats per dose group.

bioavailability is typically estimated as the ratio of the areas under the dose-normalized zero moment curves after oral and i.v. administration (Gibaldi and Perrier 1982). Hence, by definition, under the usual conditions of linear and stationary pharmacokinetics, an oral bioavailability $>100\%$ is not possible. In Ward *et al.* (1999a), SB-265123 demonstrated an apparent oral bioavailability $>100\%$. Previously, the present authors had also demonstrated that the racemate

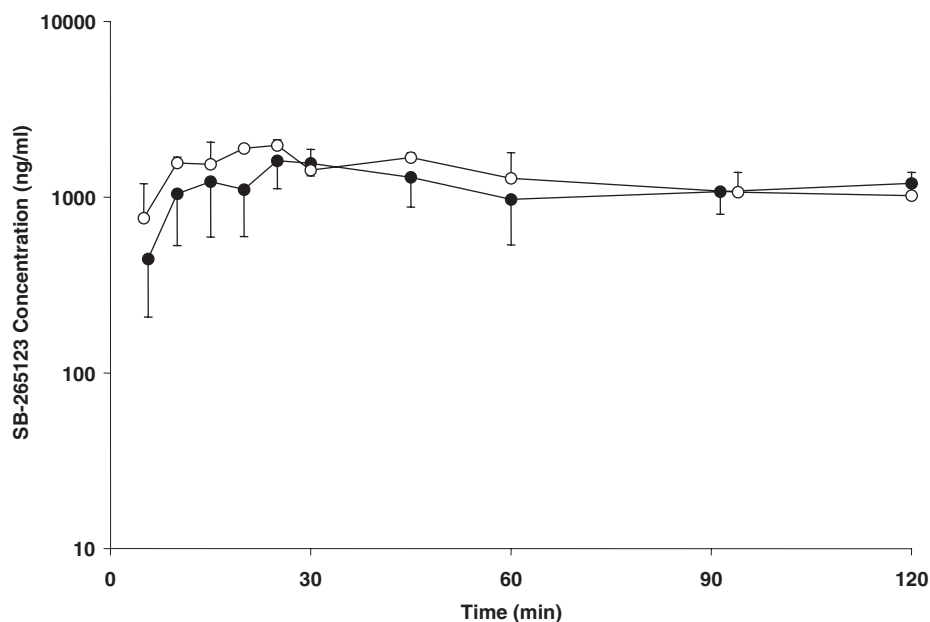


Figure 6. Plasma concentration–time profiles in the hepatic portal (open circles) and femoral (closed circles) veins for SB-265123 following an intraduodenal administration ($2 \mu\text{mol kg}^{-1}$) to the anaesthetized rat. Data are the average $\pm$ SD in $n = 3$ rats; lines represent the linear interpolation between data points.

Table 2. Simultaneous measurement of plasma concentration and pharmacokinetic parameters for SB-265123 in the hepatic portal and femoral veins following an intraduodenal administration ($2 \mu\text{mol kg}^{-1}$) to the anaesthetized rat.

Parameter	Portal vein	Femoral vein
C_{max} (ng ml^{-1})	1991 ± 130	1873 ± 163
T_{max} (min)	23 ± 3	20 ± 5
AUC(0–2 h) ($\text{min } \mu\text{g ml}^{-1}$)	154 ± 28	132 ± 16
Corrected portal DNAUC (min kg l^{-1})†	28.0 ± 16.1	not applicable
Absorption (%)‡	59.3 ± 34.1	not applicable

Values are average $\pm$ SD for $n = 3$ rats.

†Calculated according to the method of Ward *et al.* (2001a) by dividing the AUC from both the portal and femoral veins by the administered dose, and subtracting the systemic AUC from the portal AUC.

‡Calculated according to the method of Ward *et al.* (2001a) by dividing the calculated corrected portal DNAUC by the theoretical maximum portal DNAUC in the rat, 47.2.

of SB-265123 (SB-252219) also had $F_{\text{app}} > 100\%$ (Ward *et al.* 1999b). The present investigation has confirmed this observation in numerous experiments with SB-265123, and it has been established that $F_{\text{app}} > 100\%$ occurs for numerous (though not all) structural analogues of SB-265123 (figure 2). An observation of $F_{\text{app}} > 100\%$ is uncommon in the published literature, and the present investigation has endeavoured to identify potential causes of this observation and to explore likely mechanistic explanations.

First, experimental artefacts that might have resulted in the observed $F_{\text{app}} > 100\%$ were investigated. One of the major areas of investigation pursued in these studies was a rigorous evaluation of the analytical methodologies used.

The liquid chromatography coupled with tandem mass spectrometry detection used for the quantification of SB-265123 (and other molecules in this series) was optimized to be insensitive to ion suppression from the dose vehicles used (particularly for the i.v. vehicles), and would be insensitive to interference from metabolites with different molecular weights and/or different daughter ions produced in the mass spectral analysis. Furthermore, the apparent absence of any such metabolites was confirmed in this investigation using qualitative analytical techniques. However, it has been demonstrated that *in vivo* isomerization to an opposite enantiomer not discriminated by the analytical methodology might result in an $F_{app} > 100\%$ when the metabolic reaction occurs to a greater extent following oral administration (Ward *et al.* 2001b). Furthermore, SB-265123 is known to be metabolized in part via acyl glucuronidation (Ward *et al.* 1999a). Many acyl glucuronides are base labile (Janssen *et al.* 1982, Van Breeman and Fenselau 1986), and it is conceivable that post-sampling acyl glucuronide hydrolysis or rearrangement in plasma could result in alterations in the non-conjugated drug concentrations. However, in the present series of studies, interference from either *in vivo* isomerization (via a selective chiral assay) or glucuronidation (via sample acidification) were also excluded as potential influences on the F_{app} of SB-265123.

Another potential artefactual cause of the $F_{app} > 100\%$ could be non-specific binding of SB-265123 to the i.v. infusion system; such binding has been demonstrated to yield an $F_{app} > 100\%$ for quinidine (Darbar *et al.* 1996). However, the present investigation demonstrated complete recovery of SB-265123 from the i.v. infusion apparatus and from the oral gavage system, negating this potential cause. Yet another experimental variant investigated here was the effect of food on the F_{app} of SB-265123. In most of the present experiments, rats were fasted before oral dosing only, with food available *ad libitum* before i.v. infusion. Food effects on oral bioavailability are common and are usually related to altered absorption of the drug of interest (Schmidt and Dalhoff 2002). In this instance, where $F_{app} > 100\%$, administration of SB-265123 to fed animals would confound the experimental outcome, because if F_{app} decreased in the presence of food, it would be unclear whether this was due to a physiological effect or to physical binding of SB-265123 to gastric contents. Therefore, the pharmacokinetics and F_{app} of SB-265123 were investigated in an experiment in which rats were fasted identically before both the i.v. and oral portions of the experiment; no difference in pharmacokinetic parameters or F_{app} were noted. Finally, dose vehicle effects were considered. In most of the present studies with SB-265123, minimal amounts of DMSO and PEG-300 were used to aid in solubilizing the drug before administration. However, dose vehicles clearly might influence drug disposition, and although PEG-300 does not appear to alter passive permeability or paracellular transport, PEGs (at higher concentrations or volumes than used in the present studies) do have the potential to alter gastric motility or active efflux mechanisms (Basit *et al.* 2001, Hugger *et al.* 2002). Consequently, the PEG-300 used in most of the experiments was replaced with either hydroxypropyl- β -cyclodextrin or a slightly increased amount of DMSO alone; in neither experiment did F_{app} decrease to below 100%.

After eliminating obvious causes of experimental error, potential mechanistic explanations for the high observed F_{app} for SB-265123 were pursued. As noted above, the standard derivation of the bioavailability relationship assumes both stationary and linear pharmacokinetics. Therefore, both assumptions were

explored for SB-265123. Non-stationary pharmacokinetics have previously been demonstrated to increase oral bioavailability artificially. For example, dichloroacetate inhibits its own metabolism on first administration, resulting in decreased clearance and increased exposure with subsequent dosing periods (Curry *et al.* 1991). In most of the studies with SB-265123, the i.v. dose was delivered first, followed by the oral dose. Although the i.v. dosage of SB-265123 was low ($< 1 \text{ mg kg}^{-1}$), and auto-inhibition or -induction unlikely, this hypothesis was explored in a reversed dose-order experiment, and no dose-order effect was observed.

Next, non-linearities in the pharmacokinetics of SB-265123 were extensively explored. Non-linearity in pharmacokinetics has been long recognized as a potential cause for apparently erroneous pharmacokinetic parameter estimates (Thron 1974), with numerous examples available in the literature (Tozer *et al.* 1981, Ludden 1991). Although non-linear plasma protein binding has previously been associated with erroneous oral bioavailability estimates (Upton and Williams 1986, Bialer *et al.* 1987), the plasma protein binding of SB-265123 in the rat is linear over the concentration range explored in these studies (Ward *et al.* 1999a). Furthermore, Bialer *et al.* have suggested that even in instances where non-linearity is observed, administration of the i.v. dose as an infusion designed to mirror the oral absorption profile may overcome the bioavailability error introduced by non-linear protein binding; all our experiments with SB-265123 were designed in this manner. Additionally, in a series of experiments, it was demonstrated that SB-265123 does not show substantial non-linear pharmacokinetics at the concentrations achieved in our bioavailability experiments. Therefore, non-linear elimination or distribution kinetics do not appear to be responsible for the observed high F_{app} of SB-265123 in the rat.

In summary, the present experiments confirmed and extended the observation of $F_{\text{app}} > 100\%$ for SB-265123 and structural analogues in the rat. In a series of studies, likely experimental artefacts (including *in vivo* isomerization, instability of a circulating glucuronide, analytical artefacts, poor recovery from the i.v. infusion apparatus, vehicle effects and feeding status) and common mechanistic explanations (non-stationary and non-linear pharmacokinetics) for the observation of $F_{\text{app}} > 100\%$ have been excluded. Together, these experiments suggest a more complex explanation for the observed $F_{\text{app}} > 100\%$, which is further explored in a companion manuscript (Ward *et al.* 2004).

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Apparent absolute oral bioavailability in excess of 100% for a vitronectin receptor antagonist (SB-265123) in rat. II. Studies implicating transporter-mediated intestinal secretion

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1. Transporters have been increasingly identified as a factor in limiting the oral bioavailability of certain drugs. Previously, the present authors investigated a compound (SB-265123) with an apparent absolute oral bioavailability (F_{app}) consistently $>100\%$, and excluded likely artefactual causes for this observation, as well as standard considerations of non-stationary or non-linear pharmacokinetics. The data led the authors to believe that SB-265123 might be a transporter substrate in the rat, and it was hypothesized that transporter interactions might be responsible for the observed $F_{app} > 100\%$.

2. In the present study, a model was proposed incorporating rapid and complete absorption and elimination by a saturable intestinal secretory pathway. Intestinal secretion was demonstrated for SB-265123 using a rat single-pass intestinal perfusion technique. In addition, in a study employing both independent and simultaneous intravenous and oral administration of SB-265123, exposure to SB-265123 was greater than additive on joint intravenous and oral administration, lending further support to the hypothesis of a saturable transporter. Furthermore, in a study with co-administration of GF120918A, a transporter inhibitor, the observed F_{app} for SB-265123 was only $84 \pm 17\%$, providing additional evidence for transporter involvement in the $>100\%$ F_{app} phenomenon.

3. Experience with SB-265123 illustrates a counterintuitive impact of transporters on oral bioavailability and highlights the importance of considering transporter interactions in the systemic disposition of xenobiotics, even those not demonstrating low oral bioavailability.

Introduction

SB-265123 is a potent and selective $\alpha v\beta 3$ antagonist under investigation for the treatment of a variety of diseases, including cancer, restenosis and osteoporosis (Miller *et al.* 2000). Previous experiments have delineated the preclinical pharmacokinetics of SB-265123 (Ward *et al.* 1999), and SB-265123 has been shown to demonstrate consistently an apparent absolute oral bioavailability (F_{app}) $>100\%$ in the rat. There are a number of possible artefactual or mechanistic explanations for such a phenomenon, including assay deficiencies (including ion suppression by intravenous dose vehicles and an inability to differentiate parent from metabolites), poor recovery from the intravenous infusion apparatus, dose-order, feeding status, dose vehicle, non-linear hepatic extraction, and non-linear systemic elimination kinetics; each potential mechanism has been explored and discounted as a cause for

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the observed $F_{app} > 100\%$ (Ward *et al.* 2004). Such an intractable pharmacokinetic problem is not commonly reported in the literature, and the present paper has endeavoured to resolve further the underlying phenomena for the oral bioavailability of SB-265123 in the rat.

Active transport processes have been increasingly implicated as mediating the disposition of a wide variety of xenobiotics (Matheny *et al.* 2001). Based on the available data with SB-265123 (detailed below), it is hypothesized that this molecule might be a substrate for active transport processes *in vivo*. The objective of the present series of studies was to investigate *in vivo* the role of saturable active transport processes in the pharmacokinetics of SB-265123 in the rodent. A model is proposed based on the available data incorporating passive intestinal absorption, active intestinal efflux and active hepatobiliary excretion of SB-265123. This model suggests that saturation of elimination by an intestinal efflux pathway after oral administration could be responsible for the observed high F_{app} . Evidence for this phenomenon was generated *in vivo* in the rat using single-pass intestinal perfusion to demonstrate intestinal secretion of SB-265123, in experiments using dual intravenous and oral dosing of SB-265123, and in a study with GF120918A, an inhibitor of P-glycoprotein and breast cancer resistance protein (BCRP). Together, the available data indicate that the pharmacokinetic behaviour of SB-265123 in the rat is mediated by active transport processes, and that one effect of these saturable active transport systems might be the observed $F_{app} > 100\%$.

Materials and methods

Model specification

An overview of the proposed model scheme describing the pharmacokinetics of SB-265123 in the rat is shown in figure 1. Ward *et al.* (1999) demonstrated that the disposition of SB-265123 in the rat is best characterized by a two-compartment pharmacokinetic model; these two compartments are represented in figure 1 as the central and peripheral tissue compartments, with associated distributional rate constants k_{12} and k_{21} . Previously, it was also demonstrated that SB-265123 undergoes glucuronidation, and that both the parent and the glucuronide conjugate are excreted into bile. Furthermore, it was also shown that the glucuronide conjugate of SB-265123 does not circulate in detectable levels in the systemic circulation (Ward *et al.* 2004). Although not extensively characterized, conjugation and biliary excretion often occur via active processes (Kusuhara *et al.* 1998), and these processes are described in figure 1 as active, saturable processes. SB-265123 might undergo minimal Phase I metabolism; this elimination route is depicted as a first-order rate constant, k_a . Once excreted from bile into the gastrointestinal tract, glucuronide conjugates may undergo cleavage back to the parent molecule (Rowland and Tozer 1995); this potential process is represented by a first-order rate constant (k_{gp}) in the model. Finally, net flux across the intestinal wall from the gut lumen into the portal vein is described in the model by a first-order rate constant for passive absorption across the enterocyte (k_a), countered by an active efflux mechanism that transports SB-265123 from the portal circulation and enterocyte into the gut lumen, with an associated $V_{max,i}$ and $K_{m,i}$.

Model assumptions

The model makes the standard assumption that at time 0, the amount of SB-265123 in the model system described in figure 1 (including the intestinal contents) is zero for an intravenous infusion, and for an oral gavage, is equal to the dose, and is all present in the intestine. Equilibration between the enterocyte and the portal vein is assumed to be rapid and complete. Although about 20% of hepatic perfusion occurs via the hepatic artery rather than by the portal vein (Davies and Morris 1993), for the purpose of this model, hepatic perfusion is considered to be entirely via the portal circulation. As noted above, the model assumes that intestinal absorption occurs via passive diffusion, and that efflux from the portal vein/enterocyte into the intestinal lumen, formation of SB-265123 glucuronide, and biliary excretion of the parent and the glucuronide all occur via saturable, active processes. Absorption and active intestinal efflux are considered to occur to the same extent throughout the relevant segment of the gastrointestinal tract, and the glucuronide is assumed to be negligibly absorbed. The model assumes no extrahepatic metabolism or renal excretion, that non-reversible glucuronidation of SB-265123 occurs in

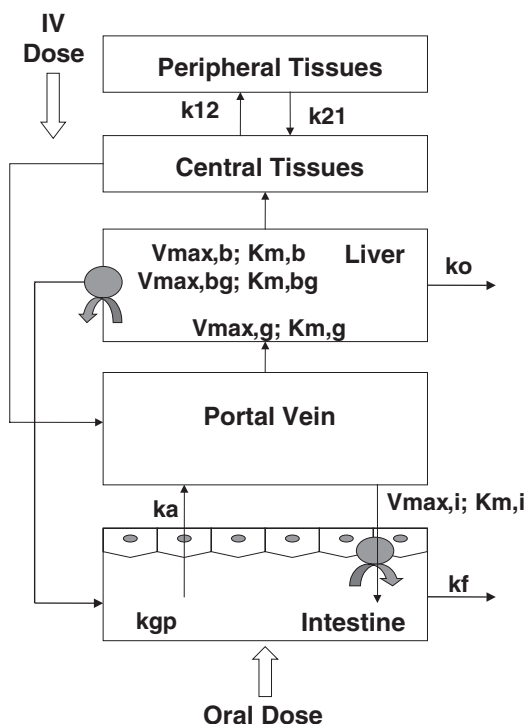


Figure 1. Proposed model for the disposition of SB-265123 in the rat. IV, intravenous; k_{12} and k_{21} , intercompartmental transfer rate constants; $V_{\max,b}/K_{m,b}$, maximum velocity and Michaelis–Menten constants for biliary excretion of SB-265123; $V_{\max,bg}/K_{m,bg}$, maximum velocity and Michaelis–Menten constants for biliary excretion of SB-265123 glucuronide; $V_{\max,g}/K_{m,g}$, maximum velocity and Michaelis–Menten constants for formation of SB-265123 glucuronide; k_o , rate of oxidative metabolism of SB-265123; k_a , absorption rate constant; $V_{\max,i}/K_{m,i}$, maximum velocity and Michaelis–Menten constants for intestinal efflux of SB-265123; k_{gp} , rate of intestinal conversion of SB-265123-glucuronide to SB-265123; k_f , rate of faecal elimination of SB-265123.

the liver, and that the glucuronide of SB-265123 can be cleaved in the intestine via a first-order process to return the parent molecule. Finally, the model assumes that any SB-265123 that is not absorbed into the portal vein is eventually eliminated via the faeces (k_f).

Model implications

When considering the intestinal disposition of a xenobiotic under these model conditions, several situations can exist. When concentrations in the intestinal lumen are low (i.e. following an intravenous dose), the total amount of drug absorbed into the portal vein is relatively small, and the saturable intestinal efflux pathway can operate as a first-order process. For molecules with a small k_a relative to the $K_m/V_{\max}$ of the active intestinal secretion process, the intestinal efflux mechanism might then act as an efficient barrier to absorption. This is the case for numerous molecules with poor oral bioavailability limited by intestinal drug transporters, especially those with a low oral dose and/or limited solubility (Fricker and Miller 2002). However, as concentrations in the intestinal lumen increase, either via delivery of drug to the intestine via biliary excretion or via an increased oral dose, the efflux mechanism may become saturated, and improved absorption may be achieved.

In the case of SB-265123, it is hypothesized that the K_m for intestinal efflux is quite small compared with the k_a for intestinal absorption. Following an intravenous dosage, the concentration of SB-265123 in the intestinal lumen due to biliary secretion is small, absorption into the enterocyte yields concentrations that are at or below the K_m for the intestinal efflux pathway, and intestinal clearance is an efficient elimination route for SB-265123. However, following oral administration, intestinal concentrations are high, and absorption yields concentrations at the transporter well above the K_m for active intestinal efflux, rendering intestinal clearance a less effective elimination mechanism. Furthermore, a substantial portion of the administered dose of SB-265123 is excreted either intact or as the

glucuronide conjugate into the bile; the amount of intestinal SB-265123 generated by this process also may be sufficient to result in saturation of the intestinal efflux pathway for SB-265123. Overall, with this working hypothesis, the systemic exposure of SB-265123 should be greatest following an oral dose in intact rats, when the intestinal 'barrier' to absorption is saturated, the entire oral dose is absorbed into the systemic circulation, and the efficiency of enterohepatic recirculation is maximized. The lowest systemic exposure of SB-265123 should be observed following an intravenous dosage to bile duct-exteriorized animals, where the only SB-265123 in the intestine is that resulting from intestinal efflux clearance, which operates at $V_{\max}$. Administration of an intravenous dose to intact rats or an oral dose to bile duct-exteriorized rats should yield systemic exposure values somewhere between these two extremes. Consequently, oral bioavailability (calculated based on the ratios of the oral and intravenous dose-normalized areas under the curves) would be $> 100\%$.

Materials

SB-265123 [(S)-10,11-dihydro-3-[3-(pyridin-2-ylamino)-1-propyloxy]-5H-dibenzo [a,d] cycloheptene-10-acetic acid; molecular weight = 402.49] and GF120918A (5-methoxy-9-oxo-9,10-dihydro-acridine-4-carboxylic acid {4-[2-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-ethyl]-phenyl}-amide HCl) were prepared by the Department of Medicinal Chemistry, GlaxoSmithKline. All other reagents were purchased from standard vendors and were of the highest available purity.

Animals

Male outbred rats (CrI:CD(SD)IGS BR; Charles River, Raleigh, NC, USA) weighing 280–410 g were used. Animals were housed according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) in individual cages in unidirectional airflow rooms with controlled temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($50 \pm 10\%$) and 12-h light/dark cycles (06.00–18.00 hours). Filtered tap water was available *ad libitum* and was periodically analysed by the Philadelphia Suburban Water Company, PA, USA. Animals were fed a standard animal diet (Purina Mills, St Louis, MO, USA); food was available *ad libitum* except for overnight periods before dosing, as described below. When overnight fasting was employed, food was provided after the 240-min blood sample was obtained on the following study day. All animal use was conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee before the study, and all surgical procedures were conducted using aseptic techniques in special-purpose operating suites.

Single-pass intestinal perfusion

To confirm the involvement of an intestinal efflux mechanism in the elimination of SB-265123 in the rat, single-pass intestinal perfusion studies were conducted according to a standard technique (Sandström *et al.* 1998). Briefly, rats were anaesthetized and femoral vein and bile duct catheters were implanted. Rats remained anaesthetized throughout the 4-h experiment, and body temperature was maintained at 37°C via a heating pad. An approximately 10-cm segment of either duodenum or ileum ($n = 4$ rats per intestinal segment) was isolated by ligation and catheterized with polyethylene tubing, with care being taken not to disturb the blood flow of the isolated segment. The lumen of the segment was perfused with prewarmed (37°C) isotonic saline for 15 min until the perfusate was clear; perfusion continued throughout the experiment at a rate of 0.1 ml min^{-1} . The experiments were initiated with a 0.3 mg kg^{-1} bolus loading dose of SB-265123 via the femoral vein catheter, followed by a continuous intravenous infusion of SB-265123 at $0.45 \text{ mg kg}^{-1} \text{ h}^{-1}$, designed to attain a target systemic SB-265123 steady-state concentration of 1000 ng ml^{-1} . Intestinal perfusate was collected at 15-min intervals, and blood samples were obtained from a lateral tail vein at regular intervals and processed as described below. At the end of the experiments, rats were euthanized with an overdose of the ketamine/xylazine anaesthetic.

Intravenous/oral crossover studies

Various permutations of a standard intravenous $\times$ oral crossover experiment were performed as described (Ward *et al.* 1999, 2004). Briefly, for each experiment, rats ($n = 3$ per experiment unless otherwise specified) received surgically implanted femoral vein catheters for drug administration. Intravenous dosages ($2 \mu\text{mol kg}^{-1}$) were administered as a 30-min infusion (4.0 ml kg^{-1}) and oral doses ($2 \mu\text{mol kg}^{-1}$) were a bolus via oral gavage (16 ml kg^{-1}); test periods were conducted at least 2 days apart. For all studies, a dose vehicle of 0.5% dimethyl sulfoxide and 5% polyethylene glycol-300 was used, unless otherwise stated. Blood samples were collected from each study animal from a lateral tail vein at various times during and after drug administration; plasma ($50 \mu\text{l}$) was isolated by centrifugation and stored at or about -70°C until analysis.

Intravenous/oral dual administration

An experiment was performed to investigate the exposure of SB-265123 during a dual intravenous infusion and oral gavage. First, a standard intravenous/oral crossover experiment was conducted, as

described above. Then on a third study day 1 week later, an identical oral and an intravenous dose were co-administered simultaneously and blood samples obtained as described above.

GF120918A study

To investigate the potential role of transporter inhibition on the F_{app} of SB-265123, a four-way crossover experiment was performed with SB-265123 and the transport inhibitor GF120918A. On study days 1 and 2, rats ($n=4$) received SB-265123 as described above; 15 min before both the intravenous and oral doses of SB-265123, the animals received an oral gavage of blank vehicle (0.5% aqueous hydroxypropylmethylcellulose with 1% Tween 80; 10 ml kg⁻¹ dose volume). On study days 3 and 4, the same rats received an identical intravenous or oral dose of SB-265123 as on study days 1 and 2, except that the pretreatment consisted of vehicle containing 2.5 mg free base ml⁻¹ GF120918A (25 mg free base kg⁻¹ target dose).

Analytical procedure

Quantitative analysis of plasma for SB-265123 was performed using a liquid chromatography/tandem mass spectrometry method as described (Ward *et al.* 1999), with a lower limit of quantitation of 2.0 ng ml⁻¹. For the GF120918A experiment, plasma was analysed similarly for GF120918, also with a lower limit of quantitation of 2.0 ng ml⁻¹.

Data analysis

Concentration–time profiles for SB-265123 (and GF120918, as appropriate) were obtained for each animal in each study. Standard non-compartmental analysis was performed on the data using WinNonlin Professional Version 3.3 (Pharsight, Mountain View, CA, USA) to recover the area under the curve (AUC) and other non-compartmental parameters. Oral bioavailability was estimated by dividing the dose-normalized AUC (DNAUC) resulting from oral administration by the DNAUC resulting from intravenous administration. All analyses were conducted using both AUC(0– t), where t is the last time point with measurable drug concentrations in the study, and AUC(0– ∞). The conclusions from each study were identical regardless of the AUC method employed; all data shown are AUC(0– t) to avoid any bias due to study-to-study differences in the extrapolated AUC. Either a two-tailed Student's t -test or a one-way analysis of variance (ANOVA) with Tukey's post-hoc comparison were used for statistical evaluation of the observed data, as appropriate. In all cases, a probability level of $p \leq 0.05$ was predetermined as the criterion of statistical significance.

Results

Application of proposed model to previous data

Based on the hypothesis described above and in the model depicted in figure 1, the data generated previously under various experimental conditions were re-evaluated (Ward *et al.* 2004) (table 1). As predicted by the model, the AUC following an intravenous dose to bile duct-exteriorized rats was significantly lower than that generated following an oral dose to intact animals, with the exposure from the other two groups being intermediate to these two and not significantly different from each other (one-way ANOVA with Tukey's post-hoc comparison).

Table 1. Area under the plasma concentration–time curves for SB-265123 in the rat under different experimental conditions (data from Ward *et al.* 2004).

Experimental condition	Dose-normalized AUC (min $\mu\text{g ml}^{-1}$ /mg kg ⁻¹)
Intravenous dose, bile duct-exteriorized rats	117 $\pm$ 22 [†]
Intravenous dose, intact rats	213 $\pm$ 29
Oral dose, bile duct-exteriorized rats	160 $\pm$ 36
Oral dose, intact rats	276 $\pm$ 82 [†]

Values are the average $\pm$ SD for $n=3$ rats.

[†]Significantly different across groups, one-way ANOVA with Tukey's post-hoc comparison.

Single-pass intestinal perfusion

To confirm the existence of an intestinal efflux mechanism for SB-265123 in the rat, single-pass duodenal and ileal perfusion experiments were conducted. Systemic infusion of SB-265123 to steady-state concentrations of approximately 1000 ng ml^{-1} in the bile duct-exteriorized rat was associated with substantial excretion of intact SB-265123 into both the ileum and duodenum (figure 2). Qualitative analysis of the intestinal perfusate revealed intact SB-265123 to be the only drug-related material detected in these samples (data not shown).

Intravenous/oral dual administration

According to the model in figure 1, the intestinal clearance of SB-265123 should be saturated in the presence of high SB-265123 concentrations in the

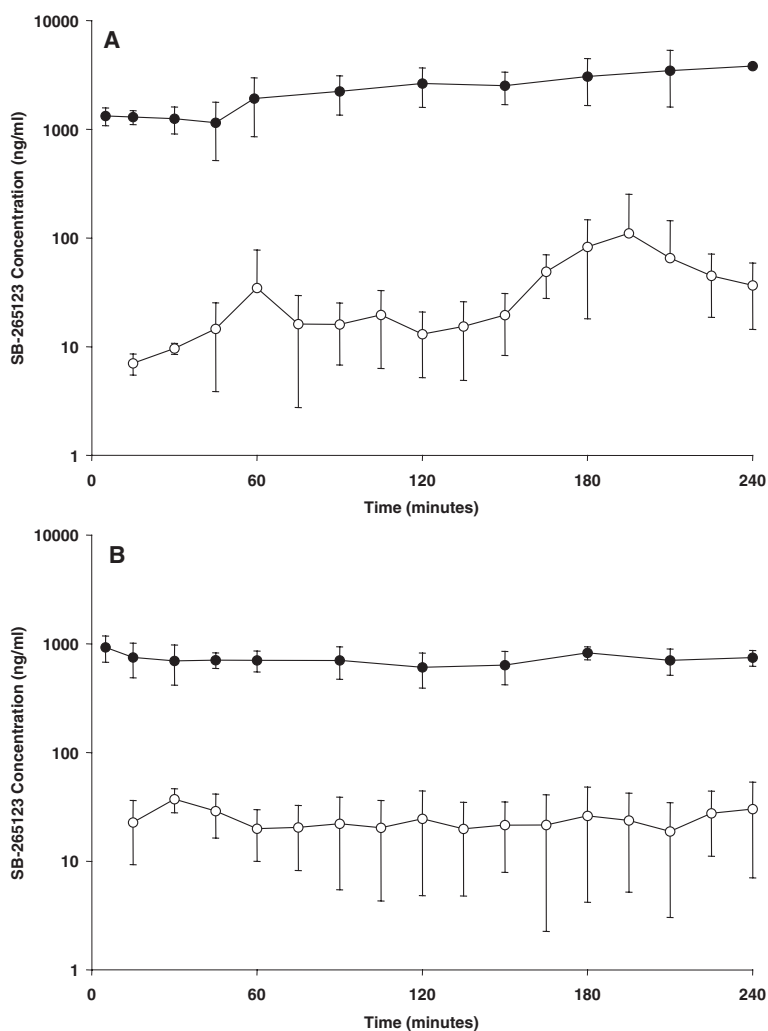


Figure 2. Plasma (●) and intestinal perfusate (○) concentrations of SB-265123 in the anaesthetized bile duct-exteriorized rat during single-pass duodenal (A) or ileal (B) experiments. Data are the average $\pm$ SD in $n = 3-4$ rats per experiment.

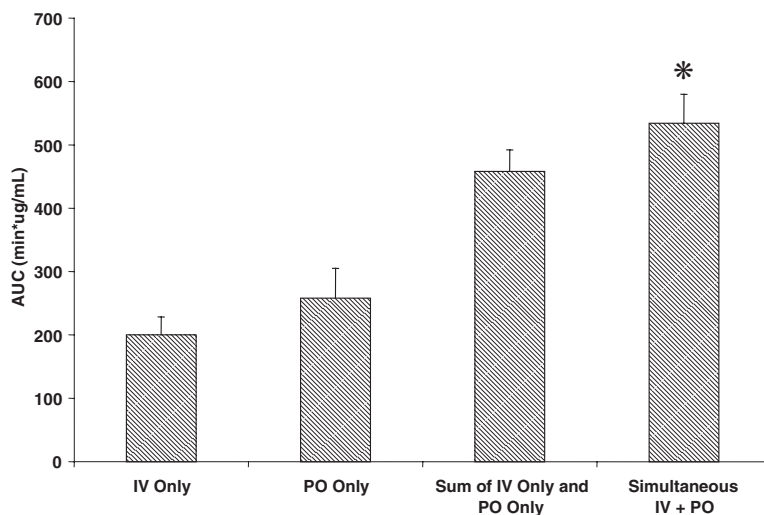


Figure 3. Comparison of the AUC generated from an intravenous and oral dose of SB-265123 administered either separately or simultaneously. Data are the average $\pm$ SD in $n=3$ rats. *Significantly different from combined AUCs from separate intravenous and oral doses of SB-265123, $p=0.041$.

intestinal lumen. To interrogate this possibility, an experiment was performed in which an intravenous and an oral dose of SB-265123 were simultaneously administered. Under these conditions, the proposed model would predict greater-than-additive exposure of SB-265123 with simultaneous administration due to saturation of the intestinal transporter with administration of the oral dose; the same rats received an identical intravenous and oral dose separately for a crossover comparison. As shown in figure 3, simultaneous co-administration of the intravenous and oral dose of SB-265123 resulted in a significantly greater-than-additive exposure compared with separate administration of the two dosages alone. Maximal systemic concentrations of SB-265123 in this experiment were $\leq 3700 \text{ ng ml}^{-1}$, and were therefore within the demonstrated range of systemic pharmacokinetic linearity for SB-265123 in the rat (Ward *et al.* 2004).

GF120918A study

To provide evidence for the involvement of P-glycoprotein and/or BCRP in the intestinal secretion of SB-265123, GF120918A was used as a tool transport inhibitor. The dosage and dose vehicle for GF120918 were selected to be similar to those that produced a significant effect on the disposition of paclitaxel and topotecan in mice (Bardelmeijer *et al.* 2000, Jonker *et al.* 2000), and that are efficacious in a murine P388 leukaemia model (Hyafil *et al.* 1993). The systemic plasma concentrations of GF120918 achieved in this experiment ($C_{\text{max}} = 112 \pm 15 \text{ ng ml}^{-1}$; $\text{AUC} = 78.5 \pm 14.6 \text{ min } \mu\text{g ml}^{-1}$) were similar to those observed by Hyafil *et al.*, confirming that adequate exposure of the modulator was achieved. As shown in table 2, co-administration of GF120918A decreased F_{app} of SB-265123 to $<100\%$, a significant decrease compared with crossover control conditions (117 ± 14 versus $84 \pm 17\%$; $p=0.0105$, paired *t*-test). As anticipated based on the proposed model, this decrease in bioavailability was not achieved by

Table 2. Pharmacokinetic parameters for SB-265123 in the presence and absence of GF120918A.

Parameter	Control	With GF120918A
Clearance ($\text{ml min}^{-1} \text{kg}^{-1}$)	4.10 ± 0.61	$3.40 \pm 0.76^\dagger$
Volume of distribution (l kg^{-1})	0.935 ± 0.142	$0.644 \pm 0.125^\dagger$
Half-life (min)	210 ± 48	176 ± 44
Mean residence time (min)	232 ± 47	195 ± 47
Intravenous AUC ($\mu\text{g min ml}^{-1}$)	203 ± 43	$244 \pm 70^\dagger$
Oral AUC ($\mu\text{g min ml}^{-1}$)	233 ± 30	240 ± 37
Oral bioavailability (%)	117 ± 14	$84.0 \pm 17^\dagger$
Oral C_{max} (ng ml^{-1})	1630 ± 521	1902 ± 233

Data are the average $\pm$ SD for $n = 4$ rats.

† Significantly different between groups, Student's two-tailed paired t -test ($p < 0.05$).

a change in oral exposure of SB-265123, as both C_{max} and AUC for SB-265123 were unchanged in the presence of GF120918A. Instead, orally administered GF120918A significantly altered only the intravenous pharmacokinetics of SB-265123, with a significant decrease in clearance (4.1 ± 0.6 versus $3.4 \pm 0.8 \text{ ml min}^{-1} \text{kg}^{-1}$; $p = 0.0158$, paired t -test) and in volume of distribution (0.935 ± 0.142 versus $0.644 \pm 0.125 \text{ l kg}^{-1}$; $p = 0.0470$, paired t -test).

Discussion

Over the past decade, the role of active transport processes in the disposition of xenobiotics has been rigorously explored. Multiple families of transporters, with overlapping expression patterns and substrate specificity, have been demonstrated to regulate pharmacokinetic processes in the intestine, blood–brain barrier, kidney and hepatobiliary system (Matheny *et al.* 2001). With respect to intestinal transporters, the conventional understanding suggests that intestinal transporters, often in conjunction with CYP3A4, generally serve to limit the absorption of orally administered substances (Benet and Cummins 2001, Wacher *et al.* 2001). In fact, several investigators have suggested the clinical use of co-administered transporter inhibitors to enhance bioavailability for therapeutic drugs with poor absorption due to active intestinal efflux (Benet *et al.* 1995, Van Asperen *et al.* 1998). Accordingly, on first consideration, the observation of $F_{\text{app}} > 100\%$ for SB-265123 was not immediately associated with a counterintuitive transporter-based interaction.

Upon further reflection and investigation, however, numerous pieces of evidence suggested the involvement of an active transport process in the pharmacokinetics of SB-265123. SB-265123 demonstrates structural characteristics commonly associated with transporter substrates, particularly the presence of a free carboxylate moiety. Interestingly, a structurally similar $\alpha\text{v}\beta 3$ antagonist has also been shown to be a substrate for P-glycoprotein (Prueksaritanont *et al.* 2002). In addition, experiments in bile duct-exteriorized animals (Ward *et al.* 1999, 2004) demonstrated that both SB-265123 and its glucuronide conjugate are excreted in bile, a phenomenon commonly associated with an active transport process (Kusuhara *et al.* 1998). However, bile duct exteriorization experiments revealed that F_{app} was still $> 100\%$, suggesting that the observed $F_{\text{app}} > 100\%$ is not defined entirely by biliary excretion. Several transporters involved with biliary excretion of xenobiotics are also expressed in the intestine, including P-glycoprotein, and

different transporters on these membranes might share substrate specificity (Van Asperen *et al.* 1998, Fricker and Miller 2002). Therefore, the possible influence of an active transport process serving as an intestinal secretion mechanism for SB-265123 was considered according to the model presented in figure 1. According to this model, the aberrantly high oral bioavailability of SB-265123 could be attributed to saturation of an active intestinal efflux process following oral, but not intravenous, administration. Such an intestinal secretion process was demonstrated in the present study to occur for SB-265123 following intravenous administration. Furthermore, evidence supporting the saturable efflux hypothesis was generated in an experiment employing separate or simultaneous oral and intravenous administration of SB-265123 in a crossover fashion; the systemic AUC generated from the simultaneous dose was significantly greater than the sum of that produced by identical separate oral and intravenous dosages.

The results from the experiment with GF120918A further implicate a specific transporter-mediated process in the oral bioavailability of SB-265123. GF120918 is a potent inhibitor of P-glycoprotein *in vitro* (Hyafil *et al.* 1993), and also significantly alters the pharmacokinetics of drugs subject to transporter-mediated disposition *in vivo*, including doxorubicin (Sparreboom *et al.* 1999), morphine (LeTrent *et al.* 1999), topotecan (Jonker *et al.* 2000) and paclitaxel (Bardelmeijer *et al.* 2000). GF120918A appears to demonstrate specificity for inhibition of P-glycoprotein over MRP1, MRP2, and the bile salt export pump (Wallstab *et al.* 1999, Evers *et al.* 2000), but is an inhibitor of BCRP (Jonker *et al.* 2000). Therefore, either BCRP or P-glycoprotein appears to be involved in the disposition of SB-265123.

P-glycoprotein and other transporters are well recognized to play important roles in the disposition of a variety of xenobiotics, and particularly to serve as 'barriers' for intestinal drug absorption (Benet *et al.* 1999). The consensus *modus operandi* of these transporter systems that has developed involves a 'futile cycling' process with absorption of the transporter substrate into the enterocyte, efflux of the substrate by the transporter back into the intestinal lumen, and re-absorption of the substrate into the enterocyte (Matheny *et al.* 2001). The model proposed for the disposition of SB-265123 in the present investigation is consistent in mechanism with this literature consensus. However, the present model incorporates an additional complexity, namely, the saturation of this process in the presence of high intestinal concentrations of SB-265123 (due to the relatively large absorption rate constant for SB-265123) that can be mimicked by the presence of a P-glycoprotein inhibitor. Typically, intestinal transporter-mediated processes are explored for molecules with poor oral bioavailability, often due to poor inherent permeability or solubility and a low absorption rate constant. However, had the F_{app} of SB-265123 in the rat been $\leq 100\%$ but still favourable, this intriguing mechanism would not have been further investigated. This observation suggests that a similar mechanism might operate for other compounds with reasonable oral bioavailability. This possibility should be further considered, particularly in light of current interest in improving oral bioavailability via transporter inhibition (Benet *et al.* 1995). The present data suggest that such approaches might result in a greater prevalence of drug interactions than might be anticipated based on the current understanding of the molecular space of transporter substrates; further investigation will be required to explore the implications of these findings.

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Comparative disposition of [^{14}C]ertapenem, a novel carbapenem antibiotic, in rat, monkey and man

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1. The disposition and metabolism of ertapenem, a carbapenem antibiotic, was examined in rat, monkey and man. Sprague–Dawley rats and Rhesus monkeys were given, by intravenous administration, radiolabelled doses of ertapenem (60 and 30 mg kg⁻¹, respectively), and healthy normal volunteers received a single fixed dose of 1000 mg. Urine and faeces were collected for determination of total radioactivity.

2. In healthy volunteers, [^{14}C]ertapenem was eliminated by a combination of hydrolytic metabolism to a beta-lactam ring-opened derivative and renal excretion of unchanged drug. Approximately equal amounts were excreted as a beta-lactam ring-opened metabolite and unchanged drug (36.7 and 37.5% of dose, respectively). A secondary amide hydrolysis product accounted for about 1% of the dose in man. About 10% of the administered radioactivity was recovered in faeces, which suggested that a minor fraction underwent biliary and/or intestinal excretion.

3. In animals, a greater fraction of the dose was eliminated via metabolism; excretion of unchanged drug accounted for 17 and 5% of dose in rats and monkeys, respectively. In monkeys, the beta-lactam ring-opened and amide hydrolysis metabolites accounted for 74.8 and 7.59% of the dose, respectively, whereas in rats, these metabolites accounted for 31.9 and 20% of dose, respectively.

4. *In vitro* studies with fresh rat tissue homogenates indicated that lung and kidney were the primary organs involved in mediating formation of the beta-lactam ring-opened metabolite. The specific inhibitor of dehydropeptidase-I, cilastatin, inhibited the *in vivo* and *in vitro* metabolism of ertapenem in rats, which suggested strongly that the hydrolysis of ertapenem in lung and kidney was mediated by this enzyme.

Introduction

Ertapenem (INVANZ, MK-826) is a new beta-lactam antibiotic with activity against Gram-positive and -negative bacteria (Gill *et al.* 1998) (figure 1). It is effective as a once-daily parenteral treatment of intra-abdominal, skin, skin-structure, community-acquired pneumonia, acute pelvic infections and urinary tract infections caused by susceptible bacteria (Anon 2002, Ortiz-Ruiz *et al.* 2002, Yellin *et al.* 2002). Following administration of a 1-g intravenous dose over 30 min, peak plasma concentrations are approximately 150 µg ml⁻¹ and the plasma half-life of ertapenem is about 4 h (Majumdar *et al.* 2002). In man, urinary excretion of

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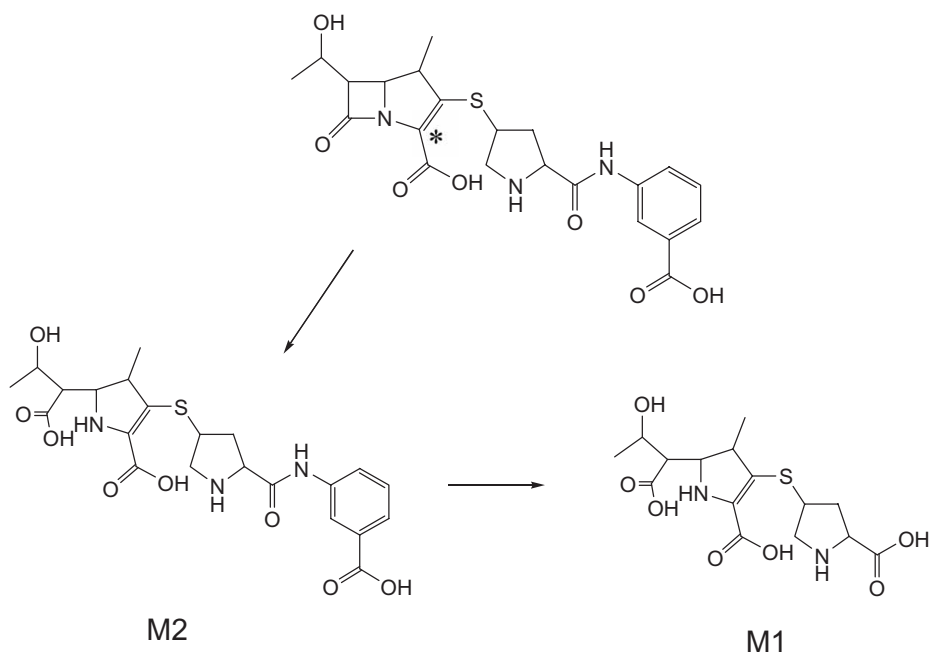


Figure 1. Proposed metabolic scheme for ertapenem. *Position of the [^{14}C]label.

unchanged drug accounts for only 40–50% of the dose, therefore clearance via metabolism appears to be important. The present studies compared the disposition of ertapenem in rats, monkeys and man.

Materials and methods

Radiolabelled and unlabelled ertapenem were prepared by the Labeled Compound Synthesis Group and Department of Process Chemistry, respectively, at Merck Research Laboratories (West Point, PA, and Rahway, NJ, USA) (figure 1). The beta-lactam ring-opened derivative of ertapenem was synthesized by the Department of Process Chemistry. The radiochemical purity of the [^{14}C]-labelled material was >97%. Dosage solutions administered to human subjects were formulated by the Pharmaceutical Research and Development Department, Merck Research Laboratories. All other reagents were of the highest commercially available grade.

Human studies

The study was conducted at the Clinical Research Unit, Thomas Jefferson University (Philadelphia, PA, USA). The study protocol was approved by the Institutional Review Board of the university. All subjects agreed to participate in the study by giving informed written consent. Seven subjects (four males, ages 23–28 years, weight 55–89 kg; three females, 45–49 years, 53–84 kg) received a 1-g dose (about 100 μCi) as an intravenous infusion dissolved in isotonic saline over 30 min. Plasma samples were obtained predose and at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24 and 48 h post-dose, and urine was collected during intervals of 0–4, >4–8, >8–12, >12–18, >18–24, >24–48, >48–72, >72–96, >96–120, >120–144 and >144–168 h. Preliminary studies established that maximal stability of ertapenem was obtained in the presence of 1 M 2-[N-morpholino]ethanesulfonate (MES) (pH 6.5) buffer and therefore this reagent was added in equal volumes to urine before storage at -70°C . Faeces were collected daily for 7 days post-dose.

Rat and monkey studies

Ertapenem dose solutions were prepared by dissolving radiolabelled and unlabelled ertapenem in isotonic saline to final specific activities of 1.23 $\mu\text{Ci mg}^{-1}$ (rat) or 0.24 $\mu\text{Ci mg}^{-1}$ (rhesus monkey). Rats, weighing about 250 g and obtained from Taconic Farms (Germantown, NY, USA), were housed under standard conditions with free access to food (PicoLab Rodent Diet 20, PMI Nutrition International,

Brentwood, MO, USA) and water. On the day before receiving drug, a silicone rubber/polyethylene cannula was implanted in a jugular vein under pentobarbital anaesthesia. A dose of 60 mg kg^{-1} ertapenem was administered to rats by a jugular vein cannula and the animals were housed in metabolism cages for the collection of urine and faeces. Urine was collected on ice, 0–4, >4–24 and >24–48 h, and faeces were collected daily for 2 days after administration of ertapenem. Male rhesus monkeys weighing an average of 5.1 kg were obtained from Charles River (Key Lois, FL, USA). For monkeys, a dose of 30 mg kg^{-1} was given by a cephalic vein and urine was collected 0–6, >6–24, >24–48 and >48–72 h, and faeces were collected daily for 4 days. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee. To assess the contribution of circulating metabolites to systemic exposure, plasma concentrations of ertapenem and total radioactivity were measured following intravenous administration of the labelled compound.

Effect of cilastatin on disposition of ertapenem in rats

The effect of cilastatin, a competitive inhibitor of dehydropeptidase-I (DHP-I), on the disposition of ertapenem was examined to assess the contribution of this hydrolytic pathway. A 40-mg kg^{-1} dose of cilastatin was given intravenously approximately 1 min before administration of the ertapenem (10 mg kg^{-1}) dose followed by four additional doses of cilastatin (25 mg kg^{-1}) at 45-min intervals. Plasma clearance and steady-state distribution volume were calculated using standard non-compartmental methods (Gibaldi and Perrier 1982). Plasma and urine were collected as described above.

In vitro studies

To assess the major organs involved in the metabolism of ertapenem, *in vitro* experiments were conducted using rat tissue homogenates. Fresh rat liver, lung and kidney tissue were homogenized in 4 vols isotonic sodium phosphate buffer (pH 7.4). Radiolabelled ertapenem was added to 0.5 ml tissue homogenate at a final concentration of $50 \mu\text{M}$. The incubation was carried out at 37°C for 30 min. Control samples were prepared under the same conditions with boiled tissues. At the end of the incubation, the pH was adjusted with 1 vol. 1 M MES (pH 6.5) buffer and proteins precipitated with acetonitrile/methanol (4/1, v/v). After centrifugation, the supernatants were transferred to fresh tubes and evaporated to dryness under vacuum. Cilastatin, a specific inhibitor of DHP-I, was used to assess the contribution of this enzyme to the metabolism of ertapenem. Rat tissue homogenates were pre-incubated for 5 min with cilastatin (1 mM) before the addition of ertapenem.

Assays

Radioactivity in urine and plasma was assayed by direct addition to Ready-Safe (Beckman, Fullerton, CA, USA) scintillation cocktail. Faeces were homogenized in water and air-dried aliquots of the resultant suspensions were combusted using a Packard Sample Oxidizer (Meriden, CT, USA). Radioactivity was measured by liquid scintillation spectrometry with quench correction by external standardization. Plasma concentrations of ertapenem were determined by direct injection column-switching liquid chromatography-ultraviolet light detection methods (Musson *et al.* 1998, Wong *et al.* 1999). The limit of quantification of these methods was $0.1 \mu\text{g ml}^{-1}$ in animal plasma and $0.125 \mu\text{g ml}^{-1}$ in human plasma.

Urinary radioactivity was profiled by gradient elution liquid chromatography with online radioactivity flow detection. The column was a Brownlee RP-18 Spheri-5 ($220 \times 4.6 \text{ mm}$) preceded by a guard column of the same material. Elution was performed with a gradient of acetonitrile in 20 mM sodium phosphate and 20 mM triethylamine (pH 6.5, linear increase from 2 to 17% from 0 to 25 min, hold at 17% acetonitrile from 25 to 35 min) at a flow rate of 1.1 ml min^{-1} . Radioactivity was monitored continuously with a Packard Flo-one using Ultimata-Flo M as scintillant at a flow rate of 3.3 ml min^{-1} . The retention times of ertapenem and the ring-opened derivative under these conditions were 22 and 18 min, respectively. Faecal radioactivity was not characterized.

Mass spectral characterization of the major urinary metabolite

Purified fractions containing radioactivity corresponding to the major urinary metabolite were analysed by liquid chromatography-tandem mass spectrometry with a Finnigan LCQ ion-trap mass spectrometer with electrospray interface operated at 5 kV and capillary temperature of 200°C . Elution was performed with a gradient of acetonitrile in 0.1% trifluoroacetic acid in water (5% acetonitrile from 0 to 5 min, linear increase to 90% acetonitrile from 5 to 20 min) at a flow rate of 0.2 ml min^{-1} . The column was a BDS Hypersil C18 ($150 \times 2.1 \text{ mm}$). Under these conditions, the retention time of the major urinary metabolite was 13 min. Mass spectra were acquired in the positive-ion mode. Mass spectral characterization of the minor amide hydrolysis product (M1) was conducted as described (Hop *et al.* 2001).

Statistical analysis

A Student's unpaired *t*-test was used to assess the effect of cilastatin on ertapenem pharmacokinetic parameters in rats. A log transformation of data was made when unequal variances were detected.

Results

In man, following administration of [^{14}C]ertapenem the radioactivity was recovered predominantly in urine (table 1). A minor fraction was excreted in faeces indicating that a small portion of the dose was eliminated by biliary and/or intestinal secretion (figure 2). Despite the extensive metabolism, plasma concentrations of ertapenem and total radioactivity were similar in man, as they were in rats and monkeys, indicating that metabolites of ertapenem contributed minimally to systemic exposure, and suggesting that the ring-opened metabolite was cleared rapidly as compared with the parent drug (figure 3).

After intravenous administration of [^{14}C]ertapenem to rats, the radioactivity was eliminated predominantly in urine as it was in man (table 1). Approximately 42% of the dose was recovered in urine during the initial 4 h, and cumulative recovery over 48 h was 72% of dose. Recovery in faeces accounted for 22.5% of dose, reflecting excretion of ertapenem or its metabolites into bile (see below). Unchanged drug in urine accounted for 17% of dose, indicating there was sub-

Table 1. Excretion of radioactivity in rats, monkeys and man following intravenous administration of radiolabelled ertapenem.

Species	Urine	Faeces	Total
Rat	71.7 $\pm$ 7.7	22.5 $\pm$ 3.0	94.2 $\pm$ 8.1
Monkey	87.3 $\pm$ 4.1	9.3 $\pm$ 1.9	96.6 $\pm$ 4.4
Man	80.5 $\pm$ 13.8	9.2 $\pm$ 7.2	89.7 $\pm$ 12.9

Values (per cent of dose) are shown as the mean $\pm$ SD of $n = 3$ for rats and monkeys, and $n = 7$ for man. Urine and faeces were collected for 48 h in rats, 72 h in monkeys and 168 h in man.

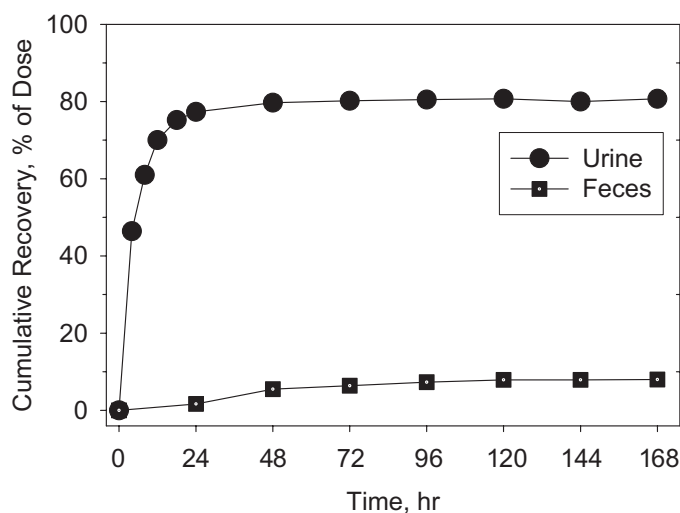


Figure 2. Cumulative urinary and faecal excretion of radioactivity in man following intravenous administration of a 1000-mg dose of radiolabelled ertapenem (shown as the mean of $n = 7$).

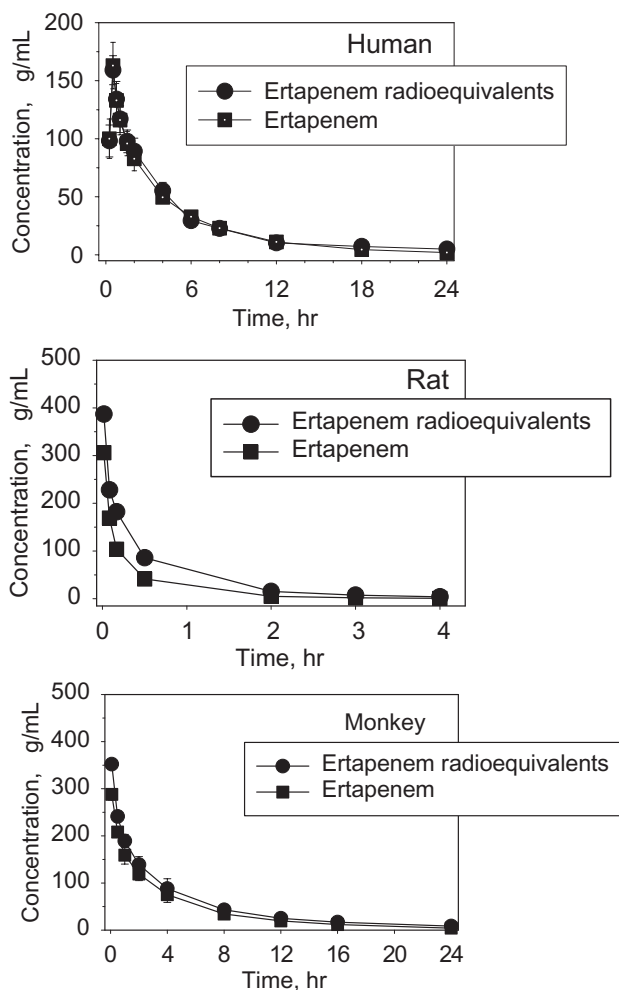


Figure 3. Mean plasma concentration–time profiles of ertapenem radioequivalents and ertapenem in man (1 g), rats (60 mg kg^{-1}) and monkeys (30 mg kg^{-1}) following intravenous administration of [^{14}C]ertapenem (shown as the mean and SD at each time point of $n = 3$ for rats and monkeys and $n = 7$ for man).

Table 2. Metabolite profile of radioactivity in rat and monkey urine following administration of an intravenous dose of [^{14}C]ertapenem.

Species	Chromatographic retention time (min)						
	2.4 M1	10.6	15 M2 (ring-opened)	21 Ertapenem	24.8	26.4	Total
	Percent of dose						
Rats	20.0 ± 2.0	n.d.	31.9 ± 4.6	17.4 ± 2.4	2.00 ± 0.33	n.d.	71.3 ± 9.0
Monkeys	7.59 ± 0.32	0.39 ± 0.15	74.8 ± 3.7	5.20 ± 2.12	2.11 ± 0.52	0.62 ± 0.23	90.7 ± 5.0

Values are the mean $\pm$ SD of $n = 3$ animals.

stantial metabolism of ertapenem (table 2). In monkeys, radioactivity was eliminated predominantly in urine, with most of the dose excreted during the initial 6 h. Cumulative recovery in urine over 72 h was 87.3% of dose and unchanged drug in urine accounted for only 5.2% of the dose (table 2).

Profile of urinary radioactivity

Profiling of human urine through 120 h showed eight components that accounted for all of the recovered radioactivity (table 3). Six of the eight components were quantitatively minor contributors (<1% of the dose). Renal excretion of unchanged ertapenem accounted for 37.5% of the dose. The major radioactive metabolic component (designated M2) in urine (36.7% of dose) corresponded to the beta-lactam ring-opened derivative of ertapenem. Together, ertapenem and this metabolite accounted for about 95% of the urinary radioactivity. Although the number of subjects was small, sex differences in the urinary metabolic profiles were not evident.

In rats and monkeys, as in man, urinary excretion was the primary mode of elimination of administered radioactivity (table 1). Although qualitatively similar, there were quantitative interspecies differences in the metabolite profiles. Analysis of rat urine revealed one major and multiple minor urinary radioactive components in addition to parent drug (table 2). The major metabolite (31.9% of dose) corresponded to the beta-lactam ring-opened derivative of ertapenem. An additional polar component accounted for a further 20% of dose. This latter component has been identified as a secondary product resulting from amide hydrolysis of the ring-opened derivative M1 (Hop *et al.* 2001). In monkeys, the radioactivity existed predominantly as the beta-lactam ring-opened form (74.8% of dose), whereas the urinary excretions of unchanged drug and M1 were minor. Additional radio-labelled components were detected in rat, monkey and human urine, and as these were quantitatively minor, they were not characterized further.

Metabolite characterization

The major urinary metabolite of ertapenem was identified as the product of hydrolysis of the beta-lactam ring. The total and reconstructed ion chromatograms of the urinary metabolite are shown in figure 4. Full-scan mass spectra of the urinary metabolite and the synthetic standard of the ring-opened derivative both gave the same protonated molecular ion at m/z 494. The product-ion mass spectrum of the metabolite contained major fragments at m/z 450, 267 and 140, and was essentially identical to that of the authentic material (latter not shown). As described, a metabolite (M1) that was the product of amide hydrolysis also was observed in rat, monkey and human urine (Hop *et al.* 2001).

Effect of cilastatin in rats in vivo and in vitro

Some carbapenem antibiotics are subject to hydrolysis catalysed by a dipeptidase, DHP-I. Inhibition of DHP-I with cilastatin decreased ertapenem clearance in the rat from 5.2 to 0.89 ml min⁻¹ kg⁻¹, and a decrease in the urinary excretion of the beta-lactam ring-opened metabolite from 31 to 9% of dose (table 4).

Conversion of ertapenem to the beta-lactam ring-opened hydrolysis product was detected in rat lung and kidney homogenates (figure 5). Under the same conditions, ertapenem was metabolized minimally by rat liver homogenates (data not shown), suggesting that the contribution of this organ to clearance was minor.

Table 3. Metabolite profile of radioactivity in human urine following administration of a 1-g intravenous dose of [^{14}C]ertapenem.

	Chromatographic retention time (min)								
	2.7 M1	11.5	15 M2 (ring-opened)	16.8	18.2	21 Ertapenem	23.4	24.3	Total
Sex	Per cent of dose								
Male	1.00 $\pm$ 0.75	0.66 $\pm$ 0.09	39.5 $\pm$ 7.6	0.81 $\pm$ 0.71	0.46 $\pm$ 0.23	35.2 $\pm$ 6.9	0.70 $\pm$ 0.48	0.97 $\pm$ 0.14	79.4 $\pm$ 13.2
Female	0.52 $\pm$ 0.15	0.54 $\pm$ 0.32	32.9 $\pm$ 10.3	0.64 $\pm$ 0.11	0.14 $\pm$ 0.11	40.6 $\pm$ 10.9	0.11 $\pm$ 0.11	1.09 $\pm$ 0.89	76.6 $\pm$ 14.7
Combined	0.79 $\pm$ 0.60	0.61 $\pm$ 0.21	36.7 $\pm$ 8.7	0.74 $\pm$ 0.52	0.33 $\pm$ 0.24	37.5 $\pm$ 8.5	0.45 $\pm$ 0.47	1.02 $\pm$ 0.53	78.2 $\pm$ 12.7

Values are the mean $\pm$ SD of $n = 3$ (females) or 4 (males).

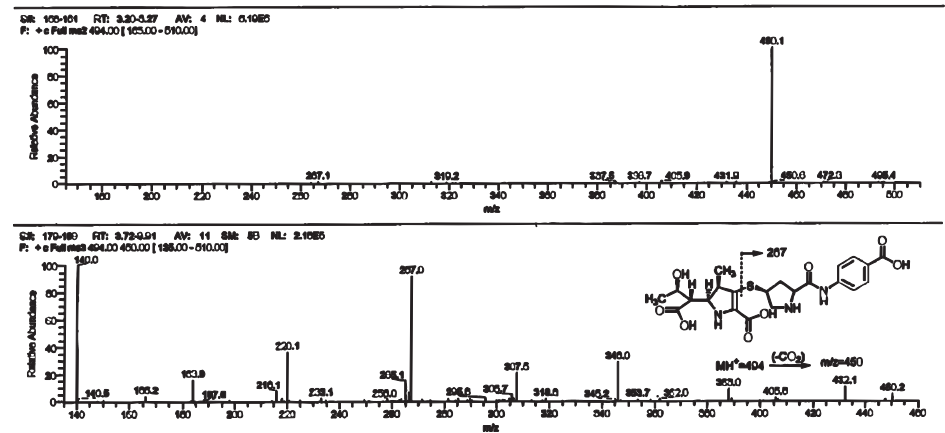


Figure 4. Product-ion mass spectrum of the major radioactive component isolated from human urine after intravenous administration of [¹⁴C]ertapenem (top panel, product-ion spectrum of *m/z* 494; bottom panel, product-ion spectrum from the MS3 experiment of precursor ion, *m/z* at 450).

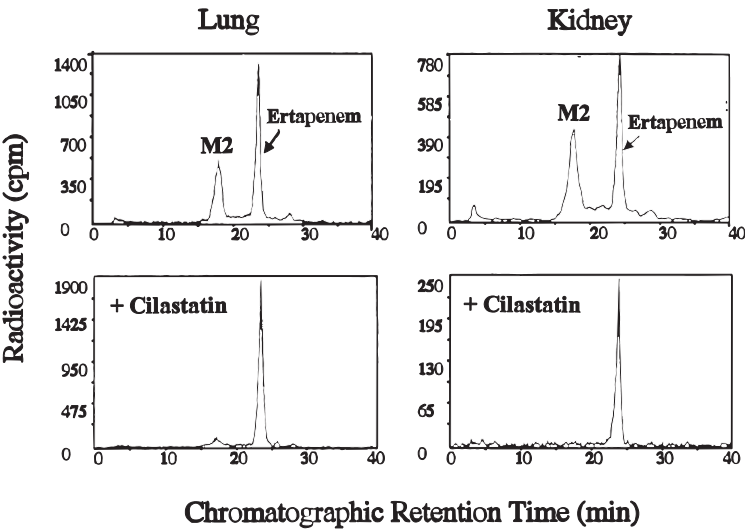


Figure 5. Effect of cilastatin on the formation of the ring-opened metabolite in rat lung and kidney homogenates.

Table 4. Effect of cilastatin on the disposition of ertapenem in rats.

Treatment	AUC ($\mu\text{g h ml}^{-1}$)	Plasma clearance ($\text{ml min}^{-1} \text{kg}^{-1}$)	Half-life (min)	V_{ss} (l kg^{-1})	Unchanged drug in urine (% of dose)	Ring-opened metabolite in urine (% of dose)
Ertapenem	2033 $\pm$ 504	5.17 $\pm$ 1.34	24.6 $\pm$ 9.0	145 $\pm$ 31	9.10 $\pm$ 1.88	31.3 $\pm$ 4.3
Ertapenem + cilastatin	11 335 $\pm$ 855*	0.886 $\pm$ 0.069*	101 $\pm$ 7*	122 $\pm$ 3	35.9 $\pm$ 5.8*	8.81 $\pm$ 2.11*

*Different from control as assessed by a Student's unpaired *t*-test (*p* < 0.001). Values are the mean $\pm$ SD of *n* = 4.

Cilastatin, a specific inhibitor of DHP-I, blocked the formation of the ring-opened metabolite in rat lung and kidney preparations. Taken with the *in vivo* effect of cilastatin on the disposition of ertapenem, coupled with the identity of the major urinary metabolite, this suggested strongly that a metabolic pathway involves DHP-I mediated hydrolysis to a beta-lactam ring-opened product.

Discussion

In man, the majority of radiolabelled ertapenem was eliminated by a combination of beta-lactam ring hydrolysis and renal excretion of unchanged drug. Together, the products of these two pathways account for more than 95% of the radioactivity recovered in urine. A second minor metabolite M1, the product of amide hydrolysis of the side chain, accounted for about 0.8% of the administered dose. Radioactivity in faeces accounted for 9.2% of dose, which suggested that a minor fraction of ertapenem or its metabolites underwent biliary and/or intestinal secretion. The metabolite pathways in man were qualitatively similar to those in animals (tables 2 and 3), although there were quantitative differences. In monkeys, the beta-lactam ring-opened metabolite accounted for about 75% of the dose and the amide hydrolysis metabolite M1 accounted for 7.6% of the dose; in rats, these metabolites accounted for 31.9 and 20.0% of the dose, respectively. In a separate study, the composition of radioactivity excreted in rat bile was shown to consist mostly of unchanged drug (data not shown). Excretion of unchanged drug in urine was the highest in man as compared with that in rat and monkey. Overall, the qualitative agreement in metabolic profile along with the balanced contribution of beta-lactam hydrolysis and renal excretion to the elimination indicated that, in general, rats were a reasonable model of the disposition of ertapenem in man.

Hydrolysis of the beta-lactam ring is a metabolic pathway characteristic of carbapenem antibiotics in man, although the relative contribution of metabolism and renal clearance of parent drug is compound- and species-dependent (Kropp *et al.* 1982, Burman *et al.* 1991, Harrison *et al.* 1993). Imipenem undergoes extensive metabolism via this pathway, and urinary excretion of unchanged drug accounts for about one-quarter of an intravenous dose (Norrby *et al.* 1983). However, when given as the marketed formulation containing an equal amount of cilastatin, the urinary excretion of unchanged drug triples to more than 70% of the dose (Norrby *et al.* 1983). In man, meropenem is excreted mostly as unchanged drug (66–79% of dose) with the remainder (10–25% of the dose) excreted as the corresponding ring-opened metabolite, and the relative extent of metabolic versus non-metabolic clearance is species dependent (Harrison *et al.* 1989, 1993). In the present studies of ertapenem in man, formation of the beta-lactam ring-opened metabolite and renal excretion of unchanged drug contributed approximately equally to elimination (table 3). Consistent with this balanced elimination, AUC from a 1-g intravenous dose increases 158 and 192% in patients with advanced (creatinine clearance of 5–30 ml min⁻¹/1.73 m²) and end-stage renal insufficiency, respectively, relative to those with normal renal function (Holland *et al.* 2001). Consequently, in these patients the recommended daily dosage is reduced by 50%.

The effect of cilastatin on the pharmacokinetics and disposition of ertapenem was examined in rats to assess enzyme systems involved in the metabolism of ertapenem. Cilastatin is a specific inhibitor of DHP-I, a microsomal dipeptidase (Kahan *et al.* 1983, Adachi *et al.* 1989). In rats, co-administration of cilastatin

decreased plasma clearance by fivefold and significantly decreased the fraction of the dose excreted as the ring-opened metabolite ($p < 0.05$; table 4). *In vitro* studies were conducted to assess the potential for different organs to mediate the metabolism of ertapenem. Lung and kidney tissue homogenates obtained from rats were capable of mediating the hydrolysis of ertapenem to its ring-opened metabolite, whereas formation of this species was minor in the corresponding liver preparation (figure 5). This is consistent with the known tissue expression levels of DHP-1 in rodents; activity is highest in lung and kidney, but relatively minor in liver (Kropp *et al.* 1982, Kudo 1994). The minor metabolite M1 likely forms by secondary hydrolysis of the beta-lactam ring-opened derivative. In contrast to DHP-I mediated hydrolysis of the beta-lactam ring, the tissue site and relative contribution of DHP-I to the hydrolysis of the amine benzoic moiety are unclear because *in vitro* formation of M1 was low and the effect of cilastatin had only a modest effect (figure 5). Other possible mediators of M1 formation include amidases, some of which are exhibit relatively high activity in rat tissue (Uchida and O'Brien 1967). In rats, M1 was a substantial metabolite; however, in man and monkeys, it was quantitatively minor; < 1 and about 8% of the dose, respectively. Taken together, the *in vitro* and *in vivo* data indicate that a major clearance pathway of ertapenem in rats is DHP-I-mediated hydrolysis to a beta-lactam ring-opened metabolite.

Plasma concentrations of ertapenem declined with a terminal half-life of about 4 h, which agrees with the half-life measured in other studies conducted in normal healthy volunteers (Majumdar *et al.* 2002). The plasma AUC(0–8) values based on total radioactivity were similar to those of ertapenem (685 ± 90 versus $627 \pm 72 \mu\text{g h ml}^{-1}$, mean $\pm$ standard deviation [SD]), indicating that the metabolites of ertapenem contributed minimally to systemic exposure. Although the number of subjects was small, sex differences in the metabolite profiles in urine were not apparent (table 3). This was in general agreement with the findings of a larger study that reported that after administration of single doses of 0.5–3 g, the overall pharmacokinetic behaviour was similar in men and women, with only small differences in some parameters (a slightly shorter half-life, a smaller distribution volume and a slightly greater renal clearance in women) that were not considered to be clinically significant (Majumdar *et al.* 2002).

In summary, in rat, monkeys and man, following administration of radiolabelled ertapenem, the radioactivity was excreted predominantly in urine as the beta-lactam ring-opened metabolite and unchanged drug. An additional urinary metabolite was a minor amide hydrolysis product. However, circulating radioactivity consisted predominantly of parent drug, indicating that metabolites contributed minimally to systemic exposure. The metabolic pathways were qualitatively similar across species.

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Comparative pharmacokinetics and metabolism of levetiracetam, a new anti-epileptic agent, in mouse, rat, rabbit and dog

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1. The pharmacokinetics and metabolism of ^{14}C -levetiracetam, a new anti-epileptic agent, in mouse, rat, rabbit and dog after a single oral dose were investigated. Moreover, the *in vitro* hydrolysis of levetiracetam to its major carboxylic metabolite by rat tissue homogenates was investigated to identify tissues involved in the production of the metabolite. Data are also presented on the induction of the enzyme(s) involved in levetiracetam hydrolysis in the rat.

2. Levetiracetam was rapidly and almost completely absorbed. The unchanged drug accounted for a very high percentage of plasma radioactivity. Levetiracetam did not bind to plasma proteins. Although brain radioactivity concentrations were lower than those of whole blood at early time points, brain-to-blood ratios increased over time. The predominant route of elimination of total ^{14}C was excretion via urine, accounting for about 81, 93, 87 and 89% of the dose in the mouse, rat, rabbit and dog, respectively. Consequently, levetiracetam was poorly metabolized. It was submitted *in vivo* to hydrolysis and/or oxidation. Hydrolysis of the amide function of levetiracetam produced the corresponding acid. However, levetiracetam could also be oxidized at positions 3 and 4 of the 2-oxopyrrolidine ring. Finally, the compound and the corresponding acid metabolite could be oxidized at position 5 of the 2-oxopyrrolidine ring and then hydrolysed with the opening of the ring.

3. All the investigated rat tissues (liver, kidney, lung, brain, small intestine mucosa) had the potential to produce the acid metabolite. By contrast, the acid was undetectable following incubation of levetiracetam with buffer alone or heat-denaturated liver fractions.

4. No marked species or sex differences were observed in the absorption, disposition and metabolism of levetiracetam.

5. The hydrolysis of levetiracetam is carried out by an enzymatic process characterized by a broad tissue distribution. In the rat, the enzyme system hydrolysing levetiracetam is not induced by phenobarbital, at least under the experimental conditions used herein, whereas the enzyme system(s) involved in the other metabolic pathways is induced.

Introduction

Levetiracetam (ucb L059) ((*S*)- α -ethyl-2-oxo-1-pyrrolidine acetamide) is a new anti-epileptic drug whose chemical structure, related to the structure of the nootropic drug piracetam, differs from all other anti-epileptic drugs (figure 1). The pharmacology of levetiracetam is distinct from other anti-epileptic drugs by a lack of anticonvulsant activity in the two classical screening tests for anti-epileptic

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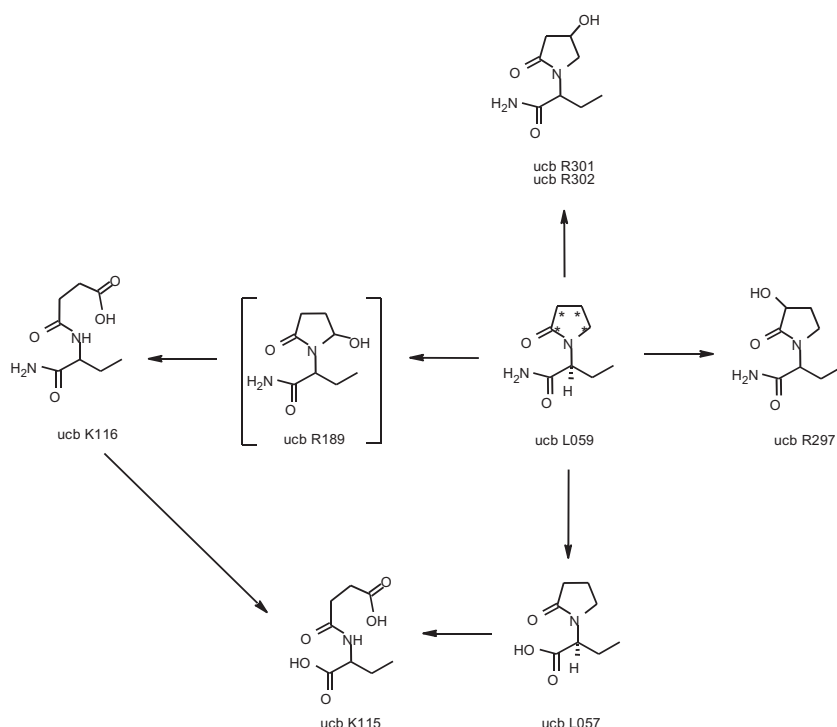


Figure 1. Suggested metabolic pathways of levetiracetam in mouse, rat, rabbit and dog. *Site of labelling of ^{14}C -levetiracetam.

drugs in rodents: the maximal electroshock seizure and pentylenetetrazole tests (Löscher and Hönack 1993, Klitgaard *et al.* 1998). This contrasts with potent seizure suppression in animal models of chronic epilepsy mimicking both partial and generalized seizures in man (Löscher and Hönack 1993, Gower *et al.* 1995, Klitgaard *et al.* 1998). Levetiracetam also displays a wide safety margin between doses inducing seizure protection and acute central nervous system adverse effects in rodents (Klitgaard *et al.* 1998) and possesses a significant ability to induce a persistent inhibition of kindling development in rats (Löscher *et al.* 1998).

Levetiracetam appears devoid of any direct interaction with conventional mechanisms involved in the action of classic anti-epileptic drugs (Margineanu and Klitgaard 2002). This parallels the discovery of a brain-specific binding site for levetiracetam, which probably contributes to its anti-epileptic activity (Noyer *et al.* 1995). Furthermore, levetiracetam has been shown to reduce moderately high voltage-activated Ca^{2+} currents (Niespodziany *et al.* 2001), due to selective inhibition of N-type Ca^{2+} channels (Lukyanetz *et al.* 2002), and also to suppress the inhibitory effect of Zn^{2+} on γ -aminobutyric acid and glycine-gated currents (Rigo *et al.* 2002).

The main aim of the present paper was to compare the pharmacokinetics and metabolism of levetiracetam in the mouse, rat, rabbit and dog, the species used for the evaluation of pharmacological and toxicological properties of this compound. Levetiracetam is a highly water-soluble (1.04 g ml^{-1}) and low molecular weight (170 Dalton) compound. Therefore, high absorption and low interspecies differences are expected. The data presented were obtained after administration of a

single 54 mg kg⁻¹ oral dose that encompasses doses effective in genetic rat models of epilepsy (Gower *et al.* 1995), in amygdala-kindling, pentylenetetrazole-induced kindling and pilocarpine-induced seizures (Gower *et al.* 1992, Klitgaard *et al.* 1998). The dose selected for the species comparison of pharmacokinetics and metabolism was also close to the lowest dose in chronic toxicity studies in rats and dogs and to the human therapeutic dose.

An additional aim was to investigate whether the main metabolic pathway of levetiracetam, hydrolysis of the amide function, occurs only in the liver or whether extrahepatic tissues may also be involved.

Finally, a preliminary investigation has been carried out in the rat to know whether the enzyme(s) involved in the hydrolysis of the drug is inducible.

Materials and methods

In vivo studies

Species comparison.

Test substance, reference compounds and other chemicals. The test substance was ¹⁴C-levetiracetam (specific activity 0.9–3 mCi mmol⁻¹ and radiochemical purity > 97%) (figure 1) and cold levetiracetam (Batch No. 12). The following reference compounds were synthesized in the Chemical Department of UCB S. A., Pharma Sector:

- ucb G025, 2-(2,2-dimethyl-5-oxo-pyrrolidinyl)propanamide.
- ucb L057, (2S)-2-(2-oxopyrrolidin-1-yl)butanoic acid.
- ucb H214, 3-hydroxy-2-(2-oxo-pyrrolidin-1-yl)-propionic acid, a racemic mixture containing one pair of enantiomers.
- ucb K115, racemate of 2-(3-carboxy-propionylamino)-butyric acid.
- ucb K116, racemate of 4{[1-(aminocarbonyl)propyl]amino}-4-oxobutanoic acid.
- ucb R297, a mixture of the 4 stereoisomers of 2-(3-hydroxy-2-oxopyrrolidin-1-yl)butanamide.
- ucb R189, a mixture of the 4 stereoisomers of 2-(5-hydroxy-2-oxopyrrolidin-1-yl)butanamide.
- ucb R301 and ucb R302, the two racemic mixtures of 2-(4-hydroxy-2-oxopyrrolidin-1-yl)-butanamide.

The chemical purity of the reference compounds was > 95%.

EmulsifierTM, Carbo-Sorb[®] and Permafluor[®] were obtained from Packard (Meriden, CT, USA), acetonitrile from Rathburn (Walkenburgh, UK), and tetrabutylammonium hydrogen sulphate from Waters (Milford, IL, USA). All other chemicals were obtained from a local commercial source and were of analytical grade.

Animals and husbandry. MRI mice were provided by the Department of Zootechny of UCB S. A., Pharma Sector. The body weights were 19.5–26.8 and 16.8–25.1 g for males and females, respectively. Mice were fasted for 16 h before dosage with levetiracetam and were not given food for 6 h after dosing.

OFA rats were obtained from Iffa-Credo (Brussels, Belgium). The body weight were 234–270 and 166–205 g for males and females, respectively. Rats were fasted for 16 h at the time of levetiracetam administration and were allowed access to food 4 h after dosing.

Female New Zealand rabbits of about 6 months were used. Their body weights were 3.71–3.91 kg. They were not allowed access to food in the 16 h predosing with levetiracetam and 6 post-dosing hours.

Male and female Beagle dogs (UCB breeding, Braine-l'Alleud, Belgium) weighing 8.3–8.9 and 6.5–7.5 kg, respectively, were used. The animals were fasted 16 h before dosage with levetiracetam and were not given food until 6 h after dosing.

Design of the studies. For mice, seven groups of six animals (three males and three females) were orally administered a single dose of ¹⁴C-levetiracetam in aqueous solution (54 mg kg⁻¹, 0.2 ml/animal) and were killed at 0.5, 1, 2, 4, 8, 24 and 32 h after dosing. Blood samples were collected into heparinized tubes from the ophthalmic venous plexus. Aliquots of 50–100 µl were used for the measurement of blood radioactivity; the remaining blood was centrifuged and the plasma stored at –18°C until analysis. Three groups of five males and three groups of five females were given an oral dose of ¹⁴C-levetiracetam (54 mg kg⁻¹). Each group was housed in a metabolic cage facilitating the separation of urine and faeces.

Urine was collected at intervals of 0–7, 7–24 and 24–48 h after dosing. The volume was measured and the samples were stored at -20°C until analysis. Faeces were collected after 24 and 48 h, immediately homogenized to a paste with an equal amount of water and stored frozen at -20°C until analysis.

For rats, 13 groups of three male and three female rats were given a 54 mg kg^{-1} oral dose of ^{14}C -levetiracetam in aqueous solution (0.5 ml/animal). Animals were killed at 10, 20, 40, 60 min, 2, 3, 4, 6, 8, 12, 16, 24 and 32 h after dosing. Blood was collected by cardiac puncture and immediately heparinized. An aliquot was used for whole-blood measurements and the remainder centrifuged to obtain plasma. Plasma was stored at -20°C until analysis. The following organs/tissues were collected: liver, spleen, heart, thymus, trachea, lung, gonad, cerebrum, cerebellum, spinal cord, salivary gland, kidney, bone marrow, muscle, adipose tissue, skin, hair, adrenal, thyroid, pituitary gland, eyeball, lens, aqueous and vitreous humour, as well as pancreas; stomach, small intestine, colon and caecum, which were collected with content. Animals killed later than 8 h after dosing were housed singly in metabolic cages for collection of urine and faeces. All the samples were weighed and stored at -20°C until analysis. An additional group of 10 rats (five males and five females) received a 70 mg kg^{-1} oral dose of ^{14}C -levetiracetam. The rats were singly housed in metabolic cages; urine was collected after 4, 8, 24, 48 and 72 h, whereas faeces were collected daily.

For rabbits, four female rabbits received a single oral dose (54 mg kg^{-1} or 4 ml kg^{-1}) of ^{14}C -levetiracetam in aqueous solution ($213\text{ }\mu\text{Ci/animal}$). The animals were placed into individual metabolic cages. Urine and faeces were collected. Blood was taken on heparin at predetermined times: at 10, 20, 40 min before dosing and at 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after dosing or up to the time of sacrifice. The four animals were killed at 1, 3, 6 and 24 h. Samples of 50 or $200\text{ }\mu\text{l}$ (24 h) whole blood were taken to determine radioactivity, the residual quantity was centrifuged and the plasma obtained stored at -20°C until analysis. The following organs/tissues were taken: ovary, uterus, liver, kidney, heart, thymus, lung, cerebrum, cerebellum, spinal cord, pituitary, spleen, adrenal, pancreas, eyeball, lens, aqueous and vitreous humour, thyroid, trachea, salivary gland, muscle, bone marrow, adipose tissue, skin, fur, lymph node, empty bladder, pancreas and oesophagus, as well as stomach, small intestine, large intestine, and caecum, which were collected with content. Each organ was weighed, homogenized and stored at -20°C until analysis.

For dogs, eight dogs received a single oral dose of ^{14}C -levetiracetam (54 mg kg^{-1} or 1 ml kg^{-1} , $200\text{ }\mu\text{Ci/animal}$) in aqueous solution. Four animals were killed: two males at 1 and 9 h, and two females at 4 and 24 h, respectively. Blood was collected into heparinized tubes from the cephalic or saphenous vein before dosing and then at 10, 20, 40 min, 1, 1.5, 2, 3, 4, 6, 9, 12 and 24 h or up to the time of sacrifice. Urine samples were collected by catheterization and from the metabolic cages before administration and then at the following intervals: 0–2, 2–4, 4–6, 6–9, 9–12 and 12–24 h after dosing, or up to the time of sacrifice. These samples were stored at -20°C until analysis. Faeces were collected daily from the metabolic cages. The following organs/tissues were taken for examination after sacrifice: liver, kidney, spleen, heart, lung, lymph node (retropharyngeal, mesenteric and popliteal), submaxillary gland, parotid, gonad, uterus, empty bladder, pancreas, thymus, adrenal, trachea, thyroid, parathyroid, eyeball, lens, vitreous and aqueous humour, cerebrum, cerebellum, pituitary gland, spinal cord, bone marrow, muscle, adipose tissue, skin, and fur, as well as stomach, duodenum, small intestine and large intestine, which were collected with content. The other four dogs (two males and two females) received the same oral dose of ^{14}C -levetiracetam. Blood was collected at 10, 20, 40 min, 1, 1.5, 2, 3, 4, 6, 9, 12 and 24 h; urine and faeces were collected for 24 h, after which the animals were submitted to repeated administration (UCB, data on file).

Plasma protein binding. The extent of plasma protein binding of radioactivity was investigated both *in vitro* and *ex vivo* in the rat, rabbit and dog by dialysis. Dialyses were carried out at 37°C for 4 h, using a Dianorm[®] (Diachema AG, Zurich, Switzerland) dialysis apparatus and Spectra/por[®] (Spectrum[®], Houston, TX, USA) membranes (membrane surface area = 2 cm^2 ; molecular weight cut-off = 6000–8000) against freshly prepared 0.1 mol l^{-1} phosphate buffer adjusted to pH 7.4. The membranes were prepared by soaking them first in distilled water for 20 min, then in the phosphate buffer. The binding of ^{14}C -levetiracetam to plasma proteins was measured *in vitro* in a range of concentrations from 1 to $1600\text{ }\mu\text{g ml}^{-1}$ plasma in rat and rabbit and from 1 to $800\text{ }\mu\text{g ml}^{-1}$ plasma in dog. The binding *ex vivo* was carried out in rat on plasma collected at 1 h, in rabbit on plasma collected at 1, 3, 6 and 24 h and in dog on plasma collected at 1 and 4 h after administration of ^{14}C -levetiracetam. The binding was calculated according to the equation:

$$\text{Per cent binding} = \frac{P - B}{P} \bullet 100,$$

where B and P are the radioactivity concentrations measured by liquid scintillation (dpm/0.05 ml) in buffer and plasma after dialysis.

Total radioactivity analysis. Total radioactivity was measured in urine, faeces, plasma, dialysed samples, whole blood and tissues. Urine, plasma and dialysed samples were mixed with the scintillation mixture EmulsifierTM. Whole blood, homogenates of faeces and tissues were burned in a combustion

apparatus (Oxidizer, Packard, Healden, USA). The $^{14}\text{CO}_2$ formed was absorbed using Carbo-Sorb® (6 ml) and mixed with the scintillation mixture Permafluor® (12 ml). Radioactivity was measured in a Berthold 5000/300 liquid scintillation spectrometer (Wildbad, Germany) with automatic quenching correction by external standardization and automatic subtraction of the background noise.

Plasma analysis. Unchanged levetiracetam was determined in plasma samples by gas chromatography using ucb G025 as internal standard (IS). Methanol (200 μl) was added to an aliquot of plasma (50 μl) containing the IS (50 μl of a methanolic solution at either 20 or 200 $\mu\text{g ml}^{-1}$). The solution was stirred, centrifuged and a portion of the clear supernatant obtained injected into the gas chromatograph. The apparatus used was a Hewlett-Packard 5880A gas chromatograph (Palo Alto, CA, USA) fitted with a nitrogen-phosphorus flame-ionization detector and with a split-splitless injector operating in split mode. A fused-silica capillary column (Supelcowax 10, Supelco, Bellefonte, CA, USA) coated with a Carbowax 20 M film or a fused-silica column coated with a film of polyethylene glycol-terephthalic acid modified (FFAP, Hewlett-Packard) stationary phase were used. The carrier gas was oxygen-free dry helium (flow rate 25 cm s^{-1}) and the make-up of the helium flow at the level of the detector was 20 ml min^{-1} . The temperature of injection was 250°C; the detector was maintained at 300°C and the oven at 230 or 235°C. The output of the chromatogram was connected to a Hewlett-Packard 5880A graphical recorder. The quantities of unchanged levetiracetam were determined by the peak areas-ratio technique. The mean accuracy, expressed as the percentage ratio of the amount found to the amount added to plasma, varied between 99 and 83%. As the concentrations of levetiracetam were calculated from calibration curves obtained from extracted plasma samples, the results were automatically corrected for possible losses occurring during the preparation of the samples. The limit of quantitation (LOQ) was 0.5 $\mu\text{g ml}^{-1}$ plasma.

Urine analysis. Urine was used to measure the amount of unchanged drug by the same method as for plasma (20 μl urine + 20 μl IS at 2 mg ml^{-1} + 900 μl methanol). The metabolic profile in the different species was also investigated in urine.

For rabbits, urine (diluted fivefold with the mobile phase) was analysed by radio-high-performance liquid chromatography (HPLC) under the following conditions:

- Apparatus: liquid chromatograph Hewlett Packard 1084B.
- Stationary phase: ODS 5 μm hypersil column (200 $\times$ 4.6 mm) (Hewlett-Packard).
- Mobile phase: A = H_2O containing $5 \times 10^{-3} \text{ mol l}^{-1}$ tetrabutylammonium hydrogen sulphate (low UV Pic A; Waters, Milford, USA); or B = $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (95/15, v/v) containing $5 \times 10^{-3} \text{ mol l}^{-1}$ tetrabutylammonium hydrogen sulphate (low UV Pic A; Waters, Milford, USA).
- Column and mobile phase temperature: 33°C.
- Flow: 1.2 ml min^{-1} .

The eluate was collected into counting vials and mixed with the scintillation cocktail Emulsifier™. Radioactivity was measured in a Berthold 5000/300 liquid scintillation counter as described above.

Mice, rats and dogs. Urine was analysed by radio-thin-layer chromatography (TLC). Aliquots of urine were filtered (Swinnex — 13, Millipore S. A., Molsheim, France) before chromatography. Urinary radioactive components were separated by TLC under the following conditions:

- Mobile phase: $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}$ (8/1/1, v/v/v).
- Support: TLC plates LK6DF linear — K silical gel 250 μm layer thickness (Whatmann Int. Ltd, Maidstone, UK).

Autoradiochromatograms were prepared by apposition of the plate with Hyperfilm® βmax (Amersham, Amersham, UK) X-ray film and subsequent photographic development of the X-ray film. The silica corresponding to regions of interest, i.e. background and radioactive peaks, was defined and then scraped. Methanol and scintillant were added to each scraped area followed by further analysis in a liquid scintillation counter. A mixture of ^{14}C -levetiracetam and all reference standards was chromatographed simultaneously on each TLC plate in order to determine and compare the R_f values.

Determination of pharmacokinetic parameters. The pharmacokinetic parameters were estimated by non-compartmental analysis. The area under the plasma drug concentration-time profile (AUC) was calculated by the linear trapezoidal rule from 0 to the last measurable concentration $C_{(t_z)}$ at time t_z and extrapolated to infinite time using the terminal rate constant λ_z (addition of the factor $C_{(t_z)}/\lambda_z$). Plasma peak drug concentration (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. The terminal elimination rate constant (λ_z) was derived by the slope of the linear regression curve obtained by fitting the natural logarithms of the terminal concentrations versus time. The terminal elimination half-life ($t_{1/2}$) was calculated as $\ln 2/\lambda_z$. In the mouse and rat, the terminal

elimination rate constant and AUC were calculated using the means of the plasma concentrations obtained at the different times. Moreover, $C_{\max}$ corresponded to the maximum mean of the plasma concentrations and $t_{\max}$ to the time of the maximum mean of the plasma concentrations.

Enzyme induction.

Test substance, reference standards and other chemicals. The test substance was ^{14}C -levetiracetam (specific activity $0.92 \text{ mCi mmol}^{-1}$ and radiochemical purity 99.7%) and cold levetiracetam (Batch No. 901). The chromatographic reference standards available for such study were ucb L057, ucb K115, ucb R297 and ucb R302. They were obtained from the Chemical Department of UCB S. A., Pharma Sector. Phenobarbital was from UCB, Braine-l'Alleud (Batch No. 2022) and antipyrine was from Aldrich (Belgium).

Animals and study design. The body weights of the rats (10 males and 10 females) used to investigate the inducibility of the esterase(s)/amidase(s) involved in the major metabolic pathway of levetiracetam were 280–330 and 178–212 g for males and females, respectively. The rats were divided in four groups of five animals each. One group of males and one group of females received NaCl 0.9% intraperitoneally (i.p.) (5 ml kg^{-1}) for 3 days and the two other groups phenobarbital i.p. ($80 \text{ mg kg}^{-1} \text{ day}^{-1}$, 5 ml kg^{-1}) for the same period. All groups were administered ^{14}C -levetiracetam orally on day 4 (1800 mg kg^{-1} , 10 ml kg^{-1}) after a fasting period of 16 h and antipyrine intravenously (i.v.) on day 5 (10 mg kg^{-1} , 2.5 ml kg^{-1}) 24 h after administration of levetiracetam. A total of 24-h urine samples were collected after administration of levetiracetam. The volume of each fraction was measured, then the individual urinary fractions were stored at -15°C or lower until analysis. After antipyrine administration, blood was collected at 0.5, 2 and 4 h in males and at 0.5, 3 and 6 h in females. Blood (approximately 0.7 ml) was collected by ophthalmic plexus puncture from each animal into dry heparinized tubes and centrifuged. The plasma obtained was stored at a temperature of -15°C or lower until analysis.

Antipyrine plasma analysis and determination of the pharmacokinetic parameters. Antipyrine was determined in plasma samples by a validated HPLC procedure (UCB, data on file). Briefly, after addition of the IS, the plasma was extracted at alkaline pH with dichloromethane (solid/liquid extraction). After evaporation of the organic layer, the dry residue was reconstituted with the mobile phase and analysed by reverse phase HPLC with UV detection. Antipyrine pharmacokinetic parameters were calculated by the Siphar Pharmacokinetic data analysis package. The structural model was the one open exponential model after intravenous administration using the minimization algorithm of Powell. The model was fitted to data using an extended least-squares algorithm. The following parameters were estimated from the fitted model and reported: rate constant k (h^{-1}), area under the curve (AUC) (mg h l^{-1}), volume of distribution V_{ss} (l kg^{-1}), plasma clearance (l) ($\text{l kg}^{-1} \text{ h}^{-1}$) and mean residence time (MRT) (h).

Measurement of urinary radioactivity, separation and quantification of the urinary metabolites of levetiracetam. Measurement of total urinary radioactivity was performed by mixing urine samples (0.05 ml) with the EmulsifierTM scintillation cocktail (10 ml; Packard, Zellik, Belgium). Radioactivity was measured in a Berthold 5000/300 scintillation spectrometer with automatic quenching correction (external standard) and automatic background subtraction. Urine samples were analysed for unchanged drug and metabolites by gradient reverse phase HPLC (Kontron pump 420, ternary gradient former 425 and autosampler 460) with radiochemical detection (Berthold radioactivity monitor LB507A) using a 0.6-ml YG600U4 solid cell. A $5 \mu\text{m}$ reverse phase C18 Hypersil column (200 mm, 4.6 mm i.d.) (both Hewlett Packard, Brussels, Belgium) were used. The mobile phase composition (%) was as follows:

- A = B = water containing 0.005 mol l^{-1} tetrabutylammonium phosphate (Pic A from Waters).
- C = water/ CH_3CN 90/10 (v/v) containing 0.005 mol l^{-1} tetrabutylammonium phosphate (Pic A from Waters).

The composition of the gradient used was as follows: 49% A + 49% B + 2% C during 9 min, 10% A + 10% B + 80% C after 19 min, 5% A + 5% B + 90% C after 29 min, and 49% A + 49% B + 2% C after 31 min.

The chromatographic signal was recorded on a Compaq 286 e mod 20 workstation and analysed via the data Pack 2SN from Kontron. Reference substances were analysed similarly, except that they were detected by an UV detector (Varichrom from Varian, Brussels, Belgium) operating at 205 nm. The total amount of levetiracetam and metabolites was obtained from liquid scintillation radioactivity measurements and the radioactivity associated with each radioactive component was estimated from the relative peak area of the radiochromatogram, assuming that all radioactivity was recovered during the chromatographic run.

In vitro metabolism

Test substance and reference standards. The test substance levetiracetam (Batch No. 901), the authentic reference used for metabolite quantification ucb L057 (Batch No. A002) and the IS ucb N167 [2-(2-oxopyrrolidin-1-yl)propanoic acid] (Batch No. A001) were obtained from UCB S. A., Pharma Sector and stored in the dark at room temperature until use.

Rat tissue homogenates. Homogenates were prepared from eight Crl:CD (SD) BR Charles River male rats (7 weeks old, fasted overnight and weighing 200–250 g on the day of sacrifice). At sacrifice, kidney, lung, brain, small intestine and liver were immediately excised. Kidney, lung, brain and liver were rinsed and individually homogenized in 0.15 mol l^{-1} KCl to give 20% w/v homogenates. Small intestine was cut from the pylorus to the caecum, slit open, rinsed with a 0.15 mol l^{-1} KCl solution and the mucosal layer was removed by scraping. The cells were then suspended in 0.15 mol l^{-1} KCl containing 10 mmol ethylenediamine tetra-acetic acid and homogenized. Homogenates were pooled according to tissue, aliquoted and stored at about -80°C until further use.

Incubation procedure. Incubation mixtures contained tissue homogenate (2 mg ml^{-1}) and levetiracetam ($500 \mu\text{M}$) in a 0.5 ml final volume of 50 mmol l^{-1} Tris buffer (pH 7.4). The protein concentration of the final incubation mixture was 2 mg ml^{-1} . The mixture was incubated in a shaking water bath at 37°C and terminated after 1 h by placing the samples in dry ice. The samples were stored at about -20°C until further analysis. In addition, assays were also performed using tissue homogenates heat denatured (10 min incubation at 100°C) before addition of levetiracetam. Control experiments included incubation of $500 \mu\text{mol l}^{-1}$ levetiracetam in a 0.5 ml final volume of Tris buffer (pH 7.4).

Extraction and analysis. A $100 \mu\text{l}$ aliquot of incubation mixture was thawed and transferred into disposable tubes, spiked with a solution of IS in water ($50 \mu\text{l}$ of a solution containing $5 \mu\text{g ml}^{-1}$ ucb N167) and extracted twice with $500 \mu\text{l}$ ethyl acetate.

Each extraction was optimized by a 10-min mixing step and a 10-min centrifugation step at about 15 000g. The organic phases were discarded. A total of $500 \mu\text{l}$ acetonitrile were added to the aqueous phase with mixing. The mixture was allowed to stand at -20°C for 10 min before a 10-min centrifugation at about 15 000g. The supernatant was transferred into a disposable tube, whereas the precipitate was washed twice with $300 \mu\text{l}$ acetonitrile. Each supernatant was collected after a 5-min centrifugation at about 15 000g. The supernatants were pooled and evaporated to dryness using a concentrator evaporator (Jouan RC10.22, Jouan, Saint-Herblain, France). The dry residue was reconstituted with $100 \mu\text{l}$ mobile phase and filtered by centrifugation at about 4000g (Ultrafree filter unit — MC Durapore $0.45 \mu\text{m}$, Millipore, Bedford, MA, USA). Fractions of $20 \mu\text{l}$ were injected onto the LC/ESI/MS system. The analytical column (Inertsil 5 ODS (2) $250 \times 2.0 \text{ mm i.d.}$, Chrompack, Middelburg, the Netherlands) was protected by a guard column (Hypersil ODS $5 \mu\text{m}$, $20 \times 2.1 \text{ mm i.d.}$, Hewlett Packard, Palo Alto, CA, USA). The column and precolumn were placed in an oven set at 40°C . The mobile phase consisted of acetonitrile/ H_2O 5/95 v/v containing 5 mmol l^{-1} ammonium acetate and the flow was adjusted to $0.250 \text{ ml min}^{-1}$. The flow was split post-column to provide a flow of approximately $0.125 \text{ ml min}^{-1}$ into the electrospray source. Electrospray mass spectra were recorded with a VG-Quattro mass spectrometer. The cone voltage was adjusted to 14 V (skimmer lens offset of 5 V) and the source temperature was kept at 150°C . The spectra were acquired at about 1 mass unit resolution (10% valley definition) in negative-ion mode. Single ion recording (SIR) was used with a dwell time and interchannel delay fixed at 0.50 and 0.02 s, respectively. The chromatographic traces corresponding to ions with m/z 170 amu $[(\text{M} - \text{H})^-]$ of ucb L057 and 156 amu $[(\text{M} - \text{H})^-]$ of the IS were recorded as a function of time. The vehicle buffer (i.e. 50 mmol l^{-1} Tris, pH 7.4) interfered with measurements of IS. Thus, the ucb L057 levels reported were not corrected for extraction efficiency. A standard calibration curve was constructed by the analyses of extracted plasma spiked with ucb L057 at nominal concentrations of 20, 50, 100, 500, 1000 and 2000 ng ml^{-1} . Peak areas were measured using MassLynx software (version 2.3).

Results

In vivo studies

Species comparison.

Plasma levels of total radioactivity and unchanged levetiracetam. The means of total radioactivity and unchanged levetiracetam concentrations in the different species are shown in figure 2. Mean peak concentrations of total radioactivity were observed at 30 min post-dose in the male and female mouse (52 and $41.3 \mu\text{g eq. ml}^{-1}$, respectively), at 1 h and 20 min in the male and female rat (56.6 and $54.8 \mu\text{g}$

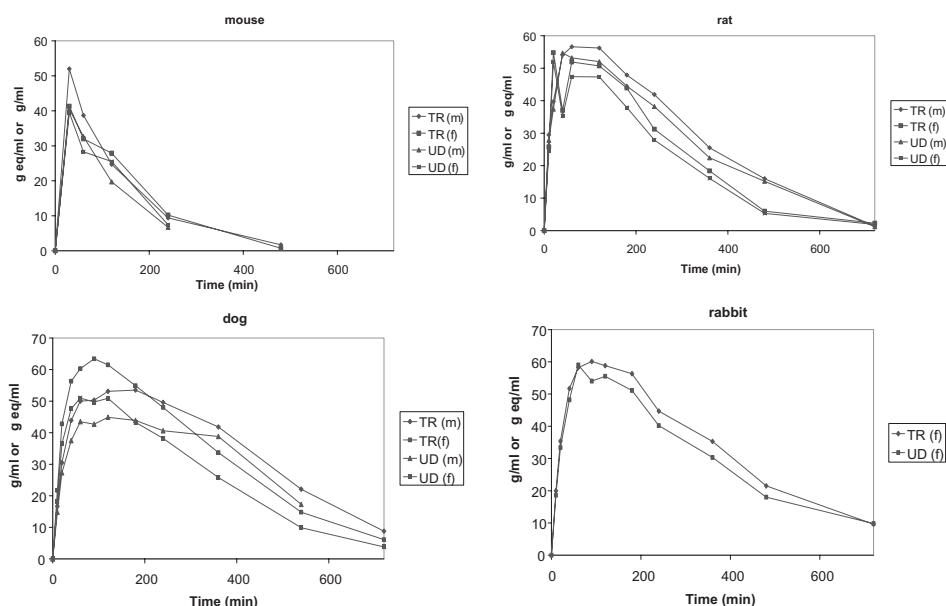


Figure 2. Plasma concentrations of total radioactivity (TR) and unchanged drug (UD) versus time after administration of a single oral dose of ^{14}C -levetiracetam (54 mg kg^{-1}) in animals (means: $n=3$ for mice and rats, $n=2-4$ for dogs, $n=1-4$ for rabbits).

eq. ml^{-1} , respectively), at 1.5 h in the female rabbit ($60.1 \mu\text{g eq. ml}^{-1}$), and at 3 and 1.5 h in the male and female dog (53.5 and $63.4 \mu\text{g eq. ml}^{-1}$, respectively). At these times, the unchanged drug accounted for approximately 78 and 96% of plasma total radioactivity in the male and female mouse, respectively, for 94 and 95% in the male and female rat, respectively, for 90% in the female rabbit and for 82 and 78% in the male and female dog, respectively.

The pharmacokinetic parameters of levetiracetam in the different species are shown in table 1. The mean C_{max} values are very similar in all the species studied and were obtained 20–40 min following administration in the mouse and rat and 1.3–2.5 h in the rabbit and dog. The plasma elimination half-life was short in all the species studied, ranging from a mean of 1.3–1.5, 1.8–2.0, 4.0 and 3.1–2.3 h following administration in the male and female mouse, male and female rat, female rabbit, and male and female dog, respectively.

The mean plasma levels in the different species as a function of time are shown in figure 3a. The same data according to the 'elementary Dedrick's plot' representation are shown in figure 3b. The Dedrick's plot is obtained after normalization of the concentration axis by dividing the observed plasma concentrations by the dose/body weight (mg kg^{-1}) and after normalization of the time axis by dividing the times after drug administration by body weight^{0.25} (Boxenbaum and Ronfeld 1983, Efthymiopoulos *et al.* 1991). For comparative purposes, data obtained until 6 h after administration were selected in figure 3 as data for the rabbit were available from only one animal after that time. From figure 3a, it is apparent that the mouse differs from the other species studied whereas the elementary Dedrick's plot (figure 3b) shows that the mouse is not an outlier and the concentration–time profiles in the different animal species are nearly superimposable. No binding of levetiracetam to plasma proteins was observed in any species studied. Moreover, *ex vivo*

Table 1. Plasma and urine pharmacokinetic parameters of unchanged levetiracetam in mouse, rat, rabbit and dog after single oral administration of the radiolabelled compound (54 mg kg^{-1}).

Parameter (unit)	Pharmacokinetic parameter values (mean or mean $\pm$ SD), model-independent						
	Mouse		Rat		Rabbit	Dog	
	Males	Females	Males	Females	Females	Males	Females
$C_{\max}$ ($\mu\text{g ml}^{-1}$)	40.5	39.5	54.7	51.9	57.8 ± 9.10	55.1 ± 12.9^f	53.7 ± 7.5^f
$t_{\max}$ (h)	0.5	0.5	0.7	0.3	1.3 ± 0.50	2.5 ± 2.4^f	1.4 ± 0.5^f
AUC ($\mu\text{g h ml}^{-1}$)	93.7	102.6	317.4	245.1	401 ^d	425.4 ^g	320.0 ^g
$t_{1/2}$ (h)	1.3 ^a	1.5 ^a	1.8 ^b	2.0 ^b	4.0 ^{d,e}	3.1 ^{g,h}	2.3 ^{g,h}
0–24-h Ae (% dose)	51.2 ± 1.9	50.1 ± 3.7	77.7 ± 4.2^c	78.0 ± 3.7^c	58.1 ^d	58.4 ^g	46.6 ^g

^aCalculated from 0.5 to 4 h.^bCalculated from 3 to 12 h.^cValues after a single oral dose of 70 mg kg^{-1} .^dValue from one animal.^eCalculated from 4 to 24 h.^fValues from single administration and from the first day of repeated administration.^gValues from the first day of repeated administration.^hCalculated from 4 to 12 h.

Data were obtained from 42 mice, 39 rats, one or four rabbits, and four or eight dogs for plasma and from 30 mice, 24 rats, one rabbit and four dogs for urine (see the Materials and methods for more details).

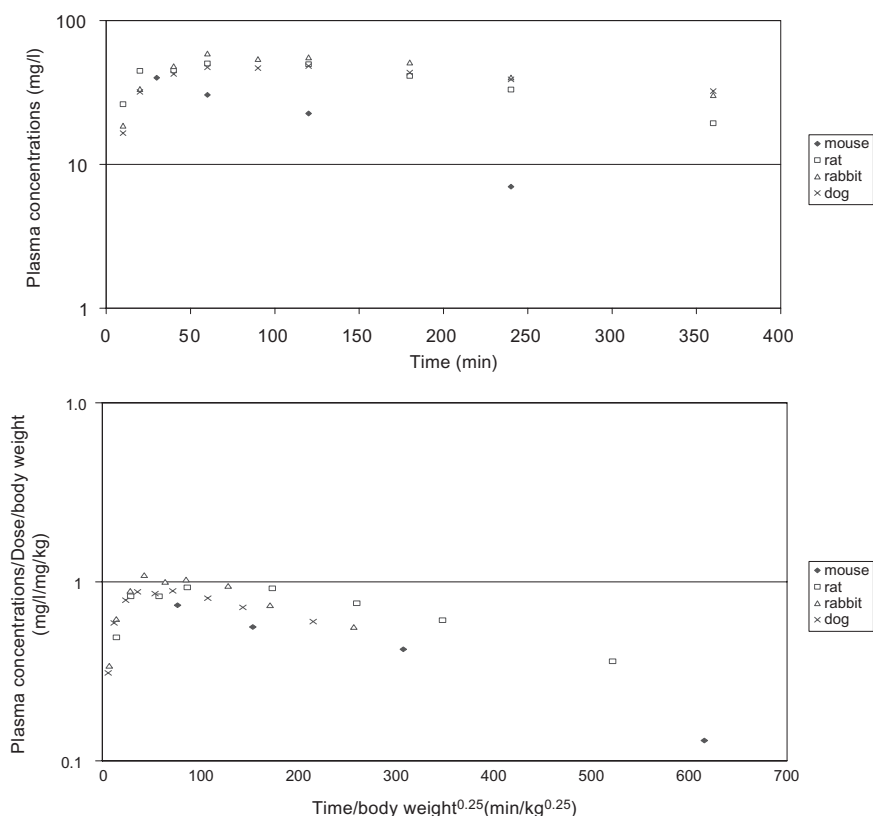


Figure 3. Plasma concentrations of unchanged drug (means: $n=3$ for mice and rats, $n=2-4$ for dogs, $n=1-4$ for rabbits) after administration of a single oral dose of ^{14}C -levetiracetam (54 mg kg^{-1}) to mice, rats, rabbits and dogs presented against time (a) and normalized to dose/body weight against time/body weight^{0.25} (b).

Table 2. Plasma protein binding of ^{14}C -levetiracetam in rat, rabbit and dog.

Type of experimentation	Percentage binding		
	Rat	Rabbit	Dog
<i>In vitro</i>	$< 10^a$	0^a	$< 10^a$
<i>Ex vivo</i>	$< 10^b$	0^c	$< 10^d$

^aTested range of concentrations: 1–1600 $\mu\text{g ml}^{-1}$ for rat and rabbit; 1–800 μml^{-1} for dog.

^bMeasured 1 h after a single dose of 54 mg kg^{-1} p.o.

^cMeasured 1, 3, 6 and 24 h after a single dose of 54 mg kg^{-1} p.o.

^dMeasured 1 and 4 h after a single dose of 54 mg kg^{-1} p.o.

Table 3. Plasma-to-blood ratio of total radioactivity concentrations in mouse, rat, rabbit and dog at different times after single oral administration of ^{14}C -levetiracetam (54 mg kg^{-1}).

Time (h)	Ratio (mean $\pm$ SD)			
	Mouse ($n=6$)	Rat ($n=6$)	Rabbit	Dog ($n=4$) ^c
0.17		1.14 ± 0.08	1.05 ± 0.04^a	0.95 ± 0.32
0.33		1.14 ± 0.07	1.04 ± 0.02^a	1.06 ± 0.05
0.5	1.10 ± 0.06			
0.67		1.14 ± 0.07	1.05 ± 0.04^a	1.06 ± 0.09
1	1.18 ± 0.13	1.24 ± 0.16	1.12 ± 0.06^a	1.10 ± 0.05
2	1.09 ± 0.06	1.22 ± 0.08	1.02 ± 0.02^b	1.08 ± 0.06
3		1.13 ± 0.09	1.02 ± 0.06^b	1.11 ± 0.06
4	0.90 ± 0.08	1.17 ± 0.04	0.99^c	1.11 ± 0.05
6		1.19 ± 0.05	0.98^c	1.11 ± 0.02
8	0.54 ± 0.19	1.08 ± 0.03	0.92^d	
9				1.04 ± 0.02
12		1.06 ± 0.05	0.76^d	0.98 ± 0.05
16		1.07 ± 0.09		
24	0.74 ± 0.56	0.93 ± 0.11	0.32^d	0.59 ± 0.18

^aValues from four animals.

^bValue from three animals.

^cValues from two animals.

^dValues from one animal.

^eValues from the first day of repeated administration.

determinations at different times after administration showed that radioactivity did not bind to plasma proteins in any species studied (table 2).

Whole blood levels of total radioactivity. The plasma-to-blood ratios of total radioactivity concentrations are shown in table 3. Radioactive concentrations were generally similar in blood cells and plasma in all species studied until 4 h after administration, whereas there was a tendency, after this time in the mouse and at later time points for the other species, for higher concentrations in the blood cells.

Tissue-to-blood ratio of total radioactivity. The tissue concentrations of total radioactivity 1 or 4 h after administration of levetiracetam to the male and female rat, to the male and female dog, and to the female rabbit are shown in figure 4 and compared with whole blood concentrations. These times have been selected taking into account the t_{max} for whole blood radioactivity concentrations observed in the different species. In all species, tissue concentrations higher than blood were

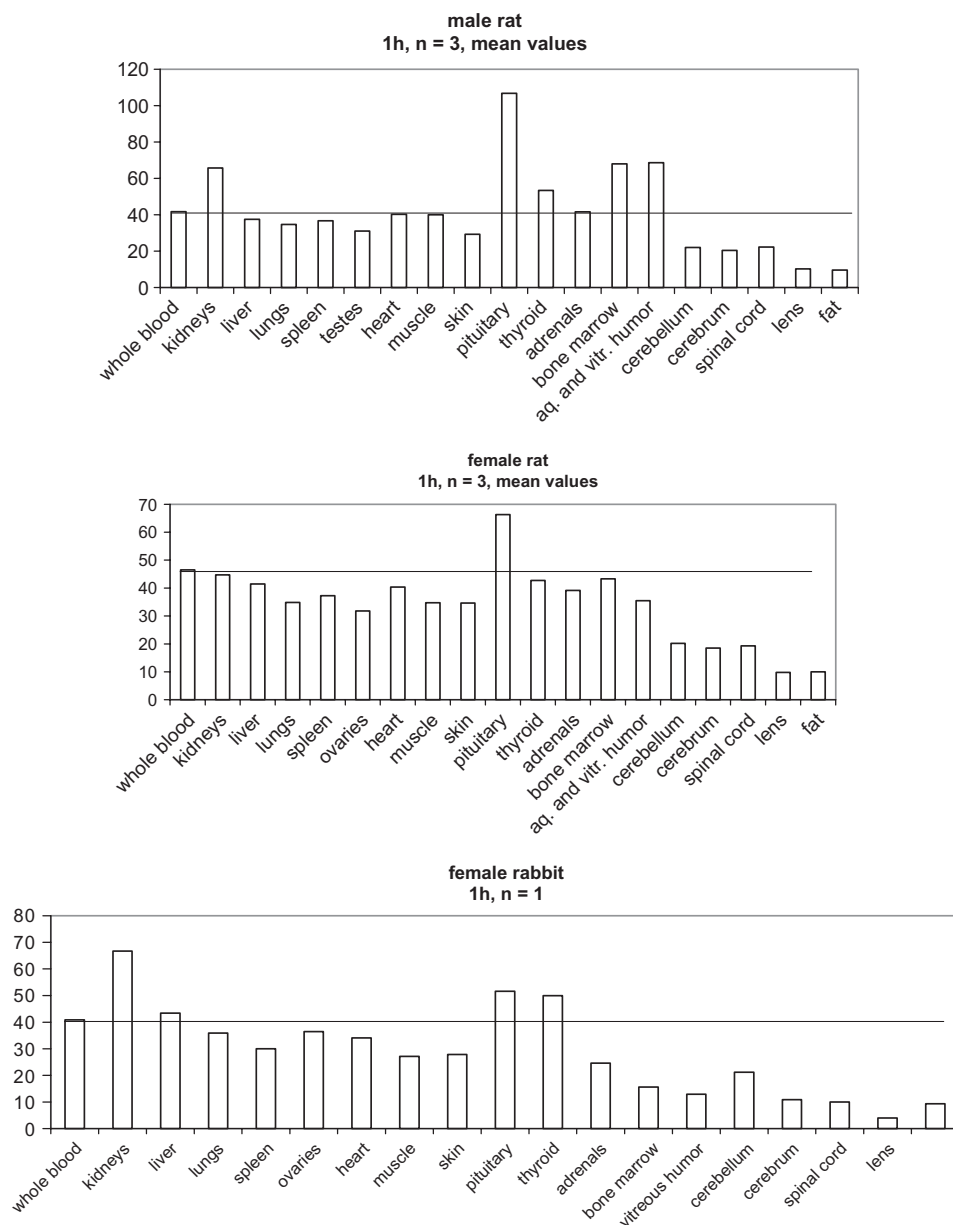


Figure 4. Tissue concentrations of total radioactivity ($\mu\text{g eq. g}^{-1}$ or ml^{-1}) after oral administration of ^{14}C -levetiracetam (54 mg kg^{-1}) to the rat, rabbit and dog. In all species, tissues are listed according to the order of decreasing radioactivity in the male dog.

generally found for kidney and pituitary gland, whereas tissue concentrations slightly lower than blood were systematically found for cerebrum, cerebellum and spinal cord, with lens and fat showing the lowest concentrations.

Brain-to-blood ratios of total radioactivity concentrations at different times after administration of levetiracetam in the male and female rats are shown in figure 5. The ratio increases as a function of time from 0.1 at 10 min to 1.0 at 6–8 h

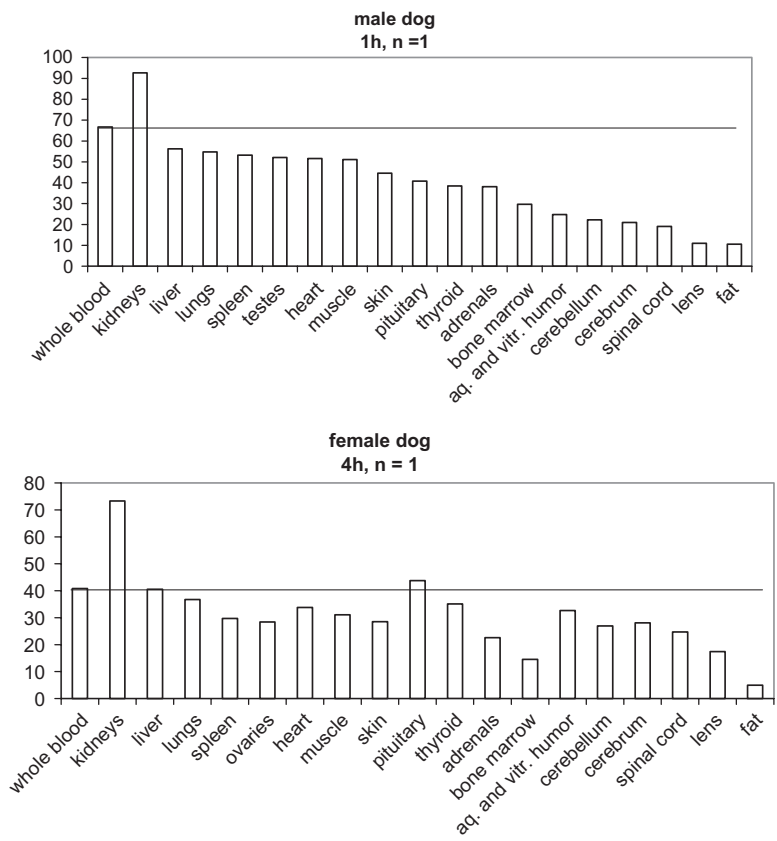


Figure 4. Continued.

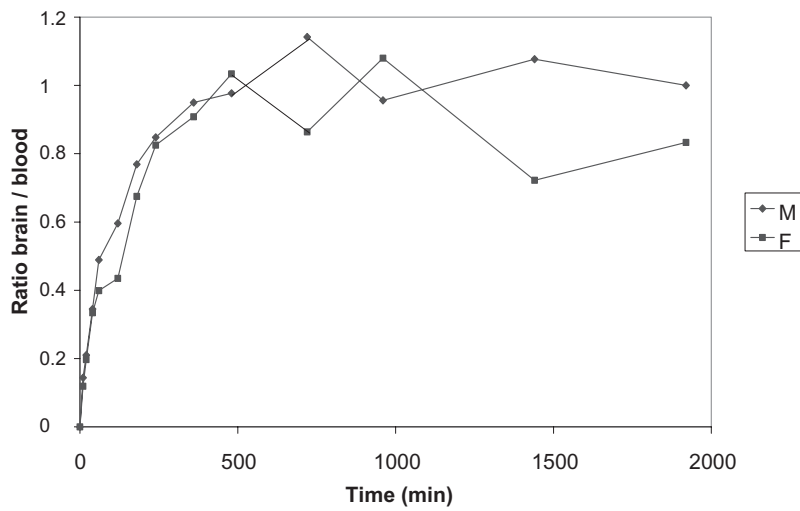


Figure 5. Mean brain-to-blood ratios of total radioactivity concentrations at different times after administration of a single oral dose of ^{14}C -levetiracetam (54 mg kg^{-1}) to male ($n=3$) and female ($n=3$) rats.

after administration and then remains practically constant until the last time point measured (32 h). The brain-to-blood ratios of total radioactivity concentrations in the rabbit and the dog also show a tendency to increase as a function of time, but the data available are sparse compared with the rat. The picture is very different in peripheral tissue such as kidney and muscle: the kidney-to-blood ratio of total radioactivity is stable over time (1.3 and 1.0 in the male and female rat, respectively, both at 10 min and 6 h after administration); the muscle-to-blood ratio shows a slight tendency to increase during the first 20 min but the value at 20 min (0.8 and 0.7 in the male and female rat, respectively) is essentially the same as at 6 h.

Excretion of total radioactivity and profile of urinary metabolites. The comparison of the urinary and faecal excretion of radioactivity in the different species 24 h after administration of levetiracetam is shown in table 4. The recovery of radioactivity was high for all the species studied: about 84–80, 92–94, 87 and 93–92% of the dose in the male and female mouse, the male and female rat, the female rabbit, and the male and female dog, respectively. The predominant route of excretion was via urine, accounting for a mean of 83.2 and 79.4% of the dose in the male and female mouse, respectively, of 91.6 and 93.8% in the male and female rat, respectively, of 86.7% in the female rabbit, and of 89.5 and 89.1% in the male and female dog, respectively, during the same interval. In the experiments where rats received 70 mg kg^{-1} , urine was collected until 72 h after administration, but most of the urinary radioactivity was already excreted after 24 h, i.e. 96% of the total cumulated urinary excretion. In the mouse, where urine was collected for 48 h, the percentage of the dose excreted at the end of this period was 87.4 and 82.4% for males and females, respectively. The unchanged drug present in the 0–24-h urine accounts on the average for 51 and 50% of the dose in the male and female mouse, respectively, for 78% in both male and female rat, for 58% in the female rabbit, and for 58 and 47% in the male and female dog, respectively (table 1).

Hydrolysis of levetiracetam generates the metabolite having the chromatographic characteristics of ucb L057 (table 5). This compound was inactive in sound-susceptible mice after acute administration of doses up to 548 mg kg^{-1} i.p., in contrast with the potent protection afforded by levetiracetam in the same model (Klitgaard *et al.* 1998). Levetiracetam can also be oxidized at position 3, 4 or 5 of the 2-oxopyrrolidine ring producing the metabolites having the chromatographic characteristics of ucb R297, ucb R301, ucb R302 and ucb R189 (table 5). Moreover, levetiracetam and/or ucb L057 can be oxidized at position 5 and then hydrolysed with the opening of the 2-oxopyrrolidine ring and the formation of the metabolites having the chromatographic characteristics of ucb K116 and ucb K115, respectively (table 5). ucb K116 can be further metabolized to ucb K115.

The suggested metabolic pathways of levetiracetam in animals are shown in figure 1, whereas the amounts of the urinary metabolites expressed as a percentage of the dose administered are shown in table 6 for the different species.

Enzyme induction.

Effect of phenobarbital pretreatment on the pharmacokinetic parameters of antipyrine. Results are summarized in table 7. Phenobarbital pretreatment increased similarly and significantly the metabolism of antipyrine in both males and females, without modification of the volume of distribution. The increase of

Table 4. Recovery of radioactivity in mouse, rat, rabbit and dog after single oral administration of ¹⁴C-levetiracetam at a dose of 54 mg kg⁻¹ in mouse, rabbit and dog and at doses of 54 and 70 mg kg⁻¹ in rat.

Biological sample	Percentage dose (mean or mean ± SD)								
	Mouse		Rat				Rabbit	Dog ^c	
			54 mg kg ⁻¹ dose		70 mg kg ⁻¹ dose				
	Males (n _c = 3) ^a	Females (n _c = 3) ^a	Males (n = 3) ^b	Females (n = 3) ^b	Males (n = 5) ^b	Females (n = 5) ^b	Females (n = 1) ^b	Males (n = 2) ^b	Females (n = 2) ^b
0–24-h urine	83.2 ± 6.3	79.4 ± 2.8	91.6 ± 3.4	93.8 ± 6.1	90.0 ± 3.6	88.3 ± 3.2	86.7	89.5	89.1
0–24-h faeces	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.3 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	0.5	3.9	3.0

^an_c, number of cages (containing five animals each).

^bn, number of animals.

^cMeans from the first day of repeated administration.

Table 5. Chromatographic characteristics of the test substance and reference compounds in the high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) methods used for species comparison.

Compound(s)	Radio HPLC, t_R (min) ^a		Radio TLC, R_f (range) ^b
	A	B	
ucb L057	> 16	17.3	0.60–0.67
ucb K115	> 16	12.5	0.17–0.21
ucb L059 (levetiracetam)	12.5	5.7	0.68–0.76
ucb K116	11.3	5.1	0.31
ucb R297	5.4, 6.2	3.1, 3.4	0.47–0.53
ucb R301	5.9	3.3	0.38–0.44
ucb R302	4.3	2.7	0.32–0.38
ucb R189	5.8, 8.5		0.55
ucb H214	3.7	2.5	

^aValues obtained for the metabolic study in rabbit.^bValues obtained for the metabolic studies in mouse, rat and dog.Table 6. Cumulative excretion (percentage dose) of levetiracetam and metabolites in urine after a single oral dose of ¹⁴C-levetiracetam (54 mg kg⁻¹).

Metabolites	Percentage dose						
	Mouse		Rat		Rabbit ^a	Dog ^b	
	(0–48-h urine)		(0–32-h urine)		(3–6-h urine)	(0–24-h urine)	
	Males	Females	Males	Females	Females	Males	Females
Unchanged drug	63.9	57.4	80.6	70.3	70	67.9	56.9
Acid metabolites (ucb L057 + ucb K115)			8.4	4.6	21	11.7	16.1
ucb L057	5.0	4.3	6.8	3.4	10	8.4	7.4
ucb K115	2.4	3.0	1.6	1.2	11	3.3	8.7
ucb K116					< 1	1.5	2.7
Hydroxylated metabolites (ucb R297 + ucb R302 + ucb R301 + ucb R189)	5.3	4.9	0.8	0.4	< 4	6	9.7
ucb R297	2.2	2.0	0.8	0.4	< 2	3.1	4.5
ucb R302	2.1	1.7			< 0.5	1.6	3.3
ucb R301						1.3	1.9
ucb R189	1.0	1.2			≤ 1.5		

^aData are percentages of the urinary radioactivity. Radioactivity present in the urinary fraction used (3–6 h) was 18% of the dose.^bValues are from the first day of repeated administration.

Data were obtained from 30 mice, 24 rats, one rabbit and four dogs (see the Materials and methods for more details).

antipyrine clearance, as measured 48 h after the third administration of phenobarbital, was on average 165% in males and 144% in females, indicating that antipyrine metabolism mediated by cytochrome P450 in phenobarbital-pretreated animals was clearly induced, during the period of urine collection following the administration of ¹⁴C-levetiracetam.

Effect of phenobarbital pretreatment on the urinary excretion of levetiracetam and its metabolites. Analysis of urine samples showed the presence of at least six radioactive components, the major component being unchanged levetiracetam. The amounts of total urinary radioactivity, of unchanged levetiracetam and

Table 7. Pharmacokinetic parameters of antipyrine (mean $\pm$ SD) in rat after administration of a single intravenous dose of 10 mg kg⁻¹.

Parameters	NaCl 0.9% pretreatment		Phenobarbital pretreatment	
	Males	Females	Males	Females
<i>t</i> _{1/2} (h)	1.26 $\pm$ 0.20	2.13 $\pm$ 0.72	0.52 $\pm$ 0.06 ^a	0.80 $\pm$ 0.13 ^a
AUC (μ g h ml ⁻¹)	19.4 $\pm$ 2.7	34.7 $\pm$ 11.7	7.53 $\pm$ 1.7 ^a	13.3 $\pm$ 2.8 ^a
Cl (l h ⁻¹ kg ⁻¹)	0.52 $\pm$ 0.07	0.32 $\pm$ 0.12	1.38 $\pm$ 0.28 ^a	0.78 $\pm$ 0.19 ^a
MRT (h)	1.81 $\pm$ 0.29	3.08 $\pm$ 1.04	0.76 $\pm$ 0.09 ^a	1.16 $\pm$ 0.19 ^a
V _{dss} (l kg ⁻¹)	0.93 $\pm$ 0.04	0.89 $\pm$ 0.05	1.02 $\pm$ 0.11 ^b	0.88 $\pm$ 0.08 ^b
<i>k</i> (h ⁻¹)	0.56 $\pm$ 0.08	0.36 $\pm$ 0.11	1.34 $\pm$ 0.14 ^a	0.89 $\pm$ 0.14 ^a

Phenobarbital- and NaCl-pretreated animals are compared:

^a*p* < 0.01.

^bNot significant, *p* > 0.05.

Data were obtained from 20 rats (see the Materials and methods for more details).

Table 8. Cumulative urinary excretion of levetiracetam and metabolites in rat 24 h after administration of a single oral dose of 1800 mg kg⁻¹.

Identification	Percentage dose (mean $\pm$ SD)			
	NaCl 0.9% pretreatment		Phenobarbital pretreatment	
	Males (<i>n</i> = 5)	Females (<i>n</i> = 5)	Males (<i>n</i> = 4)	Females (<i>n</i> = 5)
Radioactivity	89.4 $\pm$ 6.0	90.5 $\pm$ 7.3	93.6 $\pm$ 3.7 ^b	89.6 $\pm$ 5.0 ^b
ucb L059 (levetiracetam)	85.0 $\pm$ 5.2	86.9 $\pm$ 7.3	73.9 $\pm$ 1.2 ^a	80.1 $\pm$ 6.4 ^b
Component 1 (ucb R297)	1.1 $\pm$ 0.2	0.72 $\pm$ 0.10	7.7 $\pm$ 1.8 ^a	3.7 $\pm$ 1.1 ^a
Component 2 (not identified)	0.66 $\pm$ 0.24	0.43 $\pm$ 0.09	4.0 $\pm$ 0.7 ^a	1.8 $\pm$ 0.7 ^a
Component 3 (not identified)	0.15 $\pm$ 0.05	0.24 $\pm$ 0.03	1.4 $\pm$ 0.2 ^a	0.59 $\pm$ 0.26 ^a
ucb K115	0.57 $\pm$ 0.12	0.42 $\pm$ 0.07	3.4 $\pm$ 0.7 ^a	1.6 $\pm$ 0.4 ^a
ucb L057	1.9 $\pm$ 0.3	1.6 $\pm$ 0.2	1.7 $\pm$ 0.3 ^b	1.4 $\pm$ 0.1 ^b

Phenobarbital- and NaCl-pretreated animals are compared:

^a*p* < 0.01.

^bNot significant, *p* > 0.05.

metabolites (expressed as per cent of the dose given) are shown in table 8. Unchanged levetiracetam represented 85 and 87% of the dose in NaCl-pretreated male and female animals, respectively. The decrease in the excretion of parent compound was 13 and 8% in phenobarbital-pretreated male and female rats, respectively, the decrease being statistically significant in males only. The major metabolite was the acid derivative ucb L057, the percentage of which did not change significantly in males or females after phenobarbital pretreatment. The production of this metabolite was also not increased after repeated treatment with levetiracetam at 1800 mg kg⁻¹ day⁻¹ in the course of a 3-month toxicity study in the rat (UCB data on file). Therefore, the metabolic pathway involved in the production of ucb L057 is not inducible either after a repeated treatment with levetiracetam or with inducers like phenobarbital. The percentages of all the other metabolites increased after phenobarbital treatment, showing that the enzyme system(s) involved in their formation is inducible. The metabolite having the same chromatographic behaviour as the reference standard ucb K115 could be formed by oxidation followed by hydrolysis of the amide group or by hydrolysis of the amide group followed by oxidation. The statistically significant increase of this metabolite both in phenobarbital-pretreated male and female rats, together with

Table 9. Hydrolysis of levetiracetam in rat tissue homogenates.

Test systems	ucb L057 formed ($\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$)
Liver homogenate	1.12
Heat-denatured liver homogenate	n.d.
Kidney homogenate	0.73
Lung homogenate	1.17
Brain homogenate	0.93
Small intestine mucosa homogenate	0.48
50 mmol l^{-1} Tris buffer, pH 7.4	n.d.

n.d., Not detected ($\leq 0.42 \text{ pmol min}^{-1} \text{mg}^{-1} \text{protein}$).

Data are derived from a single determination.

the lack of changes of the percentage of L057 following phenobarbital pretreatment, suggests that the oxidation at position 5 of the 2-oxopyrrolidine ring is inducible and probably precedes the hydrolysis of the amide function.

In vitro metabolism. Results are shown in table 9. All the investigated rat tissue homogenates had the potential to produce ucb L057 when incubated with $500 \mu\text{mol l}^{-1}$ levetiracetam (reaction rate ranging from 0.48 to $1.17 \text{ pmol min}^{-1} \text{mg}^{-1} \text{protein}$). ucb L057 was undetectable following incubation of $500 \mu\text{mol l}^{-1}$ levetiracetam with vehicle buffers or heat-denatured liver fractions. Under the conditions used, the formation rates were the highest in lung and liver homogenates and the lowest in small intestine mucosa homogenate.

Discussion

The plasma levels of total radioactivity after administration of ^{14}C -labelled levetiracetam indicated a rapid absorption of the compound. The unchanged drug represented a very high percentage of plasma total radioactivity ($\geq 62\%$) at all times measured, in all species and in both males and females, this being an indication that the compound is poorly metabolized.

From the presentation of the plasma levels of levetiracetam using the elementary Dedrick's plot, there are no marked species differences in the plasma pharmacokinetics for this compound. An advantage of the elementary Dedrick's plot for species comparison over the use of the allometric equations relating the pharmacokinetic parameters of the drug with the body weight is that determination of the pharmacokinetic parameters in the different species is not necessary; therefore, errors in the estimation of the pharmacokinetic parameters (e.g. due to different and/or insufficient number of points in the different species) do not interfere with the analysis.

The study of plasma protein binding carried out *in vitro* at concentrations of levetiracetam covering all the possible *in vivo* concentrations of the unchanged drug showed no binding of levetiracetam to plasma proteins in all the species studied. This was confirmed by *ex vivo* data, which furthermore implies that levetiracetam metabolites are also unbound to plasma proteins.

The plasma-to-blood ratio of total radioactivity concentrations showed a tendency to decrease as a function of time. In humans, it is known that whole blood contains an amidase(s) metabolizing levetiracetam to its acid derivative ucb

L057 (Nicolas *et al.* 1998), whereas this enzyme activity in plasma is very low. If the same occurs in animals, the higher radioactive concentrations in blood cells at later times might be related to a slow release of this metabolite from the blood cells.

Although brain levels were, for most of the times examined, lower than whole blood levels, they were much higher than those the blood content of this organ (about $11 \mu\text{g g}^{-1}$ tissue in the rat) could account for (Gonzalez *et al.* 1975). For instance, the mean brain radioactivity concentration of male rats at 40 min (when whole blood concentration is the highest) once corrected for whole blood contamination is reduced from 15.8 to $15.3 \mu\text{g eq. g}^{-1}$.

The increase of the brain-to-blood ratio as a function of time observed for levetiracetam is not reported for all the new anti-epileptic agents, as, for example, for felbamate the brain-to-blood ratio of total radioactivity concentrations is constant over 24 h after administration and is around 0.5, although the compound is lipophilic (Adusumalli *et al.* 1991). It is interesting to observe that in a parallel study where the cerebrospinal fluid (CSF)-to-serum levetiracetam concentration ratio in the rat was reported as a function of time, the ratio increased up to about 120 min after i.p. administration of 80 mg kg^{-1} and then remained constant at around 1 (Doheny *et al.* 1999). It has also been reported in the case of piracetam that the uptake of this substance in the brain of mice, rats and dogs is somewhat delayed but that the substance seems to be present much longer in the central nervous system than in most of the other organs (Gobert 1975, Ostrowski *et al.* 1975, Tacconi and Wurtman 1984, Lapka *et al.* 1990). Moreover, for piracetam also, a study similar to the one done for levetiracetam (Doheny *et al.* 1999) was carried out (Doheny *et al.* 1994), showing that blood levels peaked earlier than CSF concentrations whereas the blood elimination half-life was shorter than that of the CSF.

The urinary excretion of total radioactivity versus time after administration of ^{14}C -labelled levetiracetam confirmed the plasma data, i.e. that levetiracetam is not only rapidly absorbed, but also that the absorption is almost complete. The urine analysis of the unchanged drug confirmed the plasma data, i.e. that levetiracetam is poorly metabolized.

In all the species investigated, levetiracetam is subject to hydrolysis and/or oxidation. Hydrolysis of levetiracetam to the acid derivative is mediated by an enzymatic process characterized by a broad tissue distribution, at least in the rat. This distribution pattern resembles that observed for the non-specific esterases/amidases hydrolysing *p*-nitrophenylacetate. These latter enzymes hydrolyse drugs containing an ester, thioester or amide linkage, and are present in virtually all tissues, including blood (Ali and Kaur 1983a, Morgan *et al.* 1994). Phenobarbital is an inducer of these non-specific esterases and amidases in the rat liver. However, no such induction was detectable in extrahepatic tissues (Ali and Kaur 1983b). Interestingly, in rats pretreated with phenobarbital, and in which induction of the cytochrome P450 system was demonstrated using antipyrine as a probe, the formation of the acid derivative produced by hydrolysis of levetiracetam was not increased. This suggests that the esterase(s)/amidase(s) involved in the rat in the *in vivo* hydrolysis of levetiracetam when the compound is administered orally are not inducible, at least by phenobarbital at the dose and duration of pretreatment applied in the present study. It has been shown that rat liver esterase induction by phenobarbital, when α -naphthyl acetate and *p*-nitrophenylacetate are used as

substrates, is dependent on the dose and duration of pretreatment (Schwark and Ecobichon 1968, Nousiainen and Hänninen 1981).

Although there are no qualitative differences among the species studied, some quantitative differences can be observed, e.g. the dog, the female in particular, appears to oxidize levetiracetam more extensively than the rat.

In conclusion, no marked species or sex differences were observed in the absorption, distribution, metabolism and elimination of levetiracetam after administration of a single oral dose. This is true for the four animal species presented herein, but it also applies to male human subjects (Strolin Benedetti *et al.* 2003). These data confirm the suitability of the animal species selected for pharmacological and safety studies with respect to the pharmacokinetic behaviour of levetiracetam in man and the species investigated.

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Model for the drug–drug interaction responsible for CYP3A enzyme inhibition. I: Evaluation of cynomolgus monkeys as surrogates for humans

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1. Anti-human cytochrome P450 (CYP) 3A4 antiserum completely inhibited midazolam metabolism in monkey liver microsomes, suggesting that midazolam was mainly metabolized by CYP3A enzyme(s) in monkey liver microsomes.

2. Midazolam metabolism was also inhibited *in vitro* by typical chemical inhibitors of CYP3A, such as ketoconazole, erythromycin and diltiazem, and the apparent K_i values for ketoconazole, erythromycin and diltiazem were 0.127, 94.2 and 29.6 μM , respectively.

3. CYP3A inhibitors increased plasma midazolam concentrations when midazolam and CYP3A inhibitors were co-administered orally. However, the pharmacokinetic parameters of midazolam were not changed by treatment with CYP3A inhibitors when midazolam was given intravenously. This suggests that CYP3A inhibitors modified the first-pass metabolism in the liver and/or intestine, but not systemic metabolism.

4. The drug–drug interaction responsible for CYP3A enzyme(s) inhibition was observed when midazolam and inhibitors were co-administrated orally. Therefore, it was concluded that monkeys given midazolam orally could be useful models for predicting drug–drug interactions in man based on CYP3A enzyme inhibition.

Introduction

Enzyme inhibition screening using human liver microsomes has been widely conducted at the drug discovery stage, and prediction methods for drug–drug interactions *in vivo* using *in vitro* data are currently under development (Iwatsubo *et al.* 1997, Ito *et al.* 1998, Von Moltke *et al.* 1998). Changes in pharmacokinetics caused by drug–drug interactions are thought to be one of the important factors in severe adverse drug responses (Lesar *et al.* 1997, Kremers 2002). However, some cases fail to predict drug–drug interactions because of the possibility that other metabolic pathways and pharmacokinetic process might also be altered by the inhibitor, and the accumulation of the inhibitor in the liver by active transport (Ito *et al.* 1998). On the other hand, no extrapolation method from animal to human (Higashikawa *et al.* 1999) has yet been established because of species differences in drug metabolism. However, being able to predict drug–drug interactions in humans in preclinical studies can offer very useful information for safer clinical studies. If there is available information on drug–drug interactions of new drug entities in animal *in vivo* in addition to *in vitro*/*in vivo* extrapolation methods, one can adopt a similar method for prediction in humans. Therefore, to establish a

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convenient animal model that can predict drug–drug interactions *in vivo* should be of great practical importance. The animal model considered to be of the greatest potential value is that using monkeys because the CYP enzymes in such animals are thought to be similar to those in humans (Laddison *et al.* 1995, Igarashi *et al.* 1997). In addition, monkeys are widely used for preclinical studies at the drug development stage, such as toxicology and preclinical absorption, distribution, metabolism and excretion (ADME) studies. Therefore, the prediction of drug–drug interactions using monkeys should provide clinically important information.

The present study focused on the drug–drug interaction caused by CYP3A enzyme inhibition. CYP3A enzymes are the major enzymes in the oxidative metabolism in humans, and a large number of compounds including clinically important pharmaceuticals are metabolized by the CYP3A enzymes (Spatzenegger and Jaeger 1995). Midazolam was used as a probe in the present study because it is metabolized to 1'-hydroxy midazolam and 4-hydroxy midazolam by CYP3A4 in humans and by CYP3A1 and/or CYP3A2 in rats (Kronbach *et al.* 1989, Thummel *et al.* 1994a, Ghosal *et al.* 1996, Von Moltle *et al.* 1996). It has also been used as a CYP3A probe (Thummel *et al.* 1994a, b). As typical inhibitors of CYP3A, ketoconazole, erythromycin and diltiazem were used. These compounds are well known as CYP3A inhibitors from clinical, *in vivo* and *in vitro* studies (Bjornsson *et al.* 2003).

The purpose of the current study was to establish and evaluate female monkeys, practically easier to use than male monkeys, as models suitable for predicting the drug–drug interaction responsible for CYP3A enzyme(s) inhibition.

Materials and methods

Chemicals

Midazolam and ketoconazole were synthesized at the Discovery Research Laboratories, Shionogi & Co., Ltd., and the chemical purities of midazolam and ketoconazole were 99.9 and 99.1%, respectively. Erythromycin, diazepam and diltiazem hydrochloride were from Wako Pure Chemicals (Osaka, Japan). *n*-Hexane, dichloromethane and ethylacetate for gas chromatography assay grade were obtained from Nacalai Tesque (Kyoto, Japan). Other reagents used were of the highest or high-performance liquid chromatography grade.

Animals

Female cynomolgus monkeys (*Macaca fascicularis*), 2.72–3.54 kg, were used. All procedures for the animal experiments were approved by the local committee for animal care and use.

Microsomal incubations

Monkey liver microsomes were prepared by the method of Matsubara *et al.* (1983). Midazolam metabolism activity was assayed using the method described below. Briefly, the reaction mixture consisted of 0.1 mol l⁻¹ Tris-HCl, pH 7.4, 10 mmol l⁻¹ MgCl₂, liver microsomes and midazolam. The total volume was 1.0 ml. The reaction was started by addition of 1 mmol l⁻¹ NADPH and incubated for the designated period at 37°C. The reaction was terminated by addition of 20 µl of 2 mol l⁻¹ NaOH to the reaction mixture and cooled in ice water for 15 min.

In immuno-inhibition studies, the incubations were carried out with anti-CYP antisera. The anti-human CYP antisera used were antisera against CYP1A, CYP2C9/10, CYP2C19, CYP2D, CYP2E and CYP3A. To prepare antisera, rabbits were immunized with purified recombinant CYP enzymes. These anti-human CYP1A, CYP2C9/10, CYP2C19, CYP2E and CYP3A antisera, respectively, selectively inhibited 7-ethoxyresorufin *O*-deethylation, tolbutamide 4-methylhydroxylation, aniline hydroxylation, and testosterone 6β-hydroxylation activities by human liver microsomes (data not shown). For the assay procedure, the reaction mixture was left at room temperature for 30 min after the antiserum had been added to the reaction mixture, then the reaction was initiated by addition of NADPH. The subsequent processing procedure was the same as described above. The control for these experiments was metabolic activity of the sample treated with pre-immune serum.

Preparation of dosage

The compound to be used (ketoconazole, erythromycin, diltiazem and midazolam) was ground in an agate mortar in 0.5% methylcellulose solution. The concentrations of midazolam, ketoconazole, erythromycin and diltiazem for oral administration were 2.5, 2.5, 7.5 and 2.5 mg ml⁻¹, respectively. To prepare the dosage of midazolam for intravenous administration, it was dissolved in an equal molar ratio of 0.1 mol l⁻¹ hydrochloric acid solution and diluted with saline (Otsuka Pharmaceutical, Tokushima, Japan) to prepare a 0.1 mg ml⁻¹ solution.

In vivo administration studies

Midazolam and CYP3A inhibitors were administered to monkeys under a 24-h fasted condition. When midazolam was given orally, it was given simultaneously with CYP3A inhibitor to the monkeys. When given intravenously, midazolam was administered right after oral administration of the CYP3A inhibitor. The doses of midazolam were 0.2 mg kg⁻¹ for intravenous administration and 5 mg kg⁻¹ for oral administration. CYP3A inhibitors were given at the dose of 5 mg kg⁻¹ ketoconazole, 15 mg kg⁻¹ erythromycin and 5 mg kg⁻¹ diltiazem, respectively. The monkeys of the control group were given 0.5% methylcellulose solution instead of CYP3A inhibitor suspensions. Blood samples were collected at 0.5, 1, 2, 4, 7 and 24 h after oral administration, and at 10, 20, 30 min, 1, 2, 4 and 7 h after intravenous administration. The blood samples were immediately centrifuged to obtain plasma samples. The plasma samples were kept at approximately -20°C until analysis.

Midazolam analysis

Midazolam concentration was determined by a modified gas chromatographic method (Lillsunde and Seppala 1990, Gaillard *et al.* 1993) using an electron-capture detector with diazepam as an internal standard. The instrument was a Hewlett-Packard 5890 capillary gas chromatograph with an electron-capture detector (Hewlett-Packard, Avondale, PA, USA). The HP5890 and an HP7673 automatic injector were interfaced and monitored using the software HP Chemstation 3365. The capillary column was DB-1 (30 m × 0.32 mm i.d., film thickness 0.25 µm; Hewlett-Packard), column temperature was 230°C, the carrier gas was helium, and the flow rate was 2.0 ml min⁻¹ (constant flow mode). The injection port temperature was 300°C and the detector temperature was 300°C. Midazolam was detected at the retention time of 9.0 min and diazepam was observed at the retention time of 6.3 min.

An appropriate volume of plasma (0.5 ml), 50 µl midazolam standard solutions and 50 µl internal standard solutions (100 ng ml⁻¹ diazepam) were shaken for 15 min with 0.5 ml of 0.5 mol l⁻¹ Na₂HPO₄, pH 9, and 2.5 ml *n*-hexane/dichloromethane (70:30, v/v). After centrifugation at 1880g for 15 min, the aqueous layer was discarded and the organic layer was evaporated. The residue was dissolved in 50 µl ethylacetate, and 2 µl of the solution was subjected to gas chromatography-electron capture detection. To determine the midazolam concentration in the microsomal reaction mixture, these mixtures with an internal standard (1 µg ml⁻¹ diazepam, 100 µl) were extracted with 2.5 ml *n*-hexane/dichloromethane (70:30, v/v). The subsequent procedure was the same as that described above.

The intraday coefficient of variance ranged from 0.8 to 8.0% and accuracy from -5.3 to 4.1% at concentrations ranging from 0.5 to 50 ng ml⁻¹. The interday precision was less than 11% and accuracy ranged from 1.0 to 7.0%. The lower limit of determination was 0.5 ng ml⁻¹ and the higher limit of detection was 50 ng ml⁻¹. These data supported validation of this analytical method.

Data analysis

In vitro metabolism is defined as the time-dependent decrease in midazolam concentration. The inhibition type was evaluated from the relationship of metabolic activity and the initial concentration of substrate ([S]₀) calculated using the Lineweaver-Burk plot. The apparent values of inhibition constant (*K_i*) were calculated by fitting the data to the inhibition type using a non-linear least-squares method program with WinNonlin® (Pharsight, Mountain View, CA, USA).

Pharmacokinetic parameters were estimated using WinNonlin. Plasma concentrations of midazolam after oral and intravenous administration were analysed by non-compartment analysis. The maximum concentration in plasma (*C_{max}*) of midazolam and the time to reach *C_{max}* (*T_{max}*) were used as the observed data. The area under the plasma concentration of drug versus time curve (AUC) was obtained by trapezoidal rule. The mean residence time (MRT), total clearance (CL_{tot}/*F* or CL_{tot}) and distribution volume (*V_d*) were calculated using the following equations:

$$\begin{aligned}\text{MRT} &= \text{AUMC}_{\text{inf}} / \text{AUC}_{\text{inf}} \\ \text{CL}_{\text{tot}} / F \text{ or } \text{CL}_{\text{tot}} &= \text{dose} / \text{AUC}_{\text{inf}} \\ V_d &= \text{MRT} \times \text{CL}_{\text{tot}},\end{aligned}$$

where AUMC is the area under the moment versus time curve.

The levels of statistical significance were assessed using paired *t*-test analysis. *p* values of significance were set at 0.05.

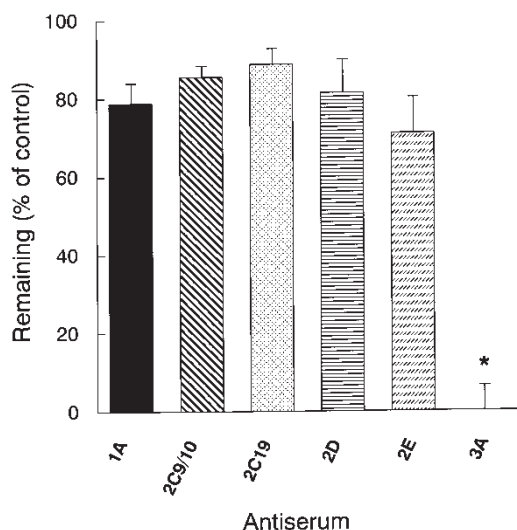


Figure 1. Effect of anti-CYP sera on midazolam metabolism in monkey liver microsomes. Values are the mean $\pm$ SD of three experiments. Absolute metabolic activity of control was 0.600 ± 0.079 nmol min⁻¹ mg⁻¹ protein. * $p < 0.05$, significantly different from the control by student's *t*-test.

Table 1. Characteristics of midazolam metabolism inhibition in monkey liver microsomes.

Compound	Type of Inhibition	K_i (μ M)
Ketoconazole	non-competitive	0.127 ± 0.062
Erythromycin	competitive	94.2 ± 29.8
Diltiazem	competitive	29.6 ± 5.9

Values are the mean $\pm$ SD of three monkey liver microsomes.

Results

Immunoinhibition studies of midazolam metabolism in monkey liver microsomes

Figure 1 shows the effect of addition of anti-human CYP antisera on midazolam metabolism in monkey liver microsomes. The immuno-inhibition study was carried out using anti-human CYP antisera (CYP1A, CYP2C9/10, CYP2C19, CYP2D6, CYP2E1 and CYP3A4). Midazolam metabolism was completely inhibited by the addition of anti-CYP3A4 antiserum. The results suggested that the enzyme responsible for midazolam metabolism was CYP3A enzyme(s) in monkey liver microsomes.

Effect of CYP3A chemical inhibitors on midazolam metabolism

The apparent inhibition constant (K_i) values and types of inhibition are summarized in table 1. Ketoconazole, diltiazem and erythromycin are typical chemical inhibitors of CYP3A enzymes (Bjornsson *et al.* 2003). Ketoconazole was shown to be a non-competitive inhibitor of midazolam metabolism with an apparent K_i of 0.127μ M (figure 2). Diltiazem and erythromycin were competitive inhibitors of midazolam metabolism and their apparent K_i values were 29.6 and 94.2μ M, respectively (figure 2). These apparent K_i values and the types of inhibition were similar to those in human microsomes, suggesting that the enzyme

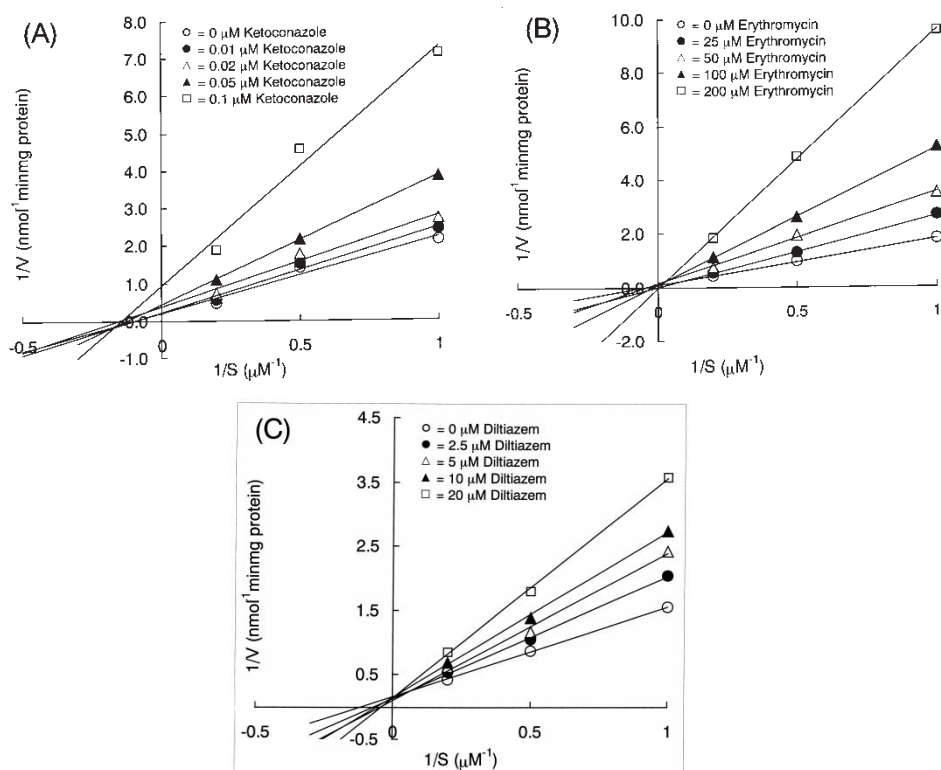


Figure 2. Lineweaver-Burk plot of midazolam metabolism in monkey liver microsomes with ketoconazole (A), erythromycin (B) and diltiazem (C).

inhibition responses in monkeys were similar to those in humans (Gascon and Dayer 1991, Wrighton and Ring 1994).

Pharmacokinetics of midazolam in female monkeys

Figure 3 shows the plasma-time concentration profiles of midazolam after intravenous administration of midazolam at a dose of 0.2 mg kg^{-1} . Midazolam rapidly disappeared from plasma with an elimination half-life ($t_{1/2}$) of $1.46 \pm 0.55 \text{ h}$, and the total plasma clearance (CL_{tot}) was $0.986 \pm 0.246 \text{ l h}^{-1} \text{ kg}^{-1}$ (table 2). After oral administration of midazolam (5 mg kg^{-1}), it was absorbed immediately. The area under the plasma concentration curve (AUC) of midazolam was $349 \pm 100 \text{ ng h ml}^{-1}$, CL_{tot}/F was $15.3 \pm 5.21 \text{ h}^{-1} \text{ kg}^{-1}$ and its bioavailability was $6.58 \pm 1.87\%$ (table 3).

Effect of CYP3A inhibitors on midazolam pharmacokinetics in female monkeys

Monkeys were given CYP3A inhibitors orally with intravenous or oral administration of midazolam. The plasma midazolam concentration profiles co-administered with CYP3A inhibitors are shown in figures 3 and 4, and the pharmacokinetic parameters are summarized in tables 2 and 3. After intravenous administration of midazolam, plasma concentrations and pharmacokinetic parameters of midazolam did not change on treatment with CYP3A inhibitors (table 2). On the other hand, plasma concentrations and the bioavailability of

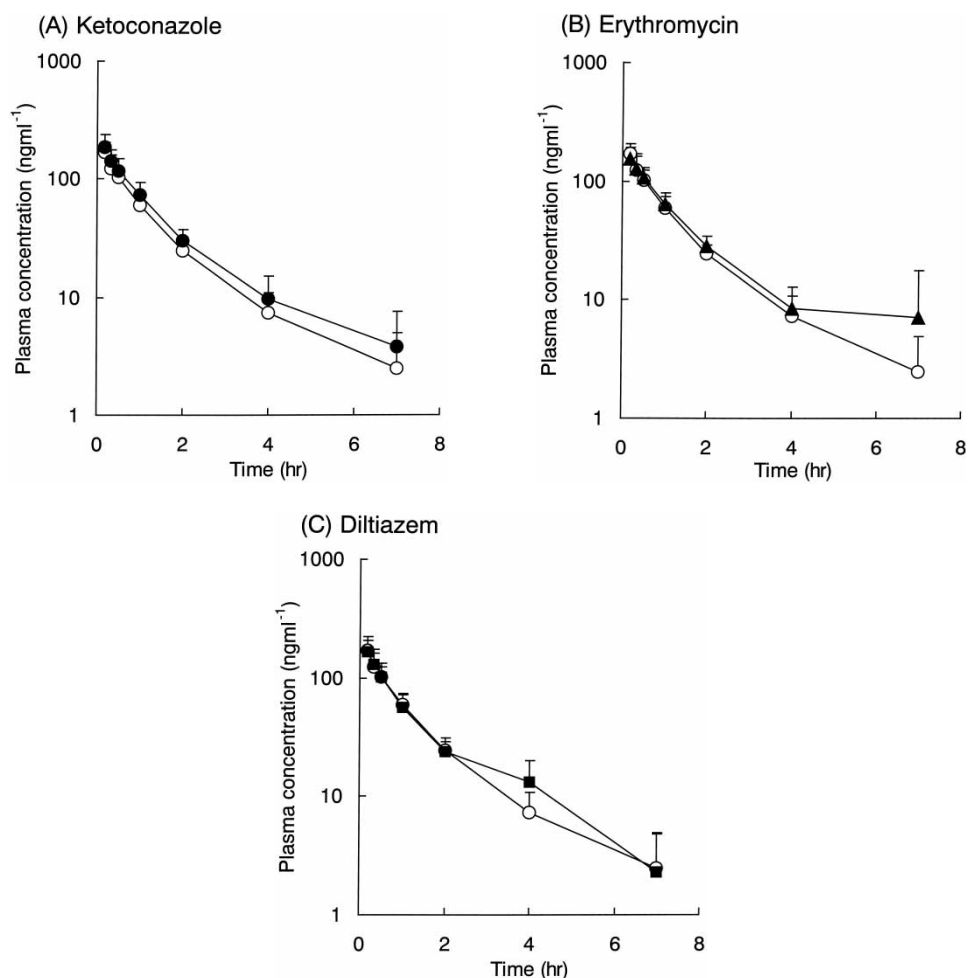


Figure 3. Effect of ketoconazole (A), erythromycin (B) and diltiazem (C) on plasma concentration of midazolam after intravenous administration of midazolam to female cynomolgus monkeys. The dosed groups were the control group (open circles), ketoconazole treatment group (closed circles), erythromycin treatment group (closed triangles), and diltiazem treatment group (closed squares). Values are the mean $\pm$ SD of three monkeys.

Table 2. Pharmacokinetic parameters estimated from the plasma concentration of midazolam after intravenous administration of midazolam with or without ketoconazole, erythromycin and diltiazem to female cynomolgus monkeys.

	AUC_{inf} (ng h ml ⁻¹)	CL_{tot} (l h ⁻¹ kg ⁻¹)	MRT (h)	V_d (l kg ⁻¹)
Control	213 $\pm$ 59	0.986 $\pm$ 0.246	1.39 $\pm$ 0.38	1.31 $\pm$ 0.01
Ketoconazole	186 $\pm$ 37	1.10 $\pm$ 0.22	1.26 $\pm$ 0.25	1.35 $\pm$ 0.00
Erythromycin	240 $\pm$ 30	0.843 $\pm$ 0.113	2.20 $\pm$ 0.94	1.80 $\pm$ 0.58
Diltiazem	221 $\pm$ 71	0.972 $\pm$ 0.311	1.56 $\pm$ 0.23	1.47 $\pm$ 0.25

Values are the mean $\pm$ SD of three monkeys.

AUC_{inf} , area under the plasma concentration of midazolam versus time curve from time 0 to infinity; CL_{tot} , total clearance; MRT, mean residence time; V_d , volume of distribution.

No significant difference was shown from the control monkeys by a paired *t*-test.

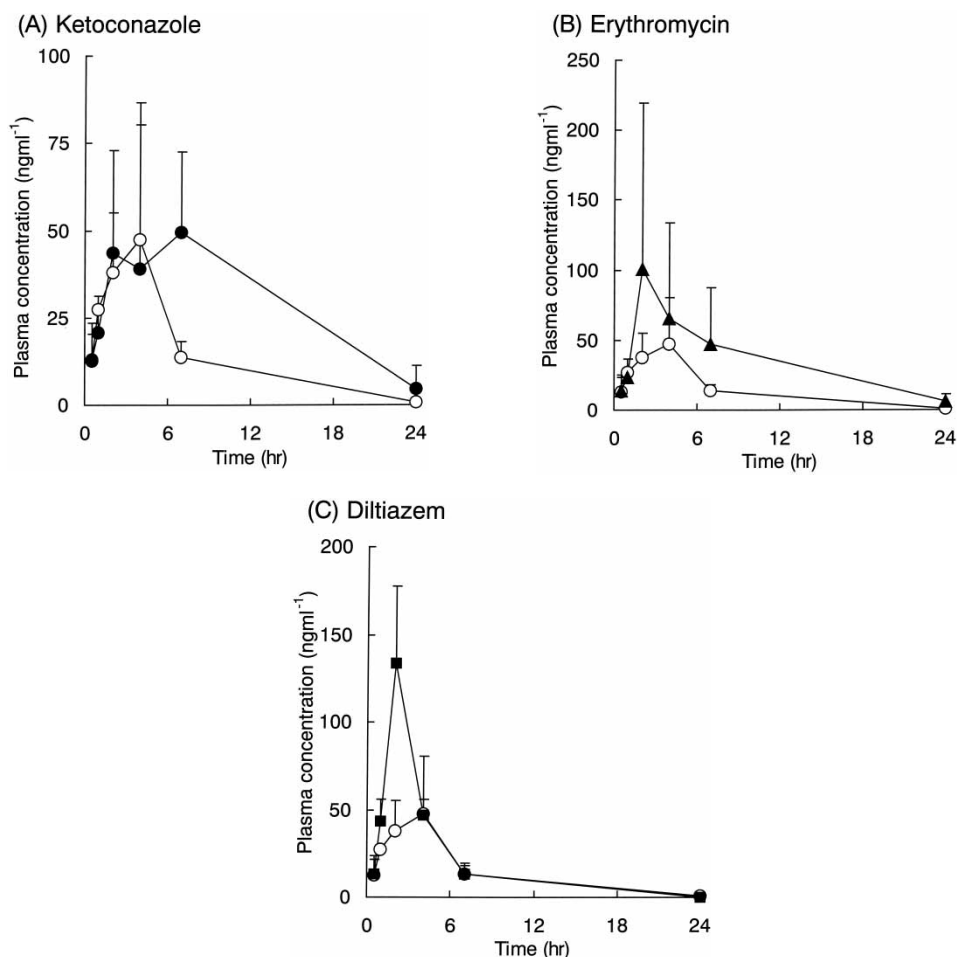


Figure 4. Effect of ketoconazole (A), erythromycin (B) and diltiazem (C) on plasma concentration of midazolam after oral administration of midazolam to female cynomolgus monkeys. The dosed

Table 3. Pharmacokinetic parameters estimated from the plasma concentration of midazolam after oral administration of midazolam with or without ketoconazole, erythromycin and diltiazem to female cynomolgus monkeys.

	AUC_{inf} (ng h ml ⁻¹)	CL_{tot}/F (l hr ⁻¹ kg ⁻¹)	MRT (h)	V_d/F (l kg ⁻¹)	Bioavailability (%)
Control	349 ± 100	15.3 ± 5.2	5.28 ± 0.27	92.3 ± 55.1	6.58 ± 1.87
Ketoconazole	776 ± 133*	6.56 ± 1.03*	9.45 ± 6.14	50.7 ± 42.4	16.7 ± 2.8*
Erythromycin	861 ± 455	6.64 ± 3.08	7.16 ± 4.30	47.7 ± 40.1	14.4 ± 7.6
Diltiazem	493 ± 146	10.9 ± 3.7	3.91 ± 0.29*	51.4 ± 29.6	8.94 ± 2.64

Values are the mean ± SD of three monkeys.

AUC_{inf} , area under the plasma concentration of midazolam versus time curve from time 0 to infinity; CL_{tot}/F , total clearance; MRT, mean residence time; V_d/F , volume of distribution; CL_{tot}/F and V_{ss}/F , where F is the fraction of dose absorbed.

* $p < 0.05$, significantly different from the control monkeys by a paired t -test.

midazolam increased after oral co-administration of midazolam and CYP3A inhibitors (table 3). CYP3A inhibitors produced increased AUC and bioavailability without a change in the elimination half-life, suggesting that CYP3A inhibitors alter the first-pass metabolism in liver and/or intestine. Thus, the drug–drug interaction that occurred *in vitro* could also be observed *in vivo* after oral administration of midazolam to monkeys.

Discussion

As drug–drug interactions are generally considered to be undesirable (Kremers 2002), information on CYP enzyme inhibition ideally should be obtained before selection of a drug candidate for development. Recently, CYP enzyme inhibitions screening using human liver microsomes has been widely conducted at an early drug-discovery stage to predict potential *in vivo* drug–drug interactions. A number of *in vitro/in vivo* extrapolation methods have been developed to assess the potential risk of drug–drug interactions based on CYP inhibition (Ito *et al.* 1998, Iwatsubo *et al.* 1997). The $[I]/K_i$ ratio has been considered to reflect the degree of inhibition, when the inhibition mechanism is reversible and the pharmacokinetics are linear. In fact, such an *in vitro/in vivo* extrapolation can provide useful information for predicting potential drug–drug interactions, although incorrect predictions can also occur (Ito *et al.* 1998). When one tries to predict the *in vivo* drug–drug interaction from *in vitro* data, the hepatic portal input concentration of total drug in plasma is generally used as the concentration of the inhibitor *in vivo* at the enzyme site ($[I]$). Since it is not possible to measure the $[I]$ value in humans, it is estimated from the following equation (Ito *et al.* 1998):

$$I_{\text{in, max, } u} = \left(I_{\text{max}} + \frac{k_a \cdot D \cdot F_a}{Q_h} \right) \cdot f_u,$$

where $I_{\text{in, max, } u}$, I_{max} , k_a , D , F_a , Q_h and f_u are, respectively, the maximum inflow concentration of the inhibitor into the liver, maximum plasma concentration of the inhibitor in the circulation, absorption constant with first-order rate, dose, absorption ratio, hepatic blood flow and free fraction of inhibitor. However, as the actual values of k_a and F_a of the inhibitors and Q_h are usually not available, these values are usually quoted from literature or the results of preclinical animal studies. Such use of non-validated values could lead to incorrect predictions with severe adverse effects.

To conduct clinical studies more safely and effectively, the present authors considered conducting on *in vivo* drug–drug interaction study using animal models before clinical study to obtain more reliable predictions. Furthermore, the combination of *in vitro/in vivo* scaling and the result of *in vivo* model studies should provide more reliable and useful predictions. This led us to try to establish an *in vivo* drug–drug interaction model using the cynomolgus monkey. Non-human primates including the cynomolgus monkey have been considered to be the most appropriate animal models for predicting drug metabolism in humans, and many publications have suggested that the monkey may provide more reliable predictions for human drug metabolism than rodents or dogs (Yasumori *et al.* 1992, Wu *et al.* 1993, Ring *et al.* 1994, Sharer *et al.* 1995, Igarashi *et al.* 1997). This general belief is based on the sequence similarity of drug-metabolizing enzymes. It

has been reported that the nucleotide and the deduced amino acid sequence of CYP enzymes (CYP2D and CYP3A) in cynomolgus monkey (*M. fascicularis*) had >90% identity with human CYPs (Laddison *et al.* 1995, Igarashi *et al.* 1997). In addition, as monkeys are used in ADME studies at the drug discovery and developmental stage, pharmacokinetic information would also be available for predicting a drug-drug interaction.

The current study focused on the drug-drug interaction based on CYP3A inhibition, because CYP3A enzymes are the most abundant constituents of the CYP enzyme in human liver (Thummel and Wilkinson 1998, Guengerich 1999). Midazolam was used as a probe because it has been reported to be metabolized by CYP3A enzymes in humans and rats (Kronbach *et al.* 1989, Gorski *et al.* 1994, Ghosal *et al.* 1996). First, we clarified the enzyme responsible for midazolam metabolism in monkey liver microsomes, as there had been no reports regarding the CYP enzymes involved in midazolam metabolism in monkeys. The results of the immuno-inhibition study showed that midazolam metabolism was completely inhibited by the addition of anti-CYP3A antiserum. Thus, midazolam can be considered to be metabolized by CYP3A enzyme(s) in monkey liver microsomes. Furthermore, midazolam metabolism was inhibited by typical CYP3A inhibitors, such as ketoconazole, erythromycin and diltiazem. Previous reports have shown that ketoconazole is a high-affinity non-competitive inhibitor of CYP3A and its apparent K_i value against testosterone 6 β -hydroxylation was 0.11 μ M in human liver microsomes (Gascon and Dayer 1991, Wrighton and Ring 1994). Erythromycin competitively inhibits midazolam metabolism with the apparent K_i = 194 μ M (Gascon and Dayer 1991, Wrighton and Ring 1994). Diltiazem also inhibits CYP3A activity and its IC_{50} against CYP3A-mediated testosterone 6 β -hydroxylation is 120 μ M (Sutton *et al.* 1997). These apparent K_i values and the types of inhibition of midazolam metabolism in monkey liver microsomes were similar to those in human microsomes, suggesting that the enzyme inhibition responses in monkeys were similar to those in humans. Thus, the results of both immunochemical and chemical inhibition studies indicate that the midazolam metabolic enzyme(s) in monkey microsomes are CYP3A enzymes. This offers evidence that monkeys could yield data close to that of humans in an *in vitro* drug-drug interaction model to assess CYP3A enzyme inhibition. From the result of our *in vitro* studies, we considered that monkeys could be used as surrogates of humans to predict *in vivo* drug-drug interactions responsible for CYP3A inhibition.

Next, it was evaluated whether the monkey could be a model for an *in vivo* drug-drug interaction model. Midazolam was administered to monkeys at a dose of 5 mg kg⁻¹ for oral administration or 0.2 mg kg⁻¹ for intravenous administration, based on the range of linear pharmacokinetics from the results of our studies (data not shown). Ketoconazole, erythromycin and diltiazem were administered at doses of 5, 15 and 5 mg kg⁻¹, respectively. Since ketoconazole is clinically administered at 200 or 400 mg day⁻¹, this corresponds to 2.9 or 5.7 mg kg⁻¹ day⁻¹, respectively, for a human body weight of 70 kg. Erythromycin is used at a dose of 800 or 1200 mg day⁻¹ and diltiazem at 30 $\times$ 3 or 60 $\times$ 3 mg day⁻¹. Thus, the doses used in the current study are equivalent to the clinical doses in humans. In addition, the T_{max} values of ketoconazole, erythromycin and diltiazem are 2, 3 and 2 h after single administration, respectively (Yakatan *et al.* 1979, Baxter *et al.* 1986, Backman *et al.* 1994), suggesting that these inhibitors should reach at the enzyme

site after the absorption phase. Thus, we judged drug–drug interaction based on the inhibition by CYP3A inhibitors could be evaluated by this method, if inhibitors have an effect on midazolam pharmacokinetics after intravenous administration of midazolam. In other words, when inhibitors have an inhibitory potential on midazolam metabolism *in vivo*, the elimination phase (half-life, $T_{1/2}$) of midazolam should be altered. When midazolam was administered intravenously, its concentration in monkey plasma was not influenced by treatment with CYP3A inhibitors and the elimination half-lives of midazolam were almost the same whether or not CYP3A inhibitors were administered. On the other hand, after oral administration of midazolam, its AUC and the bioavailability increased with oral co-administration of CYP3A inhibitors. These findings suggest that CYP3A inhibitors alter the first-pass metabolism in the liver and/or intestine, but not systemic metabolism. Thus, it was confirmed that the drug–drug interaction that occurs *in vitro* is also observed *in vivo* after oral administration of midazolam to monkeys. This suggests that the monkey may be an appropriate surrogate for humans for assessing drug–drug interactions based on CYP3A inhibition.

In conclusion, the present study has shown that monkeys can serve as a useful *in vivo* model for predicting drug–drug interactions based on CYP3A inhibition. Such drug–drug interactions were observed in monkey with oral administration of midazolam, but not with intravenous administration. Even when ketoconazole, one of the most potent inhibitors of CYP3A, was co-administered, no drug–drug interaction could be detected in monkeys after intravenous administration of midazolam. These findings suggest that intravenous administration is not an appropriate administration route for evaluating the drug–drug interaction responsible for CYP3A enzyme inhibition. We thus recommend oral administration as an appropriate route for evaluating drug–drug interactions of new drug entities, which have a high plasma clearance. The CYP3A enzymes have also been reported to exist in the small intestine where they can contribute to drug metabolism (Thummel and Wilkinson 1998, Guengerich 1999). Thus, oral administration of midazolam is probably a more suitable route for assessing the drug–drug interaction including that in the intestine and liver. Evaluating drug–drug interactions using monkeys can offer important information before drug–drug interaction trials in Phase I or Phase II clinical studies, thus enabling safer clinical trials.

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Model for the drug–drug interaction responsible for CYP3A enzyme inhibition. II: Establishment and evaluation of dexamethasone-pretreated female rats

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1. Cytochrome P450 (CYP) 3A catalysis of testosterone 6 β -hydroxylation in female rat liver microsomes was significantly induced, then reached a plateau level after pretreatment with 80 mg kg⁻¹ day⁻¹ dexamethasone (DEX) for 3 days.

2. Midazolam was mainly metabolized by CYP3A in DEX-treated female rat liver microsomes from an immuno-inhibition study, and the apparent K_m was 1.8 μ M, similar to that in human microsomes.

3. Ketoconazole and erythromycin, typical CYP3A inhibitors, demonstrated extensive inhibition of midazolam metabolism in DEX-treated female rat liver microsomes, and the apparent K_i values were 0.088 and 91.2 μ M, respectively. The values were similar to those in humans, suggesting that DEX-treated female rat liver microsomes have properties similar to those of humans.

4. After oral administration of midazolam, the plasma midazolam concentration in DEX-treated female rats significantly decreased compared with control female rats. The area under the plasma concentration curve (AUC) and elimination half-life were one–11th and one–20th of those of control female rats, respectively.

5. Using DEX-treated female rats, the effect of CYP3A inhibitors on midazolam pharmacokinetics was evaluated. The AUC and maximum concentration in plasma (C_{max}) increased when ketoconazole was co-administered with midazolam.

6. It was shown that the drug–drug interaction that occurs *in vitro* is also observed *in vivo* after oral administration of midazolam. In conclusion, the DEX-treated female rat could be a useful model for evaluating drug–drug interactions based on CYP3A enzyme inhibition.

Introduction

To predict drug–drug interactions based on CYP3A enzyme inhibition with midazolam as a probe, the female monkey model is very useful (Kanazu *et al.* 2004). However, handling monkeys can be inconvenient and, also, large inter-individual variation occurs in drug metabolism and pharmacokinetics. This led the present authors to try to establish drug–drug interaction models using laboratory animals that can be more easily handled. For example, rats are widely used for preclinical absorption, distribution, metabolism and excretion (ADME) studies, and are easier to handle and rear than monkeys. However, large differences in drug metabolism have been reported between humans and rats. For example, the contents of CYP enzymes in rats are quite different from those in humans, there being an abundance of CYP3A enzymes in humans (Thummel and Wilkinson

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1998, Guengerich 1999). In rats, the CYP3A enzymes have a lesser impact on drug oxidation (Guengerich *et al.* 1982). The most abundant CYP enzymes in male rat liver is CYP2C11 (Guengerich *et al.* 1982), and therefore plays predominant role in drug oxidation in male rats.

The CYP3A enzymes can be relatively easily induced by pretreatment with dexamethasone (DEX), clotrimazole and phenobarbital (Corcos 1992, Kiritani and Matsubara 1993, Mahnke *et al.* 1996). However, even if CYP3A enzymes are induced in male rats by DEX treatment, the contribution of CYP2C enzymes to midazolam metabolism still exists. Moreover, it has been reported that there are discrepancies in the CYP enzymes responsible for metabolism between humans and rats (Nakamura *et al.* 1999, Ishigami *et al.* 2001, Kuperman *et al.* 2001). Some compounds metabolized by CYP3A enzymes in humans are metabolized by CYP2C enzymes in rats. For instance, simvastatin is metabolized mainly by CYP3A4 in humans (Vickers *et al.* 1990, Prueksaritanont *et al.* 1997), but by CYP2C11 in male rats (Ishigami *et al.* 2001) and by CYP3A in female rats. In addition, other new drug entities are also metabolized by human CYP3A4 and rat CYP2C11 (Nakamura *et al.* 1999, Kuperman *et al.* 2001). Thus, the use of male rats might not be suitable for predicting drug–drug interactions based on CYP3A enzyme inhibition. On the other hand, CYP2C enzymes in female rats have a lesser impact on drug metabolism than in male rats, and the content of CYP3A in DEX-treated female rat liver microsomes can be increased to levels similar to those of human liver microsomes. It was therefore decided to use female rats to avoid a discrepancy between humans and rats as to the metabolic enzyme(s) involved in the metabolism under study.

The present work tried to establish a drug–drug interaction model using DEX-treated female rats. It also evaluated the DEX-treated female rat used for predicting drug–drug interactions based on CYP3A inhibition. Midazolam was used as a probe in the present study because it is metabolized by CYP3A4 in humans and by CYP3A1 and/or CYP3A2 in male rats (Ghosal *et al.* 1996a). Ketoconazole, erythromycin and diltiazem were used as typical inhibitors of CYP3A.

Materials and methods

Chemicals

Midazolam and ketoconazole were synthesized at the Discovery Research Laboratories, Shionogi & Co., Ltd. and chemical purity of midazolam and ketoconazole were 99.9 and 99.1%, respectively. Erythromycin, diazepam, diltiazem hydrochloride and dexamethasone were from Wako Pure Chemicals (Osaka, Japan). *n*-Hexane, dichloromethane and ethylacetate for gas chromatography assay grade were obtained from Nacalai Tesque (Kyoto, Japan). Other reagents used were of the highest or high-performance liquid chromatography (HPLC) grade.

Animals

The female rats used were JCL:SD strain at 10 weeks old (CLEA Japan, Tokyo) weighing 197–251 g. All procedures for the animal experiments were approved by the local committee for animal care and use.

Preparation of microsomes

Female rats were given DEX at a single daily dose of 20, 50, 80, 100 and 150 mg kg⁻¹ day⁻¹ for 4 days by intraperitoneal injection. On the next day of the last administration of DEX, the rat livers were removed and liver microsomes were prepared by the method of Matsubara *et al.* (1983).

Microsomal incubation

Testosterone 6 β -hydroxylation activity was assayed by the method of Imaoka *et al.* (1988). Midazolam metabolism activity was assayed using the method of Kanazu *et al.* (2004) and represents the decreased amount of midazolam. Briefly, the reaction mixture consisted of 0.1 mol l⁻¹ Tris-HCl, pH 7.4, 10 mmol l⁻¹ MgCl₂, liver microsomes and midazolam. The total volume was 1.0 ml. The reaction was started by addition of 1 mmol l⁻¹ NADPH and incubated for the designated period at 37°C. The reaction was terminated by addition of 20 μ l of 2 mol l⁻¹ NaOH into the reaction mixture, followed by cooling in ice water for 15 min.

In immuno-inhibition studies, the incubations were carried out with anti-CYP antisera against CYP1A2, CYP2B1, CYP2C6, CYP2C11 and CYP3A2. These antisera were prepared in the present authors' laboratories. Anti-CYP3A2, CYP2C11 and CYP2B1 antisera selectively inhibited testosterone 6 β -hydroxylation (> 90%), 2 α -hydroxylation (> 80%), 16 β -hydroxylation (approximately 70%) activities in female rat liver microsomes treated with DEX at 80 mg kg⁻¹ day⁻¹ for 4 days, respectively. Anti-CYP1A1 antiserum selectively inhibited ethoxyresorfin *O*-deethylation (approximately 70%) in β -naphthoflavone (100 mg kg⁻¹ day⁻¹ for 4 days) pretreated male rat liver microsomes. Moreover, anti-CYP2C11 antiserum did not affect testosterone 6 β -hydroxylation activity. Antiserum was added to the reaction mixture, which was then left at room temperature for 30 min, after which the reaction was initiated by addition of NADPH. The subsequent procedure was the same as described above.

Preparation of dosage

The compounds (ketoconazole, erythromycin, diltiazem, DEX and midazolam) for oral administration were ground in an agate mortar in 0.5% methylcellulose solution. The concentrations of ketoconazole, erythromycin, diltiazem and midazolam were 2.5, 7.5, 2.5 and 2.5 mg ml⁻¹, respectively. The concentrations of DEX were 10, 25, 40, 50 and 75 mg ml⁻¹.

In vivo studies

Three days before the day of the administration study, rats were given an indwelling cannula (silicone rubber/polyethylene) implanted in the right jugular vein for blood sampling under light anaesthesia with ether. DEX was administered to female rats at a daily dose of 80 mg kg⁻¹ day⁻¹ for 3 days. At 24 h after the last administration of DEX, CYP3A inhibitors were given orally to the DEX-treated female rats. At 1 h after the oral administration of CYP3A inhibitors, midazolam was administered to the DEX-treated female rats. The control group rats were given 0.5% methylcellulose solution instead of the CYP3A inhibitor suspension. The doses of the CYP3A inhibitors were 5 mg kg⁻¹ ketoconazole, 15 mg kg⁻¹ erythromycin and 5 mg kg⁻¹ diltiazem. Blood samples (approximately 0.3 ml) were collected at 5, 10, 20, 30, 45 min, 1, 1.5, 2 and 4 h. The blood samples were immediately centrifuged to obtain plasma samples. After each sampling, 0.3 ml of an aqueous heparin solution (20 units ml⁻¹) was given at every collection time to the rats via a jugular vein cannula. The plasma samples were kept at around -20°C until analysis.

Midazolam analysis

Midazolam concentration was determined by the method of Kanazu *et al.* (2004). An appropriate volume of plasma (0.1 ml), 0.4 ml blank plasma, midazolam standard solution and an internal standard (diazepam, 5 ng) solution were shaken for 15 min with an appropriate volume of 0.5 mol l⁻¹ Na₂HPO₄ (pH 9) and 2.5 ml *n*-hexane/dichloromethane (70:30, v/v). The aqueous layer was discarded and the organic layer evaporated. The residue was dissolved in 50 μ l ethylacetate and 2 μ l of the solution was subjected into gas chromatography-electron capture detection.

The midazolam concentration in *in vitro* study was determined by an HPLC-ultraviolet light method (Carrillo *et al.* 1996). The reaction mixtures containing an internal standard were extracted with 2.5 ml chloroform. The aqueous layer was discarded and the organic layer was evaporated. The residue was dissolved in 100 μ l mobile phase and 20 μ l of the solution were subjected into HPLC-ultraviolet light. The column was Symmetry C₈ (150 $\times$ 4.6 mm i.d.; Waters, Milford, MA, USA). The mobile phase was acetonitrile:0.1 mol l⁻¹ acetate buffer, pH 4.7 (40:60, v/v) at a flow rate of 1 ml min⁻¹, and monitored at 230 nm. Midazolam was detected at the retention time of 12.0 min, diazepam, which was used as an internal standard, was observed at the retention time of 14.8 min.

Data analysis

The inhibition type was evaluated from the relationship of metabolic activity and the initial concentration of the substrate ([S]₀) calculated using the Lineweaver-Burk plot. The apparent values of the inhibition constant (*K_i*) were calculated by fitting the data to the inhibition type using the non-linear least-squares method program with WinNonlin® (Pharsight, Mountain View, CA, USA).

Pharmacokinetic parameters were estimated using WinNonlin. Plasma concentrations of midazolam after oral and intravenous administration were analysed by non-compartment analysis. The *C*_{max} of midazolam and the time to reach *C*_{max} (*T*_{max}) were used as the observed data. The mean residence time (MRT), total clearance (CL_{tot}/*F* or CL_{tot}) and distribution volume (*V*_d) were calculated using the

following equations:

$$\begin{aligned} \text{MRT} &= \text{AUMC}_{0-4\text{h}} / \text{AUC}_{0-4\text{h}} \\ \text{CL}_{\text{tot}} / F \text{ or } \text{CL}_{\text{tot}} &= \text{dose} / \text{AUC}_{0-4\text{h}} \\ V_{\text{d}} &= \text{MRT} \times \text{CL}_{\text{tot}} \end{aligned}$$

Results

Effect of dexamethasone on CYP3A enzyme induction: in vitro analysis

To investigate the effect of DEX on CYP3A enzyme activity, female rats were administered DEX at a dose of 20, 50, 80, 100 or 150 mg kg⁻¹ for 3 days or 80 mg kg⁻¹ for 1, 2, 3 or 4 days. At 24 h after the last administration, the rats were killed and the female rat liver microsomes prepared. Testosterone 6 β -hydroxylation activity of rat liver microsomes significantly increased on pretreatment with DEX, and the activity reached a plateau level by pretreatment with 80 mg kg⁻¹ day⁻¹ for 3 days (figures 1 and 2).

Midazolam metabolism in DEX-treated female rat liver microsomes

Midazolam was metabolized by DEX-pretreated female rat liver microsomes with an apparent K_m for midazolam of 1.8 μ M, which is similar to that in humans (approximately 5 μ M, Gascon and Dayer 1991). Midazolam metabolism was inhibited by addition of anti-CYP3A antiserum, suggesting that it was metabolized by CYP3A isoforms (figure 3). Also, the metabolism was inhibited by typical CYP3A chemical inhibitors, such as ketoconazole, erythromycin and diltiazem. Ketoconazole demonstrated extensive inhibition of midazolam metabolism by DEX-treated female rat liver microsomes, and the apparent K_i = 0.088 μ M with non-competitive inhibition. Erythromycin also inhibited midazolam metabolism competitively, and the apparent K_i = 91.2 μ M. The apparent K_i values are similar to those in humans (Gascon and Dayer 1991, Wrighton and Ring 1994).

Midazolam pharmacokinetics in control and DEX-treated female rats

Midazolam was administered to control and DEX-treated female rats, and the estimated pharmacokinetic parameters were compared between control and DEX-treated female rats (table 1). Plasma concentration profiles following

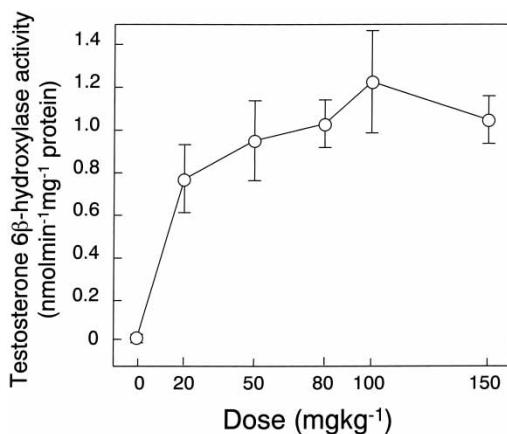


Figure 1. Effect of dexamethasone dose on the induction of hepatic testosterone 6 β -hydroxylase activity in female Sprague-Dawley rats. Values are the mean $\pm$ SD of four rats.

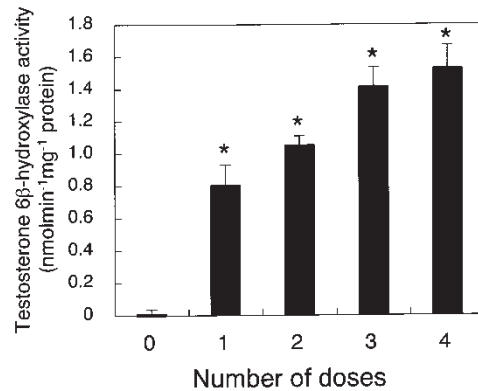


Figure 2. Effect of the number of administration times of dexamethasone on the induction of hepatic testosterone 6β-hydroxylase activity in female Sprague-Dawley rats. Values are the mean $\pm$ SD of four preparations. * $p < 0.05$, significantly different from the control by a Student's t -test.

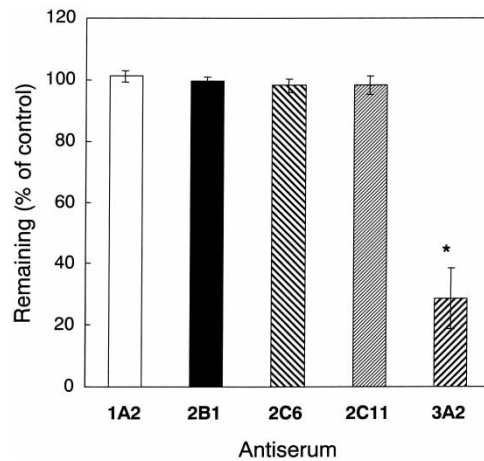


Figure 3. Effect of anti-CYP sera on midazolam metabolism in dexamethasone-pretreated female rat liver microsomes. The substrate decreased were measured by HPLC. Values are the mean $\pm$ SD of four preparations. The absolute value of 100% of control was 3.57 ± 0.19 nmol min⁻¹ mg⁻¹ protein. * $p < 0.05$, significantly different from the control by a student's t -test.

Table 1. Pharmacokinetic parameters estimated from the plasma concentration of midazolam after oral administration of midazolam to control and dexamethasone-treated female Sprague-Dawley rats.

	$C_{\max}$ (ng ml ⁻¹)	$T_{\max}$ (h)	$T_{1/2}$ (h)	AUC_{0-4h} (ng h ml ⁻¹)	CL_{tot}/F (l h ⁻¹ kg ⁻¹)	MRT (h)
Control	1190 $\pm$ 610	0.292 $\pm$ 0.159	10.5 $\pm$ 15.9	1858 $\pm$ 1205	16.9 $\pm$ 13.9	1.55 $\pm$ 0.31
DEX-treated	190 $\pm$ 77*	0.250 $\pm$ 0.204	0.559 $\pm$ 0.290	165 $\pm$ 76*	137 $\pm$ 48*	0.953 $\pm$ 0.374

Values are the mean $\pm$ SD of four rats.

* $p < 0.05$, significantly different from the control by Student's t -test.

administration of midazolam at the dose of 20 mg kg⁻¹ to control and DEX-treated female rats are shown in figure 4. The plasma concentration profile of DEX-treated female rats was significantly lower and midazolam concentrations decreased faster than in control female rats. The $C_{\max}$ values in control and

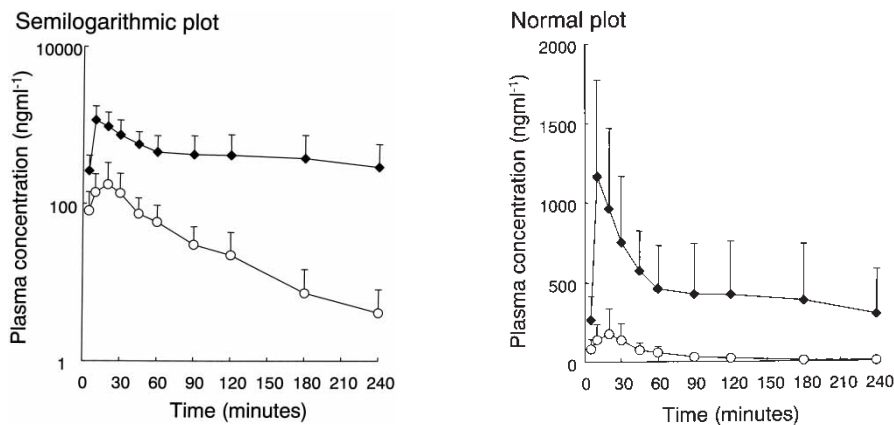


Figure 4. Plasma concentration of midazolam after oral administration of 20 mg kg^{-1} midazolam to control (closed diamonds) and dexamethasone-treated female Sprague-Dawley rats (open circles). Values are the mean $\pm$ SD of four rats.

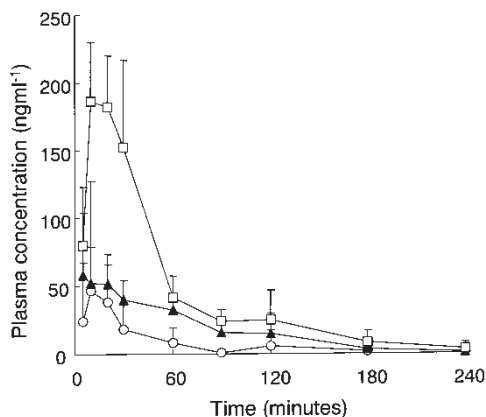


Figure 5. Plasma concentration of midazolam after oral administration of midazolam to dexamethasone-pretreated female rats. The doses of midazolam were 5 mg kg^{-1} (open circles), 10 mg kg^{-1} (closed triangles) and 20 mg kg^{-1} (open squares). Values are the mean $\pm$ SD of three rats.

DEX-treated female rats were 1190 ± 610 and $190 \pm 77 \text{ ng ml}^{-1}$, respectively; $C_{\max}$ in DEX-treated female rats was one-sixth of that in control rats. $\text{AUC}_{0-4\text{h}}$ values in control and DEX-treated female rats were 1858 ± 1205 and $171 \pm 72 \text{ ng h ml}^{-1}$, respectively; the value in DEX-treated female rats was one-11th of that in control rats. The elimination half-life in DEX-treated female rats ($0.559 \pm 0.290 \text{ h}$) was almost one-20th of that in control rats ($10.5 \pm 16.9 \text{ h}$). Thus, the results show that the pharmacokinetic parameters of midazolam in the female Sprague-Dawley strain rats were altered by pretreatment with DEX.

Dose-linearity of midazolam pharmacokinetics

$\text{AUC}_{0-4\text{h}}$ values in DEX-treated female rats after oral administration of midazolam at a dose of 5, 10 and 20 mg kg^{-1} were 39.8 ± 21.8 , 73.2 ± 18.6 and $173 \pm 18 \text{ ng h ml}^{-1}$, respectively (figure 5). The result suggests that an oral dose of 20 mg kg^{-1} of midazolam in rats is in the dose-linearity range.

Effect of CYP3A inhibitors on midazolam pharmacokinetics in DEX-treated female rats

To evaluate the effect of CYP3A inhibitors (ketoconazole, erythromycin and diltiazem) in DEX-treated female rats, midazolam was administered with or without administration of inhibitors. At 1 h after each CYP3A inhibitor had been administered orally, midazolam was orally administered to DEX-treated female rats, and blood samples collected at the designated times. The plasma midazolam concentration profiles are shown in figure 6 and the pharmacokinetic parameters are summarized in table 2. AUC and $C_{\max}$ increased 1.47- and 1.63-fold, respectively, and the elimination half-life of midazolam was increased by ketoconazole treatment (1.67 ± 0.71 h for the ketoconazole treatment group and 0.695 ± 0.323 h for the

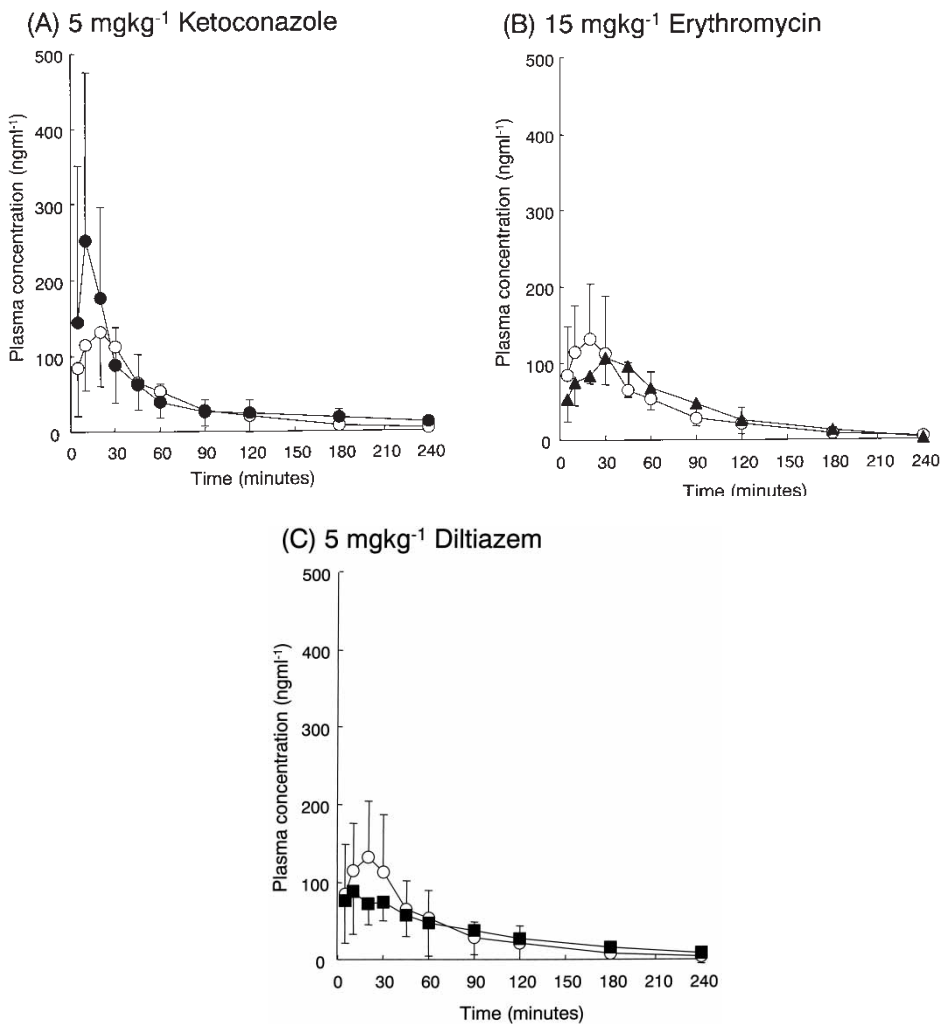


Figure 6. Effect of ketoconazole (A), erythromycin (B) and diltiazem (C) on the plasma concentration of midazolam after oral co-administration of midazolam and CYP3A inhibitor to dexamethasone-treated female Sprague-Dawley rats. The groups are the control group (open circles, $n=9$), ketoconazole treatment group (closed circles, $n=4$), erythromycin treatment group (closed triangles, $n=4$) and diltiazem treatment group (closed squares, $n=4$). Values are the mean $\pm$ SD.

Table 2. Pharmacokinetic parameters estimated from the plasma concentration of midazolam after oral administration of midazolam with or without ketoconazole, erythromycin and diltiazem to dexamethasone-treated female Sprague–Dawley rats.

	$C_{\max}$ (ng ml ⁻¹)	$T_{\max}$ (h)	$T_{1/2}$ (h)	AUC_{0-4h} (ng h ml ⁻¹)	CL_{tot}/F (l h ⁻¹ kg ⁻¹)	MRT (h)
Control	174 ± 56	0.306 ± 0.298	0.695 ± 0.323	136 ± 70	146 ± 37	1.13 ± 0.42
Ketoconazole	284 ± 223	0.188 ± 0.105	1.67 ± 0.71*	201 ± 77	112 ± 44	2.10 ± 1.11*
Erythromycin	113 ± 28	0.479 ± 0.239	0.682 ± 0.133	160 ± 68	148 ± 74	1.13 ± 0.29
Diltiazem	119 ± 35	0.333 ± 0.446	0.807 ± 0.563	146 ± 60	154 ± 58	1.62 ± 0.70

Values are the mean ± SD ($n=9$ for control group and $n=4$ for ketoconazole, erythromycin and diltiazem treatment groups).

* $p < 0.05$, significantly different from the control by Student's t -test.

control group). However, no significant change of pharmacokinetic parameters of midazolam was observed after treatment with erythromycin or diltiazem (table 2).

Discussion

Enzyme inhibition studies have been widely conducted at the drug discovery stage, and there are a number of reports of methods to predict drug–drug interactions *in vivo* from *in vitro* data (Iwatsubo *et al.* 1997, Ito *et al.* 1998, Moltke *et al.* 1998). However, *in vitro* enzyme inhibition data sometimes lead to an incorrect prediction of drug–drug interactions *in vivo* (Ito *et al.* 1998). Thus, in cases of new drug entities displaying excellent pharmacological activity but extensive ($<1\mu\text{M}$ of K_i) or moderate enzyme inhibition ($1\text{--}10\mu\text{M}$ for the K_i), it is difficult to decide whether or not to conduct drug development from only *in vitro* information.

To resolve this issue, it is suggested that making predictions based on an *in vivo* drug–drug interaction animal model might offer important information for making such decisions. Previously, we have reported an *in vivo* drug–drug interaction model using the cynomolgus monkey (Kanazu *et al.* 2004). However, the throughput of a monkey study is relatively low and the handling of monkeys is considered to be inconvenient. Thus, we tried to establish an *in vivo* assessment system for predicting drug–drug interactions using rats, which allows a much higher throughput and more facile care of the research animals. However, there are substantial species differences in drug metabolism between rats and humans (Guengerich *et al.* 1982). CYP3A enzymes play major roles in oxidative metabolism in humans but in male rats, CYP2C enzymes are abundant and play an important role in the oxidative metabolism (Guengerich *et al.* 1982). Moreover, there are some differences as to the enzymes responsible for metabolic activities in humans and rats. For example, simvastatine is metabolized by CYP2C11 in male rats but by CYP3A4 in humans (Vickers *et al.* 1990, Prueksaritanont *et al.* 1997). Rat liver CYP3A enzymes have been reported to be induced by pretreatment with DEX (Corcos 1992, Kirita and Matsubara 1993, Ghosal *et al.* 1996a). However, even if CYP3A enzymes are increased by pretreatment with DEX, CYP2C11 still substantially contributes to drug oxidation in male rats (Ghosal *et al.* 1996b). On the other hand, in female rats, CYP2C enzymes have a lesser role to play in drug metabolism, and CYP3A enzymes are present in only trace amounts (Imaoka *et al.* 1991). Thus, CYP3A enzymes in the female rat are substantially induced by DEX

treatment to reach levels close to those in humans (Ghosal *et al.* 1996b). We thus attempted to establish a model for drug–drug interactions using female rats.

CYP3A activity against testosterone 6 β -hydroxylation was significantly increased by DEX treatment, and the activity reached a plateau level on pretreatment with 80 mg kg⁻¹ day⁻¹ for 3 days. Although the activity was increased when DEX was administered to female rats at a dose of 80 mg kg⁻¹ day⁻¹ for 4 days or 100 and 150 mg kg⁻¹ day⁻¹ for 3 days, the body weight of female rats decreased. Therefore, DEX treatment at 80 mg kg⁻¹ day⁻¹ for 3 days should be an appropriate period of administration. To study the properties of DEX-treated female rat liver microsomes, we conducted an enzyme kinetic and immuno-inhibition study of midazolam metabolism. Metabolism was inhibited by addition of anti-CYP3A2 antiserum, suggesting that it was metabolized mainly by CYP3A enzyme(s). The apparent K_m for midazolam (1.8 μ M) was similar to that in humans (approximately 5 μ M, Gascon and Dayer 1991). The K_i values were estimated by fitting the data to the inhibition type using a non-linear least-squares method program. The estimated apparent K_i values against ketoconazole and erythromycin were similar to those for human liver microsomes. Thus, these results show that DEX-treated female liver microsomes display similar properties to that of human liver microsomes, suggesting that DEX-treated female rat microsomes could be used for predicting the drug–drug interaction responsible for CYP3A enzyme inhibition in humans. It may also be possible to use these DEX-treated female rats instead of monkeys to predict the drug–drug interactions responsible for CYP3A inhibition.

Midazolam was administered to control and DEX-treated female rats, and the pharmacokinetic parameters of midazolam in these rats were estimated. After oral administration of midazolam to control rats, it was absorbed rapidly, but the elimination from plasma was quite slow, with an elimination half-life of approximately 11 h. In the case of DEX-treated female rats, the elimination half-life of midazolam was only 0.7 h. In humans, the elimination half-life of midazolam has been reported to be 2.1 ± 0.5 h (Allonen *et al.* 1981). Thus, the DEX-treated female rats seem to be much closer to humans than control female rats, and it should be possible to use the former for predicting drug–drug interaction in humans.

To evaluate DEX-treated female rats for predicting the drug–drug interactions responsible for CYP3A inhibition, we investigated the effect of CYP3A inhibitors on midazolam pharmacokinetics. When ketoconazole was administered before oral administration of midazolam to DEX-treated female rats, the AUC and C_{max} of midazolam increased and the elimination half-life also increased. However, no change in the pharmacokinetic parameters of midazolam was observed by the treatment with erythromycin and diltiazem. Ketoconazole has a low apparent K_i , but erythromycin and diltiazem have high apparent K_i values. Thus, the K_i is thought to be one of the factors leading to differences in drug–drug interactions among the CYP3A inhibitors used in the current study. Multiple administration of erythromycin has been reported to cause an increase in the AUC of midazolam (Olkola *et al.* 1993, Takedomi *et al.* 2000). Single administration of erythromycin did not cause a change in the elimination half-life of midazolam (Takedomi *et al.* 2000). Erythromycin is metabolized to produce a metabolic intermediate, which binds to CYP enzymes and forms stable and inactive complexes (Delaforge *et al.* 1983). Diltiazem is also known to produce a metabolite intermediate complex

between CYP enzymes. (Sutton *et al.* 1997, Jones *et al.* 1999, Ma *et al.* 2000, Trieu and Murray 2000). Therefore, using DEX-treated female rats with oral administration of midazolam as a probe in experiments of multiple pretreatment with erythromycin and diltiazem likely demonstrates a drug–drug interaction caused by mechanism based inhibition.

In conclusion, it has been shown that DEX-treated female rats with midazolam as a probe might be a useful model for evaluating drug–drug interactions responsible for CYP3A enzyme inhibition. Predictions using DEX-treated female rats should offer important information for making and supporting decisions in drug development. As a drug–drug interaction study using rats can be conducted with relatively high throughput, this assessment system should be useful at the drug discovery stage.

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Identification and relative contributions of human cytochrome P450 isoforms involved in the metabolism of glibenclamide and lansoprazole: evaluation of an approach based on the *in vitro* substrate disappearance rate

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1. The identification and relative contributions of human cytochrome P450 (CYP) enzymes involved in the metabolism of glibenclamide and lansoprazole in human liver microsomes were investigated using an approach based on the *in vitro* disappearance rate of unchanged drug.
2. Recombinant CYP2C19 and CYP3A4 catalysed a significant disappearance of both drugs. When the contribution of CYPs to the intrinsic clearance (CL_{int}) of drugs in pooled human microsomes was estimated by relative activity factors, contributions of CYP2C19 and CYP3A4 were determined to be 4.6 and 96.4% for glibenclamide, and 75.1 and 35.6% for lansoprazole, respectively.
3. CL_{int} of glibenclamide correlated very well with CYP3A4 marker activity, whereas the CL_{int} of lansoprazole significantly correlated with CYP2C19 and CYP3A4 marker activities in human liver microsomes from 12 separate individuals. Effects of CYP-specific inhibitors and anti-CYP3A serum on the CL_{int} of drugs in pooled human liver microsomes reflected the relative contributions of CYP2C19 and CYP3A4.
4. The results suggest that glibenclamide is mainly metabolized by CYP3A4, whereas lansoprazole is metabolized by both CYP2C19 and CYP3A4 in human liver microsomes. This approach, based on the *in vitro* drug disappearance rate, is useful for estimating CYP identification and their contribution to drug discovery.

Introduction

Recently, drug metabolism and pharmacokinetic investigations have played an increasingly important role in drug discovery. Identification of cytochrome P450 (CYP) enzymes involved in candidate compound metabolism is important for pharmacokinetic studies. This is because such information has led to success in predicting potential drug–drug interactions, polymorphisms and the extent of interindividual differences in drug metabolism (Lin and Lu 1998).

CYPs are the principal drug metabolizing enzymes, and in human liver, five CYPs (1A2, 2C9, 2C19, 2D6 and 3A4) are primarily responsible for the metabolism of most drugs (Guengerich 1996).

Current methods used in CYP identification include the use of specific and inhibitory antibodies against human CYPs, the use of specific chemical inhibitors of CYPs, the use of a panel of recombinant CYPs and the estimation of correlation

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between the metabolic rate of the drug of interest and a marker activity for each CYP in individual human liver microsomes (Lu *et al.* 2003). In addition, Crespi (1995) proposed the relative activity factor (RAF), a parameter that relates metabolic activities in recombinant CYPs to those in human liver microsomes, to estimate the relative contribution of each CYP in the metabolism of a drug.

The identification and contributions of CYPs using the methods mentioned above generally have been evaluated by measuring the production of metabolites in a specific metabolic pathway. However, metabolic pathways of compounds are not sufficiently characterized in early drug discovery. Recently, Chiba and co-workers reported that an approach based on the disappearance rate of unchanged drug is useful in the identification of a major CYP involved in drug metabolism in human liver microsomes and suggested that CYP3A4 is a major enzyme involved in gallopamil, medroxyprogesterone acetate and clarithromycin metabolism (Suzuki *et al.* 1999, 2003, Kobayashi *et al.* 2000). This approach, based on the *in vitro* drug disappearance rate, might be useful in drug discovery; however, there have been only a few studies on the extent of the contribution of multiple CYPs using this approach.

Glibenclamide is a blood glucose-lowering drug widely used for the treatment of patients with type 2 diabetes mellitus. Recently, Van Giersbergen *et al.* (2002) studied the effects of CYP-specific inhibitors and a panel of recombinant CYPs in the metabolism of glibenclamide and suggested that glibenclamide is metabolized *in vitro* by CYP2C9, 2C19 and 3A4. However, they did not demonstrate the relative contributions of each CYP to the overall metabolism of glibenclamide.

Lansoprazole is a proton-pump inhibitor used for the treatment of acid-related gastric disorders. Pearce *et al.* (1996) reported that it is metabolized by CYP2C19 and CYP3A4. It is likely these CYPs contribute in specific proportions to the metabolism of lansoprazole because the major metabolites of lansoprazole (5-hydroxylansoprazole and lansoprazole sulfone) are formed by CYP2C19 and CYP3A4, respectively, at pharmacological concentration levels. However, the relative contribution of each CYP to the overall metabolism of lansoprazole has not determined.

The present study has identified the relative contributions of human CYPs involved in the metabolism of glibenclamide and lansoprazole using an approach based on *in vitro* disappearance rate of each parent drug. The RAF approach was also used to estimate their relative contributions. Further, the results of glibenclamide and lansoprazole were compared as model compounds mainly metabolized by one CYP and by more than two CYPs, respectively, and the utility of this approach based on *in vitro* substrate disappearance rate was confirmed.

Materials and methods

Chemicals

Glibenclamide was from Nacalai Tesque, Inc. (Kyoto, Japan). Lansoprazole, sulfaphenazole, quinidine and diethyldithiocarbamic acid were from Sigma Chemical Co. (St Louis, MO, USA). Furafylline, ketoconazole, *S*-(+)-mephénytoin, ($\pm$)-4'-hydroxymephénytoin, testosterone and 6 β -hydroxytestosterone were from Ultrafine Chemicals (Manchester, UK). *S*-($\pm$)-*N*-3-benzylirinanol was from BD Gentest (Woburn, MA, USA). Chemicals were of the highest grade commercially available. Anti-rat CYP3A2 rabbit serum was from Daiichi Pure Chemicals (Tokyo, Japan). The manufacturer stated that anti-rat CYP3A2 serum reacts only with human CYP3As and not other CYPs.

All other reagents and solvents used were of analytical or high-performance liquid chromatography (HPLC) grade.

Enzyme preparations

Microsomes from baculovirus-infected insect cells expressing CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (BD Gentest) were co-expressed with NADPH-CYP reductase. CYP2C9, 2C19, 2E1 and 3A4 were also co-expressed with cytochrome-b₅. CYP contents of these microsomes were provided in data sheets from the manufacturer. Human liver microsomes from 12 separate individuals (H003, H006, H023, H030, H042, H043, H056, H066, H070, H089, H093 and H112) and pooled human microsomes (H161) were purchased from BD Gentest.

Assays with human liver microsomes and recombinant human CYPs

The time course of parent glibenclamide or lansoprazole disappearance was investigated *in vitro*. A typical reaction mixture contained human liver microsomes or recombinant CYPs, an NADPH-generating system (2 mM NADP⁺, 10 mM glucose 6-phosphate, 0.4 U ml⁻¹ glucose 6-phosphate dehydrogenase and 5 mM MgCl₂), 100 mM potassium phosphate buffer, pH 7.4, and 1 µM glibenclamide or lansoprazole, in a final volume of 500 µl. The concentration of human liver microsomes used was 0.5 mg ml⁻¹. The concentration of recombinant CYP isoforms used was 20 or 40 pmol CYP ml⁻¹. After pre-incubation at 37°C for 5 min, enzyme reactions were initiated by addition of 5 µl glibenclamide or lansoprazole in methanol. After incubation at 37°C for various periods, the reactions were terminated by the addition of 500 µl acetonitrile. After stopping the metabolic reactions, the reaction mixtures were centrifuged at 10 000g for 5 min, and an aliquot of the supernatant was injected into an HPLC for the quantitation of unchanged compound concentration.

HPLC conditions

Determination of unchanged glibenclamide and lansoprazole was performed by HPLC analysis. The HPLC system used was an LC module I plus (Millipore Co., Milford, MA, USA). The column for analysis was an Inertsil ODS-3 (5 µm, 150 × 4.6 mm) (GL Science, Inc., Tokyo, Japan). The flow rate was 1.0 ml min⁻¹. The mobile phase and detection wavelength for the analysis of each drug were as follows. Glibenclamide, mobile phase: buffer A (5 mM phosphate buffer, pH 7.2)/acetonitrile (60/40), detection: ultraviolet light 225 nm; lansoprazole, mobile phase: buffer A/acetonitrile (55/45), detection: ultraviolet light 285 nm.

All assay methods were in the concentration range 0.1–2 µM. Reproducibility was evaluated by performing five replicate analyses of microsomal samples containing 0.1, 0.5 and 1 µM compound, respectively. The coefficient of variation (CV) was less than 5% and the actual concentration of the compounds ranged from 98.2 to 102%. All assay methods thus provide good accuracy and precision.

Calculation of intrinsic clearance (CL_{int})

CL_{int} values were calculated from the *in vitro* substrate disappearance rate as described (Naritomi *et al.* 2001, 2003). If substrate disappearance can be assumed to follow a first-order reaction, the unchanged drug profile as a function of time ($C(t)$) is described as follows:

$$C(t) = C_0 \bullet \exp(-k_e \bullet t), \quad (1)$$

where C_0 is the initial concentration of the compound and k_e is the disappearance rate constant of unchanged drug (min⁻¹).

Furthermore, the initial metabolic rate (V_0) (µmol min⁻¹ mg⁻¹ microsomal protein or pmol CYP) is as follows:

$$V_0 = k_e \bullet C_0 / P_{MS}, \quad (2)$$

where P_{MS} is the microsomal protein concentration (mg ml⁻¹) or CYP concentration (pmol ml⁻¹).

On the other hand, from the Michaelis–Menten equation, V_0 is described by the following:

$$V_0 = V_{max} \bullet C_0 / (K_m + C_0). \quad (3)$$

If the substrate concentration used in experiments (1 µM) is below the K_m for CYP-mediated reactions, the drug concentration can be assumed to be much smaller than K_m ($K_m \gg C_0$). Thus, V_0 can be expressed as follows:

$$V_0 = V_{max} / K_m \bullet C_0. \quad (4)$$

Consequently,

$$CL_{int} = V_{max} / K_m = V_0 / C_0. \quad (5)$$

CL_{int} was thus calculated by equation (5) based on the time course of unchanged drug concentrations by a least-square linear regression.

Contributions of CYP2C19 and CYP3A4 to the CL_{int} of glibenclamide and lansoprazole in human liver microsomes

Per cent contributions of CYP2C19 and CYP3A4 to CL_{int} of glibenclamide and lansoprazole in pooled human liver microsomes were estimated by application of RAF (Crespi 1995) using the ratio of CL_{int} of CYP marker activity (Nakajima *et al.* 1999). Marker activities used in this study were *S*-mephenytoin 4'-hydroxylase activity for CYP2C19 (Goldstein *et al.* 1994) and testosterone 6 β -hydroxylase activity for CYP3A4 (Waxman *et al.* 1988). The RAF for each CYP was determined as follows:

$$RAF_{CYPX} = \frac{CL_{int, marker} \text{ in human liver microsomes}}{CL_{int, marker} \text{ in recombinant CYP X}}, \quad (6)$$

where $CL_{int, marker}$ was estimated as the V_{max}/K_m of each marker activity.

The CL_{int} of glibenclamide and lansoprazole by each CYP in human liver microsomes ($CL_{int, CYPX}$) was estimated using equation (7):

$$CL_{int, CYPX} = CL_{int, rec-CYPX} \bullet RAF_{CYPX}, \quad (7)$$

where $CL_{int, rec-CYPX}$ is the CL_{int} of glibenclamide and lansoprazole in recombinant CYP2C19 or CYP3A4.

The contributions of each CYP to the CL_{int} of glibenclamide and lansoprazole in human liver microsomes were calculated using equation (8):

$$\text{Contribution of CYP X (\%)} = \frac{CL_{int, CYPX}}{CL_{int} \text{ in human liver microsomes}} \bullet 100. \quad (8)$$

S-mephenytoin 4'-hydroxylase activity was determined as follows: the incubation mixture contained 0.5 mg ml⁻¹ human liver microsomes or 20 pmol CYP ml⁻¹ recombinant CYP2C19, an NADPH-generating system, 100 mM potassium phosphate buffer, pH 7.4, and 5–500 μ M *S*-mephenytoin, in a final volume of 500 μ l. The reaction was started by incubating at 37°C. After incubation for 30 min, the reaction was terminated by the addition of 100 μ l 1 M HCl. A total of 100 μ l sodium phenobarbital (2 μ g ml⁻¹) was added and then extracted with 3 ml ethyl acetate/*n*-hexane (1/1). The organic fraction was evaporated under N₂ and the residue reconstituted in 5 mM KH₂PO₄ for HPLC analysis. HPLC analysis was performed using an LC module I plus (Millipore). The column for analysis was an Inertsil ODS-3 (5 μ m, 150 $\times$ 4.6 mm) (GL Science). The eluent was monitored at a wavelength of 204 nm. The mobile phase consisted of 5 mM KH₂PO₄/methanol (73/27), with a flow rate of 1.0 ml min⁻¹ and the column temperature was 45°C.

Testosterone 6 β -hydroxylase activity was determined as follows: the incubation mixture contained 0.1 mg ml⁻¹ human liver microsomes or 10 pmol CYP ml⁻¹ recombinant CYP3A4, an NADPH-generating system, 100 mM potassium phosphate buffer, pH 7.4, and 5–500 μ M testosterone, in a final volume of 500 μ l. The reaction was started by incubating at 37°C. After incubation for 5 min, the reaction was terminated by the addition of 3.5 ml ethyl acetate. The mixture was extracted. The organic fraction then evaporated under N₂. The residue was reconstituted in 50% methanol for HPLC analysis. The HPLC system and column were the same as described above and the eluent was monitored at a wavelength of 238 nm. The mobile phase was solvent A (methanol/H₂O, 1/4) and solvent B (methanol/acetonitrile, 10/1). The elution conditions were: 30% B (0–1 min), 30 to 38% B (1–18 min), 38 to 100% B (18–18.1 min), 100% B (18.1–20 min). A linear gradient was used for the solvent changes. The flow rate was 1.0 ml min⁻¹ and the column temperature was 50°C.

Correlation study

The CL_{int} values for glibenclamide and lansoprazole were correlated with the marker activities of CYPs for human liver microsomes from 12 separate individuals. Marker activities for CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 used were phenacetin *O*-deethylation (Tassaneeyakul *et al.* 1993), coumarin 7-hydroxylation (Yun *et al.* 1991), *S*-mephenytoin *N*-demethylation (Heyn *et al.* 1996), paclitaxel 6 α -hydroxylation (Rahman *et al.* 1994), diclofenac 4'-hydroxylation (Leemann *et al.* 1993), *S*-mephenytoin 4'-hydroxylation (Goldstein *et al.* 1994), bufuralol 1'-hydroxylation (Boobis *et al.* 1985), chlorzoxazone 6-hydroxylation (Peter *et al.* 1990) and testosterone 6 β -hydroxylation (Waxman *et al.* 1988), respectively. The marker activity of each CYP in human liver microsomes used was provided in the data sheets by the manufacturer.

Inhibition study

The effects of CYP-specific inhibitors on the CL_{int} values for glibenclamide and lansoprazole were evaluated using pooled human liver microsomes. Inhibitors used for CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 were furafylline (Tassaneeyakul *et al.* 1993), sulfaphenazole (Baldwin *et al.* 1995), *S*-(±)-*N*-3-benzyl-nirvanol (Suzuki *et al.* 2002, Walsky and Obach 2003), quinidine (Broly *et al.* 1989), diethyl-dithiocarbamic acid (Guengerich *et al.* 1991) and ketoconazole (Baldwin *et al.* 1995), respectively. Concentrations of inhibitors used were 0.1, 1 and 10 μ M. After adding substrate and inhibitor to the incubation mixtures, enzyme reactions were carried out as described above.

Immuno-inhibition study

The immuno-inhibition of the CL_{int} values for glibenclamide and lansoprazole was examined by pre-incubation of pooled human liver microsomes with various concentrations of anti-rat CYP3A2 rabbit serum at room temperature for 1 h. The mixtures of microsomes and anti-rat CYP3A2 serum were added to incubation medium containing the substrate, and reactions were carried out as described above. Pre-immune rabbit serum was added to maintain a constant serum concentration.

Data analysis

K_m and V_{max} values were estimated by non-linear least-squares regression analysis applying the Michaelis-Menten equation. Correlation coefficients (r) were determined by least-squares linear regression analysis.

Results

Metabolism by recombinant CYP isoforms

Table 1 shows the disappearance of glibenclamide and lansoprazole in microsomes from baculovirus-infected insect cells expressing CYPs. CYP2C19 and CYP3A4 catalysed significant disappearance of both compounds. The disappearance of glibenclamide by CYP3A4 was more extensive than that by CYP2C19. In contrast, the disappearance of lansoprazole by CYP2C19 was substantially greater than that by CYP3A4.

CL_{int} of glibenclamide and lansoprazole in recombinant CYP2C19 and CYP3A4, and pooled human liver microsomes

Figure 1 shows the time courses for unchanged drug concentrations of glibenclamide and lansoprazole in mixtures containing recombinant CYP2C19, CYP3A4 or pooled human liver microsomes. The unchanged drug profiles at 1 μ M showed that the linear log concentration declines, indicating that overall metabolism follows a first-order reaction under these conditions. The CL_{int} values

Table 1. Metabolism of glibenclamide and lansoprazole by recombinant human CYP enzymes expressed in baculovirus-infected insect cells.

	Glibenclamide	Lansoprazole
Control	101 $\pm$ 1	98.5 $\pm$ 0.8
CYP1A2	97.3 $\pm$ 8.4	93.5 $\pm$ 7.2
2A6	101 $\pm$ 1	98.8 $\pm$ 3.0
2B6	99.6 $\pm$ 1.0	98.3 $\pm$ 1.2
2C8	91.0 $\pm$ 15.8	98.1 $\pm$ 0.9
2C9	99.8 $\pm$ 0.5	98.9 $\pm$ 2.0
2C19	79.1 $\pm$ 0.5*	N.D.*
2D6	101 $\pm$ 1	101 $\pm$ 3
2E1	102 $\pm$ 1	101 $\pm$ 4
3A4	18.1 $\pm$ 0.6*	71.2 $\pm$ 3.6*

Glibenclamide and lansoprazole (1 μ M) were incubated at 37°C for 60 min with microsomes from baculovirus-infected insect cells expressing CYP isoforms (40 pmol CYP ml⁻¹). Data are the percentages of the initial concentration of the compounds (data are the mean $\pm$ SD of three experiments). * p < 0.01.

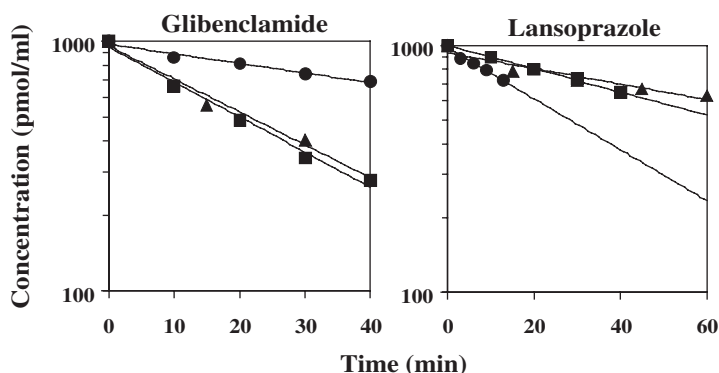


Figure 1. Time course of unchanged drug concentrations of glibenclamide and lansoprazole with recombinant CYP2C19 and CYP3A4, and pooled human liver microsomes. Glibenclamide and lansoprazole ($1\text{ }\mu\text{M}$) were incubated for various times at 37°C in a microsome mixture from baculovirus-infected insect cells expressing CYP2C19 (glibenclamide, $40\text{ pmol CYP ml}^{-1}$; lansoprazole, $20\text{ pmol CYP ml}^{-1}$) and 3A4 (glibenclamide, $20\text{ pmol CYP ml}^{-1}$; lansoprazole, $40\text{ pmol CYP ml}^{-1}$), and in pooled human microsomes (H161, 0.5 mg ml^{-1}). $\bullet$, CYP2C19; $\blacksquare$, CYP3A4; $\blacktriangle$, pooled human microsomes. The solid lines represent linear regression lines by the least-squares method. Data are the average of duplicate determinations.

for glibenclamide in recombinant CYP2C19, CYP3A4 and pooled human liver microsomes were 0.23 , $1.6\text{ }\mu\text{l min}^{-1}\text{ pmol}^{-1}\text{ CYP}$ and $67.2\text{ }\mu\text{l min}^{-1}\text{ mg}^{-1}\text{ microsomal protein}$, respectively. The CL_{int} values for lansoprazole in recombinant CYP2C19, CYP3A4 and pooled human liver microsomes were 1.21 , $0.19\text{ }\mu\text{l min}^{-1}\text{ pmol}^{-1}\text{ CYP}$ and $21.6\text{ }\mu\text{l min}^{-1}\text{ mg}^{-1}\text{ microsomal protein}$, respectively.

Contributions of CYP2C19 and CYP3A4 to the CL_{int} of glibenclamide and lansoprazole in pooled human microsomes

The contributions of CYP2C19 and CYP3A4 to the CL_{int} of glibenclamide and lansoprazole in mixtures of pooled human microsomes were determined by the RAF method, as described above. The kinetic parameters of *S*-mephenytoin 4'-hydroxylation (a CYP2C19 marker activity) for recombinant CYP2C19 and pooled human liver microsomes were as follows: recombinant CYP2C19, K_{m} : $51.1\text{ }\mu\text{M}$, V_{max} : $4.75\text{ pmol min}^{-1}\text{ pmol}^{-1}\text{ CYP}$; pooled human liver microsomes, K_{m} : $66.6\text{ }\mu\text{M}$, V_{max} : $83.2\text{ pmol min}^{-1}\text{ mg}^{-1}\text{ microsomal protein}$. Kinetic parameters of testosterone 6β -hydroxylation (a CYP3A4 marker activity) for recombinant CYP3A4 and pooled human liver microsomes were as follows: recombinant CYP3A4, K_{m} : $27.6\text{ }\mu\text{M}$, V_{max} : $110\text{ pmol min}^{-1}\text{ pmol}^{-1}\text{ CYP}$; pooled human liver microsomes, K_{m} : $57.2\text{ }\mu\text{M}$, V_{max} : $9230\text{ pmol min}^{-1}\text{ mg}^{-1}\text{ microsomal protein}$. Consequently, RAFs were 13.4 and $40.5\text{ (mg}^{-1}\bullet\text{CYP)}$ for CYP2C19 and CYP3A4, respectively. Based on the RAF and CL_{int} values in recombinant CYPs and pooled human microsomes, the relative contributions of CYP2C19 and CYP3A4 to the CL_{int} of glibenclamide and lansoprazole in pooled human microsomes were estimated (figure 2). The contributions of CYP2C19 and CYP3A4 for glibenclamide were 4.6 and 96.4% , respectively. In contrast, the contributions of CYP2C19 and CYP3A4 for lansoprazole were 75.1 and 35.6% , respectively. The sum of contributions of CYP2C19 and CYP3A4 to the CL_{int} of each compound in pooled human microsomes was almost 100% .

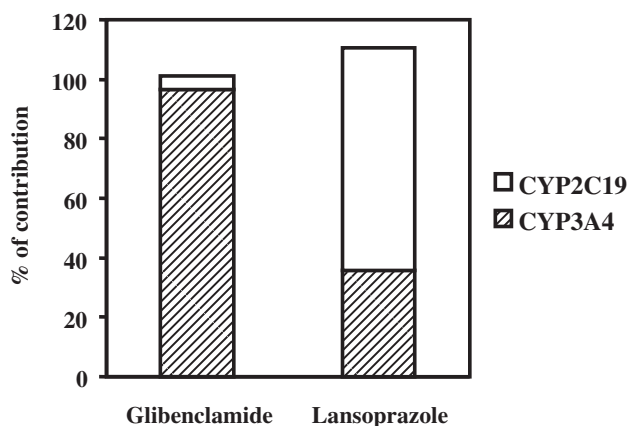


Figure 2. Contribution of CYP2C19 and CYP3A4 to the CL_{int} of glibenclamide and lansoprazole estimated by relative activity factor in pooled human microsomes.

Table 2. Correlations between the CL_{int} of glibenclamide or lansoprazole and different CYP marker activities in 12 human liver microsomes.

Marker activity	CYP	Correlation coefficient (<i>r</i>)	
		Glibenclamide	Lansoprazole
Phenacetin <i>O</i> -deethylation	CYP1A2	-0.104	0.105
Coumarin 7-hydroxylation	CYP2A6	-0.035	-0.283
<i>S</i> -mephenytoin <i>N</i> -demethylation	CYP2B6	0.804*	0.257
Paclitaxel 6 α -hydroxylation	CYP2C8	0.333	0.020
Diclofenac 4'-hydroxylation	CYP2C9	0.319	0.563
<i>S</i> -mephenytoin 4'-hydroxylation	CYP2C19	0.420	0.827**
Bufuralol 1'-hydroxylation	CYP2D6	-0.311	-0.324
Chlorzoxazone 6-hydroxylation	CYP2E1	0.298	0.281
Testosterone 6 β -hydroxylation	CYP3A4	0.971**	0.666*

* $p < 0.05$, ** $p < 0.001$.

Correlation study

Correlations between the CL_{int} values for glibenclamide and lansoprazole and the marker activities of CYPs were studied for human microsomes from 12 separate individuals. The results are summarized in table 2, where r and p are quoted for each pair of data. The CL_{int} of glibenclamide exhibited an excellent correlation with testosterone 6 β -hydroxylation, a marker activity for CYP3A4 ($r = 0.971$, $p < 0.001$), in human liver microsomes from 12 separate individuals. However, the CL_{int} of glibenclamide exhibited no significant correlation with *S*-mephenytoin 4'-hydroxylation, a marker activity of CYP2C19 ($r = 0.420$) (figure 3 and table 2). Although the CL_{int} of glibenclamide also significantly correlated with *S*-mephenytoin *N*-demethylation, a marker activity for CYP2B6 ($r = 0.804$, $p < 0.05$), there were no significant correlations between the CL_{int} of glibenclamide and other marker activities (table 2). The CL_{int} of lansoprazole exhibited a significant correlation with both *S*-mephenytoin 4'-hydroxylation and testosterone 6 β -hydroxylation ($r = 0.827$, $p < 0.001$ for *S*-mephenytoin 4'-hydroxylation; $r = 0.666$, $p < 0.05$ for testosterone 6 β -hydroxylation) (figure 3 and table 2). There were no significant correlations between the CL_{int} of lansoprazole and other marker activities (table 2).

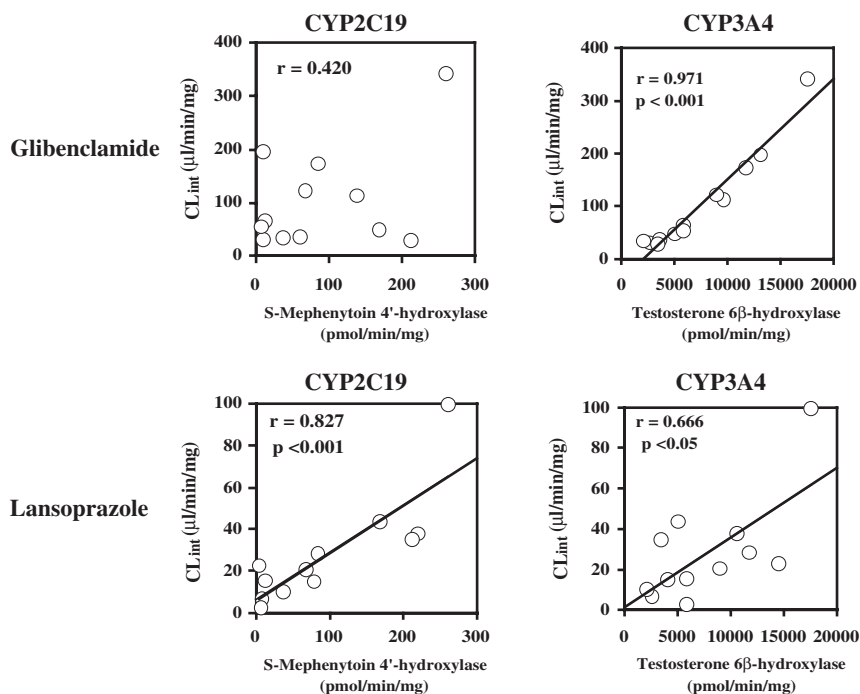


Figure 3. Relationship of the CL_{int} of glibenclamide and lansoprazole to marker activities of CYP2C19 and CYP3A4 in human liver microsomes from 12 individuals.

Inhibition study

CYP-specific inhibitors were evaluated for the effect on CL_{int} of glibenclamide and lansoprazole in pooled human liver microsomes (figure 4). Ketoconazole, a specific inhibitor of CYP3A4, reduced the CL_{int} of glibenclamide in a concentration-dependent manner, producing complete at $10\text{ }\mu\text{M}$. Other inhibitors showed a weak or no effect on the CL_{int} of glibenclamide. *S*-($\pm$)-*N*-3-benzylrivanol, a specific inhibitor of CYP2C19, and ketoconazole inhibited the CL_{int} of lansoprazole to 49.1 and 70% of the control, respectively, at $10\text{ }\mu\text{M}$. Other inhibitors showed no effect on the CL_{int} of lansoprazole.

Immuno-inhibition study

Figure 5 shows the inhibition of the CL_{int} of glibenclamide and lansoprazole by anti-rat CYP3A2 serum in pooled human liver microsomes. The CL_{int} of glibenclamide was inhibited by anti-rat CYP3A2 serum in a concentration-dependent manner, with 82.5% inhibition at $200\text{ }\mu\text{g mg}^{-1}$ microsomal protein. In contrast, the CL_{int} of lansoprazole was inhibited by anti-rat CYP3A2 serum with 27.7% inhibition at $200\text{ }\mu\text{g mg}^{-1}$ microsomal protein.

Discussion

The present study investigated the identification and relative contributions of the human CYPs involved in the metabolism of glibenclamide and lansoprazole in human liver microsomes using an approach based on the *in vitro* disappearance rate of unchanged drug. As a result: (1) of nine recombinant CYPs, CYP2C19 and

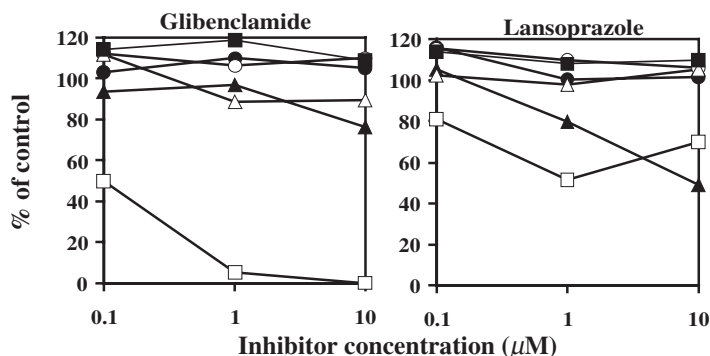


Figure 4. Effect of CYP-specific inhibitors on CL_{int} of glibenclamide and lansoprazole in pooled human liver microsomes. ●, Furafylline (CYP1A2); ○, sulfaphenazole (CYP2C9); ▲, *S*-(±)-*N*-3-benzylnirvanol (CYP2C19); □, quinidine (CYP2D6); ■, diethyldithiocarbamic acid (CYP2E1); ◻, ketoconazole (CYP3A4). Data are the average of duplicate determinations.

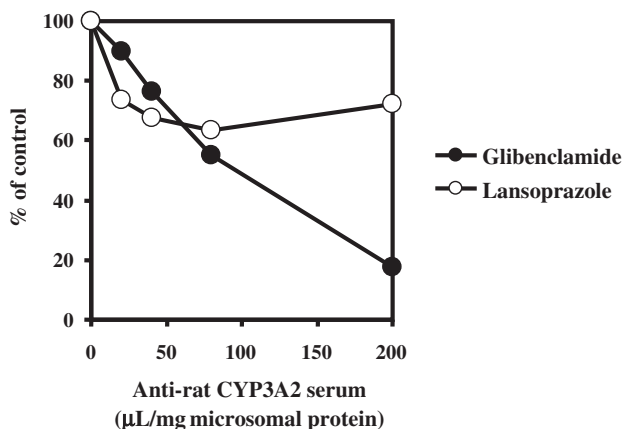


Figure 5. Immuno-inhibition of CL_{int} of glibenclamide and lansoprazole by anti-rat CYP3A2 serum in pooled human microsomes. Data are the average of duplicate determinations.

CYP3A4 catalysed the metabolism of glibenclamide and lansoprazole; (2) CYP3A4 was the major enzyme and CYP2C19 a minor enzyme involved in the metabolism of glibenclamide, whereas lansoprazole is metabolized to some extent by both CYP2C19 and CYP3A4, although the drug is more extensively metabolized by CYP2C19 than CYP3A4 in pooled human liver microsomes; (3) the CL_{int} of glibenclamide correlated very well with testosterone 6 β -hydroxylation, a CYP3A4 marker activity, whereas the CL_{int} of lansoprazole significantly correlated with *S*-mephenytoin 4'-hydroxylation, a CYP2C19 marker activity and testosterone 6 β -hydroxylation in human liver microsomes from 12 separate individuals; and (4) the CL_{int} of glibenclamide was markedly inhibited by ketoconazole, a CYP3A4 inhibitor, and anti-CYP3A serum, whereas the CL_{int} of lansoprazole was only mildly inhibited by *S*-(±)-*N*-3-benzylnirvanol, a CYP2C19 inhibitor, ketoconazole and anti-CYP3A serum in pooled human liver microsomes. These results suggest that glibenclamide is mainly metabolized by CYP3A4, whereas lansoprazole is metabolized by both CYP2C19 and CYP3A4 in human liver microsomes. In addition, the present study confirmed the utility of an approach based on

in vitro substrate disappearance rate for estimating CYP identification and relative contributions using RAF in the metabolism of compounds mainly metabolized by one or more CYPs.

The estimation of CYP identification and relative contributions have generally been performed by measuring the production of metabolites. The identification and relative contributions of CYPs were evaluated using an approach based on the disappearance rate of the parent drug. Compared with the conventional approach, the advantages of the approach used were as follows: (1) since metabolites do not need to be known, the approach is useful in drug discovery when metabolic profiles are not sufficiently characterized; and (2) it is useful in estimating overall metabolism of a drug. The prediction of human hepatic clearance using *in vitro* CL_{int} data obtained from this approach were also reported (Naritomi *et al.* 2001, 2003).

The relative contributions of CYP2C19 and CYP3A4 to the CL_{int} of glibenclamide and lansoprazole in pooled microsomes were estimated by application of RAF. RAF, proposed by Crespi (1995), was determined as the ratio of V_{max} of a CYP marker activity. Nakajima *et al.* (1999) estimated contributions of CYPs to azelastine *N*-demethylation in human liver microsomes and concluded that RAF as the ratio of CL_{int} is more appropriate for estimating contributions than the ratio of V_{max} , as when using RAF as the ratio of CL_{int} , the predicted CL_{int} of azelastine *N*-demethylation best reflected the observed CL_{int} . The current study used RAF as the ratio of CL_{int} , and the sum of predicted CL_{int} of CYP2C19 and CYP3A4 reflected the CL_{int} of glibenclamide and lansoprazole in pooled human microsomes (figure 2). In contrast, when using RAF as the ratio of V_{max} , the sum of the predicted CL_{int} was approximately 200% of observed values for each drug, showing marked differences between the CL_{int} values (data not shown).

The CL_{int} of glibenclamide significantly correlated with *S*-mephenytoin *N*-demethylation, a marker activity of CYP2B6, in addition to testosterone 6 β -hydroxylation (table 2). However, recombinant CYP2B6 did not show metabolism of glibenclamide (table 1). This correlation might result from the significant correlation between the marker activities of CYP2B6 and CYP3A4 in the 12 individual sets of human liver microsomes used in this study ($r=0.833$, $p<0.05$).

To date highly potent and selective CYP2C19 inhibitors have not been available. Recently, it was reported that *S*-($\pm$)-*N*-3-benzyl nirvanol possesses appropriate potency and selectivity as a CYP2C19 inhibitor (Suzuki *et al.* 2002, Walsk and Obach 2003). Therefore, *S*-($\pm$)-*N*-3-benzyl nirvanol was used as a specific inhibitor of CYP2C19. The inhibitory effect of *S*-($\pm$)-*N*-3-benzyl nirvanol on the CL_{int} of glibenclamide and lansoprazole accurately reflected the contribution of CYP2C19 to the CL_{int} of drugs in human liver microsomes (figure 4). Thus, *S*-($\pm$)-*N*-3-benzyl nirvanol appears to be useful for estimating the involvement of CYP2C19 in drug metabolism.

The extent of inhibition by anti-CYP3A serum was similar to the contribution of CYP3A4 in metabolism of each drug (figure 5). The present study did not estimate immuno-inhibition by anti-CYP2C19 antibody because it could not obtain an antibody specific and highly inhibitory to CYP2C19. Recently, Krausz *et al.* (2001) reported CYP identification with monoclonal antibodies specific and inhibitory to CYP2C8, 2C9 and 2C19. Such monoclonal antibodies might be a useful tool for estimating identification of CYPs in drug metabolism.

Van Giersbergen *et al.* (2002) reported that glibenclamide is metabolized by CYP2C9 in addition to CYP2C19 and CYP3A4. However, the present study did not detect any involvement of CYP2C9 in the metabolism of glibenclamide. This is likely due to the metabolic rate being very low, making it difficult to detect metabolism using an approach based on the disappearance rate. On the other hand, Van Giersbergen *et al.* investigated CYPs involved in the metabolism of glibenclamide using an approach based on the metabolite formation rate. This approach can detect a very low metabolic rate in comparison with the approach based on the parent compound disappearance rate. Indeed, the latter authors showed that metabolite formation of glibenclamide by recombinant CYP2C9 is much lower than those catalysed by recombinant CYP2C19 and CYP3A4. However, the overall metabolism of glibenclamide in human liver microsomes was evaluated using the approach based on disappearance rate. Consequently, CYP3A4 is the major enzyme, whereas CYP2C9 is a minor enzyme in comparison with CYP3A4 and CYP2C19 in the overall metabolism of glibenclamide.

It has been reported that CYP2C9 polymorphism markedly affects the pharmacokinetics of glibenclamide in humans *in vivo* (Niemi *et al.* 2001), suggesting that glibenclamide is metabolized by CYP2C9. The present authors cannot offer any reasonable explanation for the discrepancy between the results of the present *in vitro* study and those of the previous *in vivo* study. Furthermore, it is necessary to examine correlations between glibenclamide pharmacokinetics and *in vivo* CYP3A4 probes for clarifying the role of CYP3A4 in metabolism of glibenclamide in humans.

Pearce *et al.* (1996) reported the identification of CYPs involved in each major metabolic pathway of lansoprazole. However, there have been few reports about relative contributions of CYPs in the overall metabolism of lansoprazole. The current study estimated the relative contributions of CYPs in the overall metabolism of the drug using RAF and disappearance rate approach. Consequently, lansoprazole is primarily metabolized by CYP2C19 rather than by CYP3A4 in human liver microsomes *in vitro*. Metabolism of lansoprazole is associated with CYP2C19 polymorphism in humans *in vivo* (Katsuki *et al.* 1997), suggesting that CYP2C19 plays an important role in lansoprazole metabolism, and the results of the present *in vitro* study reflect those of the *in vivo* study.

In conclusion, the present study investigated the identification and relative contributions of human CYPs involved in the metabolism of glibenclamide and lansoprazole in human liver microsomes using an approach based on the *in vitro* disappearance rate of the parent drug. It is suggested that glibenclamide is mainly metabolized by CYP3A4, whereas lansoprazole is metabolized by both CYP2C19 and CYP3A4 in human liver microsomes. This approach, based on the *in vitro* substrate disappearance rate, appears to be useful for estimating CYP identification and their contributions to the metabolism of compounds in drug discovery.

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Potential of pranlukast and zafirlukast in the inhibition of human liver cytochrome P450 enzymes

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1. The potential of zafirlukast to inhibit several human cytochrome P450 enzymes is well known. However, pranlukast, a structural analogue of zafirlukast, has not been studied. Accordingly, the inhibitory potential of pranlukast was evaluated and compared with that of zafirlukast, a known CYP2C9 inhibitor, in *in vitro* microsomal incubation studies.

2. Both pranlukast and zafirlukast showed moderate inhibition of CYP2C9-catalysed tolbutamide 4-methylhydroxylation, competitively inhibiting tolbutamide 4-methylhydroxylation with estimated mean K_i values of 3.82 ± 0.50 and $5.86 \pm 0.08 \mu\text{M}$, respectively.

3. Pranlukast had no effect on CYP2C19-catalysed *S*-mephenytoin 4'-hydroxylation or CYP3A4-catalysed midazolam 1-hydroxylation. However, zafirlukast showed minor inhibition of these reactions. Neither pranlukast nor zafirlukast inhibited CYP1A2-catalysed phenacetin *O*-deethylation, CYP2D6-catalysed dextromethorphan *O*-demethylation or CYP2E1-catalysed chlorzoxazone 6-hydroxylation.

4. The results suggest that like zafirlukast, pranlukast also has the potential moderately to inhibit CYP2C9-catalysed tolbutamide 4-methylhydroxylation. Therefore, the inhibitory potential of pranlukast should be considered when it is co-administered with CYP2C9 substrates with narrow therapeutic ranges (e.g. *S*-warfarin, phenytoin).

Introduction

The leukotriene receptor antagonists (LTRAs) represent the first novel group of drugs for the treatment of asthma to be introduced in over 20 years, and the use of these agents in the management of persistent asthma has been supported by the results of placebo-controlled clinical studies (Brocks *et al.* 1996, Dunn and Goa 2001). The cysteinyl leukotrienes (LTC_4 , LTD_4 and LTE_4) are generated from arachidonic acid via the 5-lipoxygenase enzyme pathway and have been implicated as key mediators in the progression of asthma (Samuelsson 1983, Dunn and Goa 2001). These leukotrienes are highly potent mediators of inflammation, producing microvascular permeability, inflammatory cell chemotaxis, mucus hypersecretion, neuronal stimulation, and bronchial smooth muscle constriction and proliferation (Dempsey 2000). Montelukast, pranlukast and zafirlukast are LTRAs with a demonstrated clinical efficacy in the treatment of asthma, thus validating intervention at the leukotriene receptor as a therapeutic target (McMillan 2001).

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LTRAs decrease airway oedema and smooth-muscle constriction in patients with asthma (Wenzel 1998, Drazen *et al.* 1999, Reinus *et al.* 2000).

It is not unusual for patients with asthma to take combinations of several different drugs, including corticosteroids, antihistamines and antibiotics (Brocks *et al.* 1996, Garey *et al.* 1999). These patients are at high risk for drug interactions that might be masked by the positive effects or side-effects of the LTRAs themselves. Some authors have reported that zafirlukast increases the plasma concentrations of *S*-warfarin, a known substrate of CYP2C9 (Suttle *et al.* 1997, Vargo *et al.* 1997) and theophylline, a known substrate of CYP1A2 (Katial *et al.* 1998). Plasma zafirlukast concentrations fall by 30–40% with co-administration of theophylline or erythromycin (Katial *et al.* 1998, Dunn *et al.* 2001). Montelukast and zafirlukast have substantial inhibitory effects on CYP2C9-catalysed tolbutamide methylhydroxylation and, to a lesser extent, on testosterone 6 β -hydroxylation (CYP3A4) and *S*-mephenytoin hydroxylation (CYP2C19) *in vitro* (Chiba *et al.* 1997, Shader *et al.* 1999). Although zafirlukast is a known inhibitor of CYP3A4, it does not appear to exert a clinically significant pharmacokinetic effect on the drugs metabolized by CYP3A4, such as azithromycin or clarithromycin (Garey *et al.* 1999). However, the inhibitory effects of pranlukast on human CYPs have not been examined.

Pranlukast is a novel, potent and selective LTRA currently being used for the treatment of asthma. Early studies in humans have shown it to be safe in healthy Japanese and European volunteers at repeated doses of up to 675 mg (Fujimura *et al.* 1993). Brocks *et al.* (1996) reported that the pharmacokinetics of pranlukast are influenced by the timing of doses and that pranlukast at therapeutic concentrations does not inhibit the CYP3A family of enzymes based on the analysis of urinary 6 β -hydroxycortisol excretion.

To assess further the probability of drug interactions with pranlukast through the inhibition of human CYP activity and to compare its potential with that of zafirlukast, a known CYP2C9 inhibitor, the effects of pranlukast and zafirlukast on six human CYPs *in vitro* were evaluated.

Materials and methods

Chemicals

Pranlukast (purity > 98%) and zafirlukast (purity > 98%) were kindly provided by Ono Pharmaceutical (Osaka, Japan) and AstraZeneca Pharmaceuticals (Mölndal, Sweden), respectively (figure 1). Acetaminophen, carbamazepine, chlorpropamide, dextromethorphan, diethyldithiocarbamate, furafylline, lansoprazole, omeprazole, phenacetin, tolbutamide, quinidine, sulfaphenazole, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH) and β -nicotinamide adenine dinucleotide (β -NADP⁺) were from Sigma Chemical Co. (St Louis, MO, USA). Dextrophan, 6-hydroxychlorzoxazone, 4'-hydroxymephenytoin, 1-hydroxymidazolam, hydroxytolbutamide, levallorphan and *S*-mephenytoin were from Ultrafine Chemicals (Manchester, UK). Midazolam was from Roche Pharmaceutical Co. (Seoul, Korea). High-performance liquid chromatography (HPLC)-grade solvents were used for HPLC analysis (Fisher Scientific Co., Pittsburgh, PA, USA). All other reagents and chemicals were of analytical or HPLC grade.

Preparation of human liver microsomes

Microsomes were prepared from human liver tissue samples (HL-10, 14 and 18) obtained, with informed consent, from patients undergoing partial hepatectomy for removal of metastatic tumour at the Department of General Surgery, Busan Paik Hospital (Busan, Korea). The samples were from non-tumour-bearing parenchymal tissue confirmed to be histopathologically normal. Tissue obtained from patients who had taken any known CYP inhibitors or inducers in the week before surgery were not used. The Institutional Review Board of the present authors' hospital approved the use of human liver

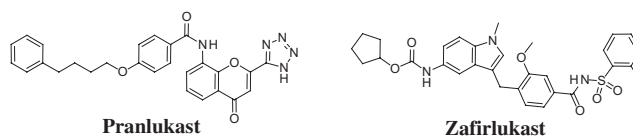


Figure 1. Chemical structures of the leukotriene antagonists pranlukast and zafirlukast.

tissues. The preparation of human liver microsomes was based on the method of Ko *et al.* (1997) with slight modifications. The liver was homogenized with 4 vols ice-cold 100 mM sodium phosphate buffer (pH 7.4) containing 1.15% potassium chloride and 0.1 mM ethylenediamine tetra-acetic acid (EDTA). Liver homogenates were centrifuged at 9200 *g* for 20 min at 4°C; the resulting post-mitochondrial supernatants were centrifuged again at 105000 *g* for 60 min at 4°C. The microsomal pellets were resuspended at a final protein concentration of 10 mg ml⁻¹, in 100 mM sodium phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 5.0 mM MgCl₂. Aliquots of liver microsomes were stored at -80°C until use and were thawed before studies. Microsomal protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Microsomal incubations

For incubation studies designed to determine the effects of pranlukast or zafirlukast on the activities of the six CYP enzymes, the incubation mixture contained 0.5 mg ml⁻¹ microsomal protein, 50 mM phosphate buffer (pH 7.4), an NADPH-generating system (3.3 mM G6P, 1.3 mM β-NADP⁺, 3.3 mM MgCl₂, and 1 unit ml⁻¹ G6PDH), one of six CYP specific substrates, and a leukotriene D4 receptor antagonist (pranlukast or zafirlukast, 1–100 μM) in a final volume of 0.25 ml. Substrates were used at concentrations approximately equal to their respective *K_m* values: 50 μM phenacetin, 100 μM tolbutamide, 50 μM *S*-mephenytoin, 50 μM dextromethorphan, 50 μM chlorzoxazone and 25 μM midazolam. Pranlukast and zafirlukast were dissolved in methanol/dimethylsulfoxide (95/5); substrates were dissolved in methanol. The final concentration of organic solvents did not exceed 1%.

Enzyme assays

The effects of leukotriene receptor antagonists on the metabolism of six different CYP specific substrates were studied: phenacetin *O*-deethylation for CYP1A2 (Tassaneeyakul *et al.* 1993), tolbutamide 4-methylhydroxylation for CYP2C9 (Relling *et al.* 1990), *S*-mephenytoin 4'-hydroxylation for CYP2C19 (Wrighton *et al.* 1993), dextromethorphan *O*-demethylation for CYP2D6 (Broly *et al.* 1989), chlorzoxazone 6-hydroxylation for CYP2E1 (Court *et al.* 1997), and midazolam 1-hydroxylation for CYP3A4 (Von Moltke *et al.* 1996). Selective inhibitors of each CYP (furafylline for CYP1A2, sulfaphenazole for CYP2C9, omeprazole for CYP2C19, quinidine for CYP2D6, diethyldithiocarbamate for CYP2E1 and ketoconazole for CYP3A4) were used as positive control index inhibitors to evaluate the inhibitory potentials of pranlukast and zafirlukast for each CYP.

All incubation mixtures contained substrate, inhibitor and human liver microsomes. After a 5-min pre-incubation at 37°C, the reactions were initiated by the addition of the NADPH-generating system. For the assays with mechanism-based inhibitors, the reaction mixture was pre-incubated for 10 min at 37°C in the presence of inhibitor and an NADPH-generating system before the addition of substrate. Incubations were performed at 37°C in a shaking water bath in an atmosphere of air. Reactions were terminated by adding 100 μl 10% (v/v) perchloric acid after 30 min for phenacetin *O*-deethylation, 100 μl 10% (v/v) perchloric acid after 60 min for tolbutamide 4-methylhydroxylation and *S*-mephenytoin 4'-hydroxylation, and 100 μl cold acetonitrile after 30 min for chlorzoxazone 6-hydroxylation, dextromethorphan *O*-demethylation and midazolam 1-hydroxylation. Samples for each assay were spiked with 25 μl internal standard (100 μM chlorpropamide for phenacetin and tolbutamide, 50 μM carbamazepine for *S*-mephenytoin, 20 μM levallorphan for dextromethorphan, 100 μM quinidine for chlorzoxazone, 100 μM lansoprazole for midazolam) and vortexed before centrifugation. Reaction rates were linear with incubation time under these conditions.

Samples for each enzyme assay were centrifuged at 20000 *g* for 10 min; the supernatant was removed and analysed by HPLC. Inhibitors were also incubated without substrate under the same conditions to ensure that the presence of inhibitors in the incubation did not interfere with the quantitation of the respective metabolites in the assays. Calibration curves were established using the authentic metabolites acetaminophen, 4-methylhydroxytolbutamide, 4'-hydroxymephenytoin, dextrophan, 6-hydroxychlorzoxazone and 1-hydroxymidazolam for phenacetin, tolbutamide, *S*-mephenytoin, dextromethorphan, chlorzoxazone and midazolam, respectively.

Analytical procedures

The HPLC system consisted of a model 307 pump, a model 234 automatic sample injector, a model 118 ultraviolet light/VIS spectrophotometric detector (Gilson Co., Villiers Le Bel, France), and a model 2475 spectrofluorometric detector (Waters, Milford, MA, USA). A Unipoint analysis system (Gilson

Table 1. Experimental conditions for inhibition studies.

CYP	Substrate	Mobile phase	Analytical column ^a	Flow rate (ml min ⁻¹)	Detection λ (nm)
1A2	Phenacetin	0.01% phosphoric acid/ 25 mM KH ₂ PO ₄ /CH ₃ CN (750/185/65, v/v/v, pH 8.0)	A	1.3	UV 245
2C9	Tolbutamide	1% acetic acid/CH ₃ CN (7/3, v/v, pH 4.0)	B	1.0	UV 235
2C19	S-mephenytoin	1% acetic acid/CH ₃ CN (72/28, v/v, pH 4.0)	B	0.6	UV 225
2D6	Dextromethorphan	10 mM KH ₂ PO ₄ /CH ₃ CN/CH ₃ OH (57/23/20, v/v/v, pH 4.0, 0.2% triethylamine)	C	0.8	fluorescence, excitation 200 nm, emission 304 nm
2E1	Chlorzoxazone	10 mM ammonium acetate (pH 5.0)/CH ₃ CN (65/35, v/v)	B	0.7	UV 287
3A4	Midazolam	0.4 M acetate buffer/CH ₃ OH/ CH ₃ CN (560/48/392)	D	0.9	UV 245

^aAnalytical columns: A, Chromolith RP-18e (100 × 4.6 mM, 5 μ m; Merck, Darmstadt, Germany); B, Lichrosorb RP-8 (250 × 4 mM, 10 μ m; Merck); C, μ -Bondapak phenyl (300 × 3.9 mM, 10 μ m; Waters, Milford, MA, USA); D, μ -Bondapak C18 (300 × 3.9 mM, 10 μ m; Waters, Milford, MA).

UV, ultraviolet light.

Co.) was used to calculate the analyte concentrations from the peak area ratios. The respective products were analysed, as described (Thummel *et al.* 1994, Shin *et al.* 1999), with some modifications. Table 1 gives further analytical details for each of the index reaction procedures.

Data analysis

The cytochrome P450-mediated activities in the presence of leukotriene receptor antagonists were expressed as a percentage of the corresponding control values in the presence of methanol alone. The apparent kinetic parameters from individual experiments were calculated using a nonlinear regression analysis program (WinNonlin v.4.0, Pharsight, Mountain View, CA, USA). These data were fitted to different models of enzyme inhibition: pure and partially competitive inhibition, non-competitive inhibition, mixed-type inhibition and uncompetitive inhibition (Segel 1975). The type of inhibition was determined by several criteria: visual inspection of Lineweaver–Burk double reciprocal plots, Dixon plots and secondary plots of Lineweaver–Burk plots versus inhibitor concentrations; the size of the sum of the squares of the residuals, Akaike information criteria, and Schwartz criteria values; the standard errors and 95% confidence intervals of the parameter estimates; and the random distribution of the residuals from the nonlinear regression analysis (Shin *et al.* 1999).

To compare the relative inhibitory potentials of the drugs tested, the ratio $[I]/K_i$ was calculated as an index of the potency of inhibition relative to the therapeutic concentration and the per cent *in vivo* inhibition was predicted using the following relationships:

$$\text{For competitive inhibition, } v = V_{\max} \cdot [S] / \{K_s(1 + [I]/K_i) + [S]\}.$$

$$\text{For partially competitive inhibition, } v = V_{\max} \cdot [S] / \{K_s(1 + [I]/K_i)/(1 + [I]/\alpha \cdot K_i) + [S]\},$$

where v is the velocity of the reaction at substrate concentration S , $V_{\max}$ is the maximum velocity of the enzyme, $K_s (=K_m)$ is the substrate concentration at which the reaction velocity is 50% of $V_{\max}$, and α is the factor by which K_m changes when an inhibitor occupies the enzyme; the values of α and K_i entered into these formulae were generated from the present study. For these calculations, the substrate concentration $[S]$ was assumed to be 1/10 of the K_m because the therapeutic range of a plasma drug concentration is usually much less than its K_m . The concentrations of LTRAs used in these calculations were the medians of the therapeutic ranges of plasma drug concentrations.

Results

Selectivity of pranlukast for the inhibition of CYP enzymes

The inhibition of CYPs by pranlukast was investigated using six CYP-specific probe substances (figure 2). The IC₅₀ values determined with the individual CYP

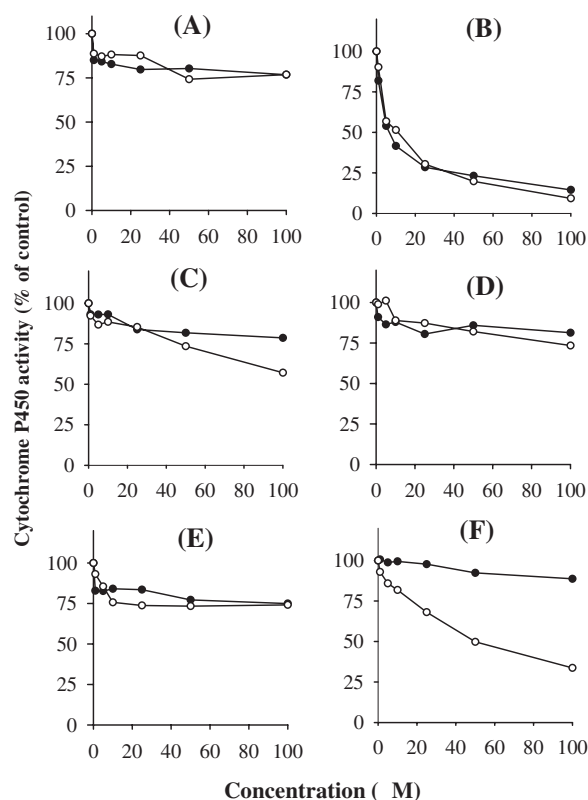


Figure 2. Inhibitory effect of pranlukast (●) and zafirlukast (○) on phenacetin *O*-deethylation (A), tolbutamide 4-methylhydroxylation (B), methenytoin 4'-hydroxylation (C), dextromethorphan *O*-demethylation (D), chlorzoxazone 6-hydroxylation (E), and midazolam 1-hydroxylation (F) in human liver microsomal incubations. Each datum point is the average obtained from three different liver microsomal preparations (HL-10, 14, 18). The representative control activities of phenacetin *O*-deethylation, tolbutamide 4-methylhydroxylation, methenytoin 4'-hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone 6-hydroxylation and midazolam 1-hydroxylation were 0.21, 0.12, 0.05, 0.16, 0.21 and 0.45 nmol min⁻¹ mg⁻¹ protein, respectively.

inhibitors were in good agreement with the range of values reported in the literature (Testino and Patonay 2003). Pranlukast at concentrations of up to 100 μ M did not significantly inhibit the reactions mediated by CYP1A2, 2C19, 2D6, 2E1 or 3A4; zafirlukast at concentrations up to 100 μ M did not significantly inhibit the reactions mediated by CYP1A2, 2D6 or 2E1 (figure 2). CYP2C19-mediated *S*-mephenytoin 4-hydroxylation and CYP3A4-mediated midazolam 1-hydroxylation were slightly inhibited by zafirlukast in a concentration-dependent manner. Tolbutamide 4-methylhydroxylation, a marker activity of CYP2C9, was moderately inhibited in the presence of either pranlukast or zafirlukast with IC₅₀ values of 6.31 or 6.90 μ M, respectively (table 2).

Mechanism of inhibition by pranlukast and zafirlukast

To examine the mechanism by which pranlukast and zafirlukast inhibit CYP2C9-mediated metabolism, kinetic studies were performed using human liver microsomes. Figure 3 shows a Dixon plot of inhibitor concentration versus tolbutamide 4-methylhydroxylation by human liver microsomes. Incubation of

Table 2. Inhibition of six human cytochrome P450 enzymes by pranlukast and zafirlukast and by representative inhibitors.

CYP	Substrate ^a	IC ₅₀ values (μM) ^b		
		Pranlukast	Zafirlukast	Index inhibitor
1A2	Phenacetin	–	–	Furafylline: 4.35 (± 0.53)
2C9	Tolbutamide	6.31 (± 2.2)	6.90 (± 2.4)	Sulfaphenazole: 0.77 (± 0.08)
2C19	S-mephenytoin	–	139.4 (± 2.9)	Omeprazole: 3.28 (± 8.17)
2D6	Dextromethorphan	–	–	Quinidine: 0.68 (± 0.12)
2E1	Chlorzoxazone	–	–	Diethyldithiocarbamate: 19.82 (± 4.65)
3A4	Midazolam	–	58.0 (± 30.7)	Ketoconazole: 0.16 (± 0.33)

^aSubstrate concentration in the incubation system was near to the K_m for each CYP.

^bData are the means ± standard deviation obtained from three different liver microsomal preparations (HL-10, 14, 18).

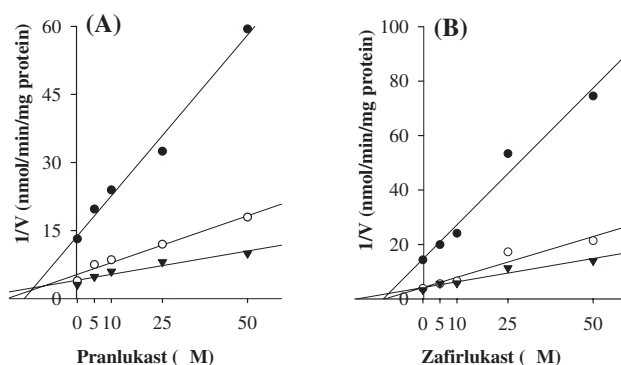


Figure 3. Representative Dixon plots for inhibition by pranlukast (A) and zafirlukast (B) of CYP2C9-catalysed tolbutamide 4-methylhydroxylation in human liver microsomal incubations. Each datum point was obtained by incubation with 25 (●), 100 (○), or 150 (▼) μM tolbutamide with 0 to 50 μM pranlukast or zafirlukast.

human liver microsomes with different inhibitor concentrations resulted in a concentration-dependent loss of tolbutamide 4-methylhydroxylation activity. The Dixon plot intersected above the x -axis, indicating that pranlukast and zafirlukast inhibited the reaction in a competitive mode over the substrate range of 25–150 μM tolbutamide. The apparent K_i values of pranlukast and zafirlukast were estimated to be 3.82 and 5.86 μM, respectively, in human liver microsomes (table 3).

Discussion

CYP monooxygenases are probably the most important enzymes for hepatic drug metabolism, which is crucial for the elimination of many therapeutic drugs. The activity of this group of enzymes can determine a patient's response to drug therapy. The inhibition of a CYP can increase systemic exposure to a drug metabolized by that CYP, possibly causing severe toxic side-effects (Ludwig *et al.* 1999, Shin *et al.* 2001). When two or more drugs are administered concurrently, the possibility of metabolism-based drug interactions exists if the drugs are metabolized by the same CYP. Therefore, to assess the *in vivo*

Table 3. *In vitro* inhibition of CYP2C9-catalysed tolbutamide 4-methylhydroxylation and the estimation of the inhibitory potency of CYP2C9 by pranlukast and zafirlukast.

Inhibitor	Type of inhibition	K_i (μM) ^a	Concentration [I] (μM)	Inhibitory potency ^b	Predicted % inhibition ^c
Pranlukast	Partially competitive ^d	3.82 ($\pm$ 0.50)	1.45	0.380	25.7
Zafirlukast	Competitive	5.86 ($\pm$ 0.08)	0.5	0.085	7.2

^aData are the means $\pm$ standard deviation obtained from three different liver microsomal preparations (HL-10, 14, 18).

^bIntrinsic inhibitory potency = $[I]/K_i$.

^cPer cent inhibition = $100 \times [I]/([I] + K_i(1 + [S]/K_m))$ for competitive inhibition, = $100 \times [I](1 - 1/\alpha)/([I](1 + [S]/\alpha \cdot K_m) + K_i(1 + [S]/K_m))$ for partially competitive inhibition, which is calculated with the assumption of substrate concentration 1/10 of K_m .

^dMean ($\pm$ standard deviation) of α , a factor by which K_m changes when an inhibitor occupies the enzyme: 6.40 ± 0.65 .

significance of drug–drug interactions involving CYP inhibition, based on *in vitro* data, it is necessary to identify the overall elimination of the drug and to characterize the inhibitory effects (Galetin *et al.* 2002).

The human liver microsomal studies described in the present experiments demonstrate that pranlukast and zafirlukast competitively inhibit CYP2C9-catalysed tolbutamide 4-hydroxylation. This is not an unusual finding, given that this CYP is involved in the metabolism of LTRAs such as montelukast (Chiba *et al.* 1997), zafirlukast (Katial *et al.* 1998, Dunn and Goa 2001) and zileuton (Machinist *et al.* 1995). Based on the data obtained in this study and in others (Chiba *et al.* 1997, Shader *et al.* 1999), it seems clear that nearly all LTRAs have the potential to inhibit CYP2C9. Because LTRAs inhibit the hepatic microsomal enzyme CYP2C9, the concurrent use of other drugs metabolized by this enzyme can result in altered clearances and changes in plasma drug concentrations. Pranlukast and zafirlukast were not found to be potent inhibitors of CYP2C9, with IC_{50} values of 6.31 and 6.90 μM , respectively, as compared with the inhibition produced by sulfaphenazole ($\text{IC}_{50} = 0.8 \mu\text{M}$), a selective inhibitor of this isoform (Newton *et al.* 1995). The interaction between montelukast metabolism and tolbutamide 4-hydroxylation has been reported to be competitive, with a K_i value of about 15 μM (Chiba *et al.* 1997).

To estimate the clinical relevance of CYP2C9 inhibition by LTRAs, the expected relative inhibitory potency was compared *in vivo* with the known therapeutic plasma concentrations of LTRAs (Brocks *et al.* 1996, Dollery 1999, Knorr *et al.* 2000). For this purpose, the potency of inhibition was calculated relative to the therapeutic concentration ($[I]/K_i$), a measure of the specificity as well as of the potency of an inhibitor (Boobis 1995), and the predicted per cent inhibition, as described. Pranlukast showed the highest $[I]/K_i$ with a value of 0.38. The predicted per cent inhibition by each LTRA, calculated using the K_i values, is shown in table 3. According to the prediction, the pranlukast and zafirlukast seems to be expected to have mild-to-moderate inhibitory drug interaction potential *in vivo*. Zafirlukast has been reported to increase the plasma concentrations of *S*-warfarin and prolong the prothrombin times, which indicates the inhibitory potential in human *in vivo* (Suttle *et al.* 1993). In the case of pranlukast, however, no report has been addressed of the inhibitory interaction of pranlukast with any substrates of CYP2C9 so far, even though the present data predicted a

larger inhibitory potential of pranlukast compared with that of zafirlukast. Therefore, *in vivo* interaction studies of pranlukast remain to be further evaluated to rule out the possible inhibitory potential of pranlukast with CYP2C9 substrates with a narrow therapeutic range.

It is well known that CYP2C9 shows genetic polymorphism. Previous results indicated that subjects with the CYP2C9*3 allele exhibited higher maximum plasma concentration and area under the curve of tolbutamide than those in the wild-type subjects (Shon *et al.* 2002). In subjects with the CYP2C9*3 variant, CYP2C9 inhibition by pranlukast and zafirlukast could increase plasma concentrations of tolbutamide further, which might result in a hypoglycemic effect.

The inhibition patterns of CYP2C19-catalysed *S*-mephenytoin 4-hydroxylation and CYP3A4-catalysed midazolam hydroxylation were different for each drug. Pranlukast did not inhibit these enzyme activities, but zafirlukast did inhibit their activities. This is an unusual finding because LTRAs such as montelukast and zafirlukast are known to inhibit CYP2C19 and CYP3A4 (Chiba *et al.* 1997, Shader *et al.* 1999, Dempsey 2000). Pranlukast is unlikely to cause significant pharmacokinetic interactions with drugs metabolized primarily by CYP2C19 and CYP3A4. The present results are supported by Brocks *et al.* (1996), who reported that pranlukast is not an inducer or inhibitor of CYP3A enzymes based on the analysis of urinary 6 β -hydroxycortisol excretion. Neither drug had a significant effect on the activities of CYP1A2, 2D6 or 2E1; all of the IC₅₀ values for these enzymes were > 100 μ M, which is much higher than the values reported for the inhibition of the metabolism of each CYP-specific substrate (Shader *et al.* 1999, Baek *et al.* 2001, Kim *et al.* 2001). These findings suggest that clinical interactions between pranlukast or zafirlukast and these CYPs would not be expected.

In conclusion, a comprehensive evaluation of the effects of pranlukast and zafirlukast on six CYPs was conducted across a wide range of substrate and LTRA concentrations using *in vitro* human liver microsomal preparations. CYP2C9 activity was selectively inhibited by pranlukast and zafirlukast during incubation with NADPH in microsomes. The findings suggest that pranlukast and zafirlukast have the potential to cause pharmacokinetic drug interactions with other co-administered drugs metabolized by CYP2C9. However, the clinical relevance of the inhibitory interaction of pranlukast with CYP2C9-substrate drugs has not been investigated. Clinical trials to evaluate the inhibitory effects of pranlukast on CYP2C9 remain to be conducted.

Acknowledgements

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Pharmacokinetic analysis of factors determining elimination pathways for sulfate and glucuronide metabolites of xenobiotics. III: mechanisms for sinusoidal efflux of 4-methylumbelliferone sulfate

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1. To elucidate the mechanisms involved in the sinusoidal efflux of sulfate and glucuronide metabolites of 4-methylumbelliferone (4MU), isolated rat liver perfusion studies were performed under several conditions.

2. The effect of sodium azide on the hepatic handling of both conjugates was examined. The net sinusoidal efflux clearance (CL_{eff}) based on the unbound concentration in the liver did not change for 4MU glucuronide (4MUG) or significantly increase for 4MU sulfate (4MUS), suggesting that the sinusoidal efflux of both conjugates is not mediated by the transport systems dependent on adenosine triphosphate.

3. Under Cl^- -depleted conditions, the CL_{eff} of 4MUG significantly decreased, but the saturation of its sinusoidal efflux rather than the transport system dependent on Cl^- might be involved because the hepatic concentration of 4MUG was extensively higher than that of the control study due to the extremely attenuated biliary excretion. The CL_{eff} of 4MUS also significantly decreased, but its hepatic concentration was not different from that in the control study, suggesting that the transport system using Cl^- is involved in the sinusoidal efflux of 4MUS.

4. The effect of glutathione was examined. CL_{eff} of 4MUG was not affected by the additional glutathione, but CL_{eff} of 4MUS decreased significantly, suggesting that some transport system sensitive to glutathione is involved in the sinusoidal efflux of 4MUS, but not of 4MUG.

5. Transporters such as Oatp1, Oatp2 and/or Npt1 might be involved in the sinusoidal efflux of 4MUS, but 4MUG is secreted from the sinusoidal membrane via the systems that are totally different from those for 4MUS.

Introduction

Most xenobiotics and/or their metabolites (Guarino and Anderson 1976, Fleck and Braunlich 1986) are eliminated from the body via both biliary and urinary excretion (Fleck and Braunlich 1990). It has been reported that glucuronides and sulfates of xenobiotics can also be handled by both the liver and kidney (Fleck and Braunlich 1990). However, glucuronides are usually excreted into both the urine (Moller and Sheikh 1983) and bile (Klaassen and Watkins 1984), whereas sulfates are extensively excreted into urine (Mulder *et al.* 1985, De Vries *et al.* 1985). Although there is an empirical rule for compounds to be excreted into the bile or urine (Bessegghir and Roch-Ramel 1987), the specific determinants of the preferential excretion pathway of xenobiotics including glucuronides and sulfates have

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not been fully clarified. Transporters that could be involved in the biliary (Kusuhara *et al.* 1998) and/or renal (Burckhardt and Wolff 2000) excretion of xenobiotics have been reported. However, quantitative pharmacokinetic analysis is needed to elucidate factors determining their preferential routes of excretion for glucuronide and sulfated conjugates.

The present authors 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). Here, it was recognized 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. It was also found that the biliary excretion clearance (CL_b) and renal clearance do not necessarily reflect the actual excretion rate of conjugates, and that sulfates generated in the liver are subject to an extensive sinusoidal efflux. Furthermore, the biliary excretion and net sinusoidal efflux of conjugates of 4MU and pNP were examined by using an isolated liver perfusion technique (Higaki *et al.* 2003). It has been reported that the biliary excretion of their glucuronides can be ascribed not to their CL_b values based on the unbound concentration in the liver, but to their high concentration in the liver. On the other hand, the sinusoidal efflux, leading to urinary excretion of sulfates, depends on the extensively higher values of the net sinusoidal efflux clearance (CL_{eff}) than those of CL_b .

In the present study, to elucidate the mechanisms involved in the sinusoidal efflux of glucuronide or sulfate of 4MU, isolated rat liver perfusion studies were performed in the presence of sodium azide (NaN_3) or glutathione (GSH) or chloride ion (Cl^-)-depleted conditions.

Materials and methods

Materials

4MU, 4MU glucuronide (4MUG), and 4MU sulfate (4MUS) (chemical purities > 98%), were from Sigma Chemical Co. (St Louis, MO, USA). As the internal standard for the determination of 4-MU and their conjugates, umbelliferone (Tokyo Chemical Co., Tokyo, Japan) was 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. Investigations were performed after approval by the authors' 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 (1961) method, as described (Higaki and Nakano 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. $\times$ 0.038 inch o.d.) followed by cannulation of the portal vein and the inferior vena cava with polyethylene tubing PE 160 (0.045 inch i.d. $\times$ 0.062 inch o.d.) and PE 240 (0.066 inch i.d. $\times$ 0.095 inch o.d.; all Division of Becton Dickinson, Parsippany, NJ, USA), respectively. The perfusate consisted of approximately 20% washed human erythrocytes, kindly supplied by the Japanese Red Cross Society (Okayama, Japan), and 0.1% D-glucose in Krebs–Ringer bicarbonate buffer and was delivered at 13.5 ml min^{-1} . Bile flow rate in the control study was $109 \pm 9 \text{ } \mu\text{l/5 min}$, demonstrating that the viability of perfused livers was maintained (Květina and Guaitani 1969).

To examine the contribution of adenosine triphosphate (ATP) to xenobiotic transport, sodium azide (NaN_3 , Ishizu Pharmaceutical Co., Osaka, Japan), a respiratory inhibitor (5 mM), was added

to the perfusate. Depletion of Cl^- was performed by using a perfusate containing gluconate ion instead of a chloride ion. GSH was added by perfusing the perfusate containing GSH (10 mM).

Estimation of unbound fraction in liver homogenate

Unbound fraction of compounds in 16.7% liver homogenate (f_T) was determined by an ultrafiltration method (1300 g for 20–30 min) using an MPS-1 membrane (Amicon Co., Tokyo, Japan). Liver homogenate (16.7%) was prepared by homogenizing liver with a 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 high-performance liquid chromatography (HPLC). 4-MU and its conjugates were assayed according to the methods of Zimmerman *et al.* (1991) with minor modifications. Briefly, the perfusate obtained after centrifugation was deproteinized with methanol after the addition of an internal standard, umbelliferone. Bile was diluted with the mobile phase used in the HPLC analysis after the addition of the 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 the internal standard. An aliquot of each supernatant was introduced onto the HPLC system, consisting of a model LC-6A HPLC pump, a model SIL-9A system controller and a model SPD-2A ultraviolet light detector (all Shimadzu, Kyoto, Japan) set at 313 nm. 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 consisted of 25% methanol in 0.05 M KH_2PO_4 , which 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} were calculated based on the unbound concentration of conjugates in the liver using the unbound fraction obtained in 16.7% liver homogenate as follows:

$$\text{CL}_b = \frac{\text{biliary excretion rate at steady state}}{f_T \cdot C_{\text{liver,ss}}} \quad (1)$$

$$\text{CL}_{\text{eff}} = \frac{\text{Net efflux rate at steady state}}{f_T \cdot C_{\text{liver,ss}}}, \quad (2)$$

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. The achievement of steady-state was assessed in the time course of biliary excretion rate or the net efflux rate for each perfusion experiment. Biliary excretion rate was calculated by multiplying the concentration in bile by the bile flow rate. 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 ± SD of four 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

The urinary excretion of 4MUS can be ascribed to the extensive sinusoidal efflux, and the bile-oriented excretion of 4MUG can be explained by its high concentration in the liver, but not by the CL_b as reported by Higaki *et al.* (2003). Furthermore, the higher hepatic concentration of 4MUG than 4MUS was generated by much lower CL_{eff} of 4MUG than that of 4MUS (Higaki *et al.* 2003) as well as a higher capacity for glucuronidation than that of sulfation (Benet *et al.* 1995), whereas CL_b is comparable between 4MUG and 4MUS (Higaki *et al.* 2003). These results obviously reveal that the sinusoidal efflux must be one of the most important factors for determining the elimination pathways of both conjugates. Therefore, a single-pass perfusion study was performed using an

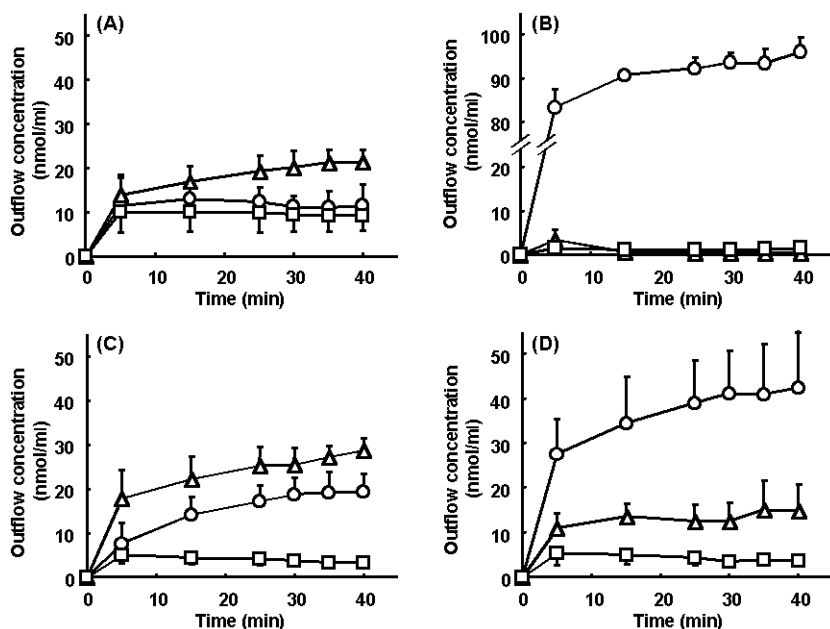


Figure 1. Effect of NaN_3 , Cl^- depletion and addition of GSH on the appearance of 4MUG and 4MUS in outflow perfusate during single-pass perfusion of 4MU through isolated liver preparations. (A) Control, (B) NaN_3 augmented, (C) Cl^- depleted, (D) GSH augmented. Perfusion of $20 \mu\text{g ml}^{-1}$ 4MU ($113.5 \text{ nmol ml}^{-1}$) was performed at 13.5 ml min^{-1} . Results are means with a vertical bar showing the SD of four experiments. $\circ$, 4MU; Δ , 4MUG; $\square$, 4MUS.

isolated rat liver preparation under several conditions to elucidate the mechanisms involved in the sinusoidal efflux of 4MUS and 4MUG.

During the perfusion of 4MU, the outflow concentration–time profiles (figure 1) and the biliary excretion rate–time profiles (figure 2) of 4MU and its conjugates were determined under several conditions such as (A) control, (B) NaN_3 augmented, (C) Cl^- depleted and (D) GSH augmented. The obtained parameters at steady-state are summarized in table 1.

To study the involvement of ATP for the transport process, NaN_3 (5 mM) was added to the perfusate. Generation of both conjugates dramatically decreased because both conjugation pathways require ATP. Therefore, net sinusoidal efflux rates, biliary excretion rates and hepatic concentrations at steady-state of both conjugates were significantly decreased compared with the control study (figures 1B, 2B and table 1). CL_b was also lowered, although the decrease in bile flow rate ($14 \pm 6 \mu\text{l/5 min}$) might partly be responsible for the decrease in the biliary excretion rates of conjugates. Extensive decrease in bile flow is reasonable because ATP is needed for bile formation via bile acid-dependent and even bile acid-independent mechanisms (Klaassen and Watkins 1984). Although functions that need ATP, such as bile formation and generation of conjugates, are suppressed by NaN_3 , the transport activity could be estimated by employing the clearance parameter such as CL_b and CL_{eff} , because this parameter is independent of bile flow and the generation of conjugates. Significant decrease in CL_b for both conjugates supports the conclusion that they are excreted into bile via Mrp2 in an ATP-dependent manner (König *et al.* 1999, Keppler *et al.* 2000, Hooiveld *et al.* 2001). On the other hand, CL_{eff} of 4MUS significantly increased, and that of

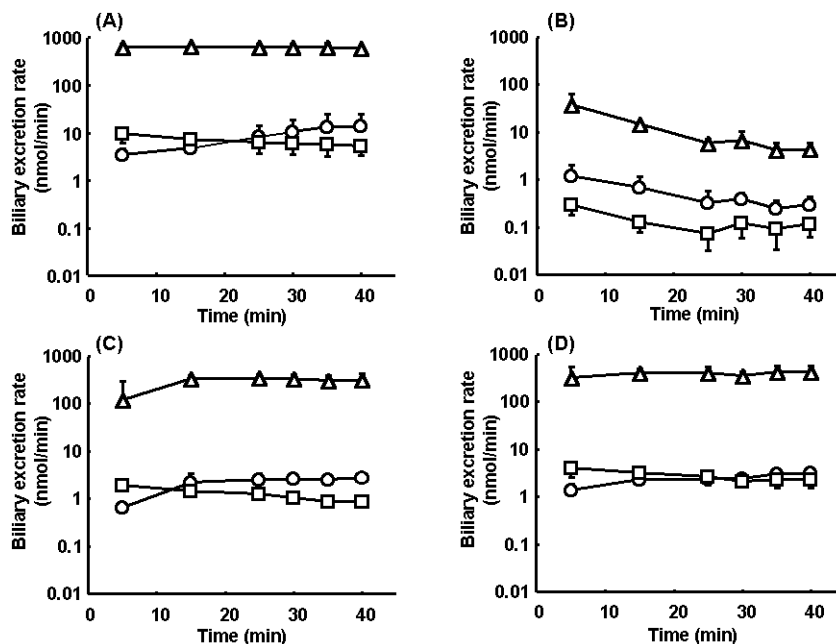


Figure 2. Effect of NaN_3 , Cl^- depletion and addition of GSH on the biliary excretion of 4MUG and 4MUS during single-pass perfusion of 4MU through isolated liver preparations. (A) Control, (B) NaN_3 augmented, (C) Cl^- depleted, (D) GSH augmented. Perfusion of $20 \mu\text{g ml}^{-1}$ 4MU ($113.5 \text{ nmol ml}^{-1}$) was performed at 13.5 ml min^{-1} . Results are means with a vertical bar showing the SD of four experiments. $\circ$, 4MU; Δ , 4MUG; $\square$, 4MUS.

4MUG was not affected significantly, suggesting that the sinusoidal efflux of 4MUS and/or 4MUG is not via the transport systems dependent on ATP. The sinusoidal efflux of dibromosulfophthalein (DBSP) has also been suggested to be mediated by a mechanism that is independent of ATP (Nijssen *et al.* 1991). The reason why CL_{eff} of 4MUS increased might be the non-linearity of the efflux of 4MUS, that is to say, the sinusoidal efflux of 4MUS would already be saturated under normal condition because the hepatic concentration of 4MUS is much higher than that in the presence of NaN_3 . Our previous study also showed that CL_{eff} of 4MUS decreased with the increase in hepatic concentration of 4MUS (Higaki *et al.* 2003). f_T for both conjugates also decreased due to very low hepatic concentrations of both conjugates.

Although Mrp3, multidrug resistance-associated protein 3, has been demonstrated to mediate the sinusoidal efflux of anions dependent on ATP (Xiong *et al.* 2002), this transporter might not be responsible for the extensive efflux of 4MUS under physiological conditions, because the constitutive expression level of Mrp3 is quite low. It might be possible that the sinusoidal uptake of 4MUS, dependent on ATP, was inhibited, but the contribution of the decrease in the uptake to the increase in CL_{eff} of 4MUS would not be so large because the sinusoidal influx permeability of 4MUS as well as 4MUG is quite small (Miyauchi *et al.* 1988).

The transport of some anions has been suggested to relate to Cl^- movement across the membrane (Wolkoff *et al.* 1987, Nijssen *et al.* 1991, Roch-Ramel 1998, Yabuuchi *et al.* 1998). Cl^- was, therefore, replaced with the gluconate ion in the perfusate and the effect of this replacement on the hepatic handling of both

Table 1. Pharmacokinetic parameters describing hepatic handling of 4MU and its conjugates in the isolated perfused liver under normal, NaN_3 -augmented, Cl^- -depleted or glutathione (GSH)-augmented condition.

Condition	Xenobiotic	Pharmacokinetic parameters							
		$C_{\text{p,ss}}$ (nmol ml ⁻¹)	Net efflux rate (nmol min ⁻¹)	Biliary excretion rate (nmol min ⁻¹)	$C_{\text{liver,ss}}$ (nmol g ⁻¹)	f_{T}	CL_{eff} (ml min ⁻¹)	CL_{b} (ml min ⁻¹)	$\text{CL}_{\text{eff}}/\text{CL}_{\text{b}}$
Control	4MU	11.5 ± 4.8	—	13.4 ± 11.2	195.8 ± 89.4	0.37 ± 0.01	n.c.	n.c.	n.c.
	4MUG	21.4 ± 2.9	289.0 ± 38.9	605.8 ± 48.7	416.9 ± 24.5	0.67 ± 0.05	1.03 ± 0.15	2.19 ± 0.40	0.48 ± 0.09
	4MUS	9.3 ± 3.4**	125.1 ± 46.0**	5.3 ± 1.9**	23.6 ± 10.5**	0.42 ± 0.09**	13.6 ± 1.6**	0.57 ± 0.12*	24.5 ± 5.5**
NaN_3 augmented	4MU	96.2 ± 3.2 [‡]	—	0.30 ± 0.13 [†]	478.0 ± 107.2 [‡]	0.36 ± 0.09	n.c.	n.c.	n.c.
	4MUG	0.36 ± 0.14 [‡]	4.84 ± 1.93 [‡]	4.16 ± 1.13 [‡]	11.6 ± 5.4 [†]	0.53 ± 0.10 [†]	0.84 ± 0.21	0.77 ± 0.25 [‡]	1.15 ± 0.33 [†]
	4MUS	1.54 ± 0.55** [‡]	20.9 ± 7.4** [‡]	0.12 ± 0.06** [‡]	3.74 ± 1.49** [‡]	0.19 ± 0.02** [‡]	30.5 ± 10.7** [†]	0.21 ± 0.17** [‡]	247.2 ± 223.2 [†]
Cl^- depleted	4MU	19.4 ± 4.2	—	2.85 ± 0.42	276.4 ± 18.6	0.38 ± 0.02	n.c.	n.c.	n.c.
	4MUG	28.7 ± 2.9 [†]	387.9 ± 38.9 [†]	324.5 ± 102.3 [‡]	2065.5 ± 389.8 [‡]	0.69 ± 0.03	0.28 ± 0.07 [†]	0.23 ± 0.08 [‡]	1.32 ± 0.52 [†]
	4MUS	3.18 ± 0.78** [‡]	43.0 ± 10.5** [‡]	0.88 ± 0.22** [‡]	31.7 ± 7.9**	0.33 ± 0.02**	4.26 ± 1.48** [‡]	0.09 ± 0.03** [‡]	54.1 ± 28.2*
GSH augmented	4MU	42.4 ± 13.1 [‡]	—	3.24 ± 0.82	332.8 ± 42.0	0.34 ± 0.02	n.c.	n.c.	n.c.
	4MUG	14.9 ± 5.8	201.1 ± 77.9	422.3 ± 146.7 [†]	435.0 ± 53.8	0.63 ± 0.07	0.74 ± 0.30	1.57 ± 0.60	0.52 ± 0.30
	4MUS	3.72 ± 0.96** [‡]	50.3 ± 13.0** [‡]	2.36 ± 0.81** [‡]	29.7 ± 4.6**	0.33 ± 0.02**	5.09 ± 1.12** [‡]	0.23 ± 0.04** [‡]	22.1 ± 5.0**

4MU, 4-methylumbelliferone; 4MUG, 4MU glucuronide; 4MUS, 4MU sulfate.

Single-pass perfusion of 20 µg ml⁻¹ 4MU (113.5 nmol ml⁻¹) was performed using Krebs–Ringer bicarbonate buffer. All parameters were calculated at steady-state. Bile flow rates at steady-state were 109 ± 9 µl/5 min (control), 14 ± 6 µl/5 min (NaN_3 augmented), 20 ± 3 µl/5 min (Cl^- depleted) and 68 ± 12 µl/5 min (GSH augmented). CL_{b} and CL_{eff} , biliary clearance and sinusoidal efflux clearance from the liver. Both clearances were calculated based on the free concentration of each substrate in the liver, using f_{T} , the unbound fraction in 16.7% liver homogenate. NaN_3 was added to the perfusate at 5 mM. Depletion of Cl^- was performed by the replacement of Cl^- with the gluconate ion. GSH was added to the perfusate at 10 mM.

Results are the mean ± SD of four experiments. n.c., Not calculated. ** p < 0.01, * p < 0.05 compared with 4MUG; [‡] p < 0.01, [†] p < 0.05 compared with the corresponding conjugate in the control study.

conjugates was examined (figures 1C, 2C and table 1). The net efflux of 4MUG was enhanced, but CL_{eff} , on the contrary, significantly decreased. CL_b of 4MUG also significantly decreased, but the transport of 4MUG might be saturated because $C_{\text{liver,ss}}$ of 4MUG extensively increased. Although the possibility that a transporter dependent on Cl^- is involved in both processes cannot be excluded, saturation has already been observed for the biliary excretion of 4MUG (Higaki *et al.* 2003). This extensive increase in $C_{\text{liver,ss}}$ of 4MUG can be ascribed to the decrease in biliary excretion due to the very low rate of bile flow ($20 \pm 3 \mu\text{l}/5 \text{ min}$). This decrease in bile flow is also reasonable because Cl^- is well known to be required for bile formation particularly via a bile acid-independent mechanism (Klaassen and Watkins 1984, Kullak-Ublick *et al.* 2000). As for 4MUS, although both CL_{eff} and CL_b significantly decreased as in the case of 4MUG, $C_{\text{liver,ss}}$ of 4MUS did not change significantly whereas that of 4MUG extensively increased. This finding infers that the transport system dependent on Cl^- is possibly involved in the sinusoidal efflux and biliary excretion of 4MUS. The possible sinusoidal efflux of organic anions where Cl^- is involved, has been reported for DBSP (Nijssen *et al.* 1991), sulfobromophthalein, bilirubin (Wolkoff *et al.* 1987) and several β -lactam antibiotics. Npt1 was suggested to play a role in the sinusoidal efflux of organic anions including β -lactam antibiotics (Yabuuchi *et al.* 1998).

GSH is a ubiquitous tripeptide, of which the antioxidant function, detoxifying function and maintenance of essential thiol status are well known (Lu 1999). Liver is one of the major organs that synthesize GSH and its hepatic concentration is around 10 mM. On the other hand, the plasma concentration of GSH is only 10–20 μM , which is mainly derived from the liver (Kaplowitz *et al.* 1985), meaning that a large concentration gradient from cytosol to plasma is maintained under physiological conditions. It has been reported that the sinusoidal efflux of GSH itself is via a transporter such as rat sinusoidal GSH transporter (Yi *et al.* 1995) and it has also been suggested that the concentration gradient of GSH is a driving force for the sinusoidal uptake of organic anions (Li *et al.* 1998, 2000) and that GSH is a possible substrate of an antiporter of organic anions (Li *et al.* 2000). Therefore, we examined the effect of GSH on the hepatic handling of 4MUS and 4MUG in an isolated perfused liver (figures 1D, 2D and table 1). Since GSH was perfused at 10 mM, which is 500–1000 times larger than its plasma concentration under physiological conditions, it was expected that the hepatic concentration of GSH was higher than that under normal conditions.

As for 4MUG, there was not any significant change in CL_{eff} comparing with the control study, although the biliary excretion rate significantly decreased and CL_b was slightly decreased. On the other hand, both CL_{eff} and CL_b for 4MUS were significantly decreased by the addition of GSH, although its hepatic concentration and the unbound fraction were almost equivalent to the control study. These results suggest that the sinusoidal efflux and biliary excretion of 4MUS were possibly *cis*-inhibited by higher hepatic concentration of GSH, whereas the hepatic handling of 4MUG was hardly affected. This *cis*-inhibition in biliary excretion would at least partly occur in the transport via Mrp2, which recognizes GSH as well as these conjugates as a substrate (Li *et al.* 1998).

Organic anion transporting polypeptide 1 (Oatp1) and Oatp2 recognize GSH as a substrate. Oatp1 is thought to function as an exchanger with organic anions (Li *et al.* 1998, 2000) and Oatp2 is suggested to function as bidirectional transporter for organic anions (Li *et al.* 2000) on the hepatic sinusoidal membrane.

Furthermore, both Oatps recognize glucuronides and sulfates of several compounds as substrates (Kakyo *et al.* 1999, Kouzuki *et al.* 1999). The driving force for the two transporters has not been clarified yet (Li *et al.* 2000), but it has been suggested that the concentration gradient of GSH from cytosol to plasma contributes as a driving force (Li *et al.* 1998, 2000) and that they are independent of ATP (Li *et al.* 2000). Taken collectively, Oatp1 and 2 might partly be involved in the extensive efflux of 4MUS and they could be *cis*-inhibited by higher concentration of GSH in the liver. On the other hand, the *trans*-stimulation was not observed for the sinusoidal efflux of conjugates, when the concentration of GSH was higher in the perfusate than that in plasma under physiological condition. It has also been suggested that GSH may play a catalytic role to stimulate the transport of anions (Lee *et al.* 2001), but any stimulating effect by GSH was not observed for the transport of both conjugates. The present study examined the hepatic handling kinetics only at steady-state, so one cannot refer to each process of the uptake and efflux precisely. However, the sinusoidal efflux of 4MUS is substantial, considering that the sinusoidal uptake of these conjugates is quite low (Miyauchi *et al.* 1988). Therefore, the information obtained at steady-state is adequate to examine the mechanisms involved in the sinusoidal efflux.

Figure 3 summarizes the hepatic handling of both conjugates under the conditions examined. In terms of biliary excretion, the transporter that is ATP-dependent and can partly be inhibited by GSH is suggested to contribute to the excretion of both conjugates. Then it is possible that both conjugates are excreted via Mrp2. However, further study should be needed to conclude that the involved transporters are GSH sensitive. Some other transporter requiring Cl^- might be

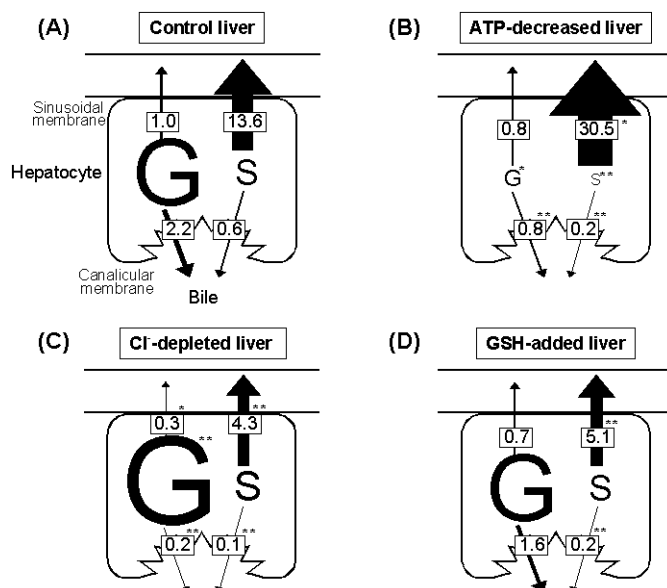


Figure 3. Schematic summary of hepatic handling of 4MUG and 4MUS under (A) normal, (B) NaN_3 -augmented, (C) Cl^- -depleted or (D) GSH-augmented conditions. G and S, 4MUG and 4MUS, respectively. The size of the letters represents the hepatic concentration of each conjugate. Figures indicate CL_{eff} or CL_b (ml min^{-1}) of each conjugate. Statistically significant differences from control are indicated as follows: ** $p < 0.01$, * $p < 0.05$.

involved in the case of 4MUS. The decrease in CL_b for 4MUG as well as that in its CL_{eff} might be attributed to the saturation of this process due to the extremely high concentration of 4MUG in the liver.

As for the sinusoidal efflux, it is suggested that the extensive efflux of 4MUS is mediated by the transport systems that are dependent on the presence of Cl^- , are inhibited by GSH and/or are independent of ATP. These results suggest that transporters such as Oatp1 and 2 and Npt1 might be involved in the sinusoidal efflux of 4MUS, although further studies in detail will be needed for the identification of the specific transporters responsible for the extensive efflux of 4MUS. On the other hand, the efflux of 4MUG across the sinusoidal membrane is much lower than that of 4MUS and might be insensitive to ATP, Cl^- and GSH.

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Stereoselective glucuronidation and hydroxylation of etodolac by UGT1A9 and CYP2C9 in man

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1. *In vitro* metabolic studies with etodolac were performed. *S*- and *R*-etodolac were converted to the acylglucuronide and hydroxylated metabolites by UDP-glucuronosyl-transferase (UGT) and cytochrome P450 in microsomes. However, the stereoselectivities of UGT and P450 for the isomers were opposite. *S*-etodolac was glucuronidated preferentially than *R*-etodolac by UGT. In contrast, *R*-etodolac was hydroxylated preferentially than *S*-etodolac by P450.

2. Of several human P450 enzymes, CYP2C9 had the greatest activity for hydroxylation of *R*-etodolac. Sulfaphenazole, an inhibitor of CYP2C9, and anti-CYP2C9 antibody inhibited the hydroxylation of *R*-etodolac in human liver microsomes. CYP2C9 therefore contributes to the stereoselective hydroxylation of *R*-etodolac.

3. Of several human UGT enzymes, UGT1A9 had the greatest activity for glucuronidation of *S*-etodolac. Propofol and thyroxine, inhibitors of UGT1A9, inhibited the glucuronidation of *S*-etodolac in human liver microsomes. Therefore, UGT1A9 is mainly responsible for the stereoselective glucuronidation of *S*-etodolac.

4. Because *S*-etodolac was metabolized more rapidly than *R*-etodolac in human cryopreserved hepatocytes, the stereoselectivities of UGT1A9 for etodolac substantially influenced the overall metabolism of *S*- and *R*-etodolac in man.

Introduction

Etodolac, (±)-1,8-diethyl-1,3,4,9-tetrahydropyrano [3,4-*b*] indole-1-acetic acid, is a non-steroidal anti-inflammatory drug (NSAID) of the pyranocarboxylic acid class. Because etodolac has a chiral centre, it is a racemic mixture of *S*- and *R*-isomers. Although only the *S*-isomer has cyclooxygenase inhibitory activities (Dermerson *et al.* 1976), etodolac is clinically used as the racemate.

There have been several reports on the pharmacokinetics of etodolac in man (Cayen *et al.* 1981, Ferdinandi *et al.* 1986, Jamali 1988, Jamali *et al.* 1988, 1989, Kraml *et al.* 1988, Brocks *et al.* 1991, 1992). After oral administration to man, etodolac is rapidly absorbed and then converted to several metabolites (figure 1) including an acylglucuronide, and 6- and 7-monohydroxylated etodolac by P450 and UDP-glucuronosyltransferase (UGT) (Ferdinandi *et al.* 1986). Previous studies have reported marked differences in the values of the pharmacokinetic parameters of *S*- and *R*-etodolac in man. The area under the curve of *R*-etodolac after oral administration of etodolac was greater than that of *S*-etodolac. In contrast, the plasma concentrations of *S*-etodolac glucuronide were higher than

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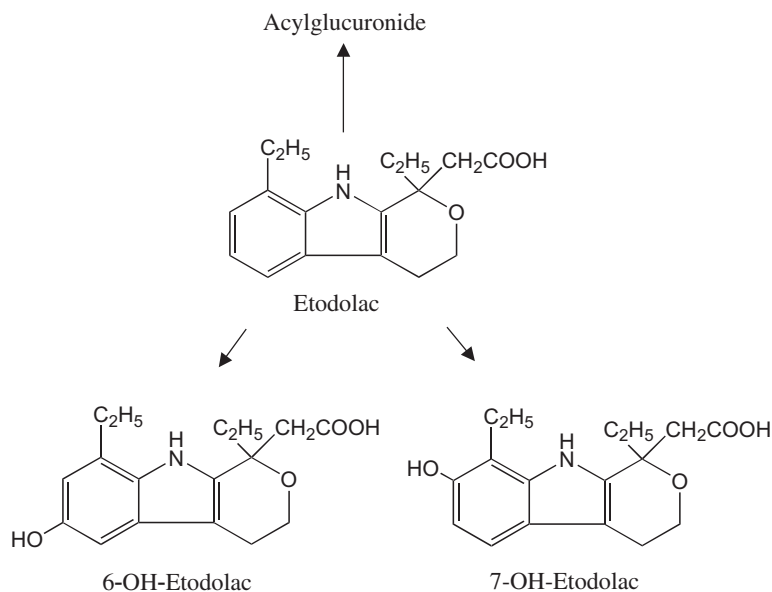


Figure 1. Metabolic pathways of etodolac in man.

those of *R*-etodolac glucuronide (Jamali *et al.* 1988). Thus, the stereoselective disposition of *S*- and *R*-etodolac in man is probably due to stereoselective glucuronidation. The *in vitro* stereoselective glucuronidation of etodolac has been reported in rats (Brocks and Jamali 1991, Honda *et al.* 1991) and the $V_{\max}$ of *S*-etodolac was about threefold greater than that of *R*-etodolac (Brocks and Jamali 1991). However, there have been few studies on the UGT enzymes responsible for the stereoselective glucuronidation of etodolac in man. The purpose of the present study was to examine the *in vitro* stereoselective metabolism of etodolac and to identify the enzymes involved in the glucuronidation and hydroxylation of etodolac in man.

Materials and methods

Compounds

^{14}C -etodolac (specific radioactivity, 661 kBq mg^{-1}), unlabelled etodolac and its hydroxylated metabolites (6-OH-etodolac and 7-OH-etodolac) were synthesized in the present authors' laboratories. The radiochemical purity of ^{14}C -etodolac determined by thin-layer chromatography and high-performance liquid chromatography (HPLC) was $> 99\%$.

Reagents

Glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH) and NADPH were from Oriental Yeast Co. (Tokyo, Japan). The chemical inhibitors of P450 and UGT were from the following manufacturers: sulfaphenazole (Sigma-Aldrich Japan, Tokyo, Japan), propofol (ICN, Aurora, OH, USA) and L-thyroxine (Tokyo Kasei, Tokyo, Japan). Uridine 5'-diphosphoglucuronic acid (UDPGA) and D-1,4-saccharic acid 1,4-lactone were from Nacalai Tesque (Kyoto, Japan). Alamethicin was from Wako Pure Chemicals Industries Ltd (Osaka, Japan). The other chemicals and solvents used were all of analytical grade. Recombinant human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT2B7 and UGT2B15 expressed in baculovirus-infected insect cells and control microsomes from insect cells infected with wild-type baculovirus were from BD Gentest (Woburn, MA, USA). Recombinant UGT1A7 and UGT1A10 expressed in baculovirus-infected insect cells were from Oxford Biomedical Research, Inc. (Oxford, MI, USA). Antibodies against human P450 (anti-CYP1A2,

2A6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4) were obtained from Nikou Nosan Co. (Yokohama, Japan). These antibodies have been reported to be highly specific for their corresponding antigen (Ng *et al.* 2003).

Human liver microsomes and cryopreserved hepatocytes

Pooled human liver microsomes (H0610 and HHM-0282) were obtained from XenoTech LLC (Kansas City, KS, USA) and Tissue Transforming Technologies, Inc. (Exton, PA, USA), respectively. Cryopreserved human hepatocytes (Lot No. 272, 293, 301B) were from XenoTech LLC. Cell viabilities were estimated to be 90–96% by the trypan blue exclusion test.

6- and 7-Hydroxylation of etodolac by human liver microsomes

The assay mixture consisted of human liver microsomes (1.0 mg ml^{-1}), ^{14}C -*S*- and *R*-etodolac ($1\text{--}500 \mu\text{M}$), an NADPH-generating system (0.25 mM NADP^+ , 2.5 mM G6P , 0.4 unit G6PDH and 1 mM MgCl_2) and 0.1 M potassium phosphate buffer containing 0.1 mM EDTA ($\text{pH } 7.4$) in a final volume of 0.5 ml . After incubation of the assay mixture at 37°C for 5 min , the reaction was started by the addition of the NADPH-generating system and stopped by the addition of 5 ml ethyl acetate after 60 min . The formation rate of 6-OH or 7-OH etodolac was linear for incubation times up to 90 min . A total of $100 \mu\text{l}$ of a solution of 4-hydroxybenzoic acid methyl ester (50 mM) as an internal standard compound and $100 \mu\text{l}$ 1 M HCl were added to the mixtures. The metabolites formed were extracted with ethyl acetate and the ethyl acetate layer was evaporated to dryness. The residue was dissolved in $100 \mu\text{l}$ methanol and a portion of this solution was injected into an HPLC column (Inertsil ODS-3, $4.6 \times 150 \text{ mm}$; GL Sciences, Tokyo, Japan). The column temperature was 40°C . The metabolites were monitored with a spectrophotometer at 225 nm . The mobile phase consisted of 15% acetonitrile in 0.1% ammonium acetate. The flow rate was 1.0 ml min^{-1} .

6- and 7-Hydroxylation of etodolac by human P450 enzymes

The assay mixture consisted of baculovirus-infected insect cells expressing human P450 (50 pmol ml^{-1}), the NADPH-regenerating system, ^{14}C -*S*- or ^{14}C -*R*-etodolac ($50 \mu\text{M}$), and 0.1 M potassium phosphate buffer containing 0.1 mM EDTA ($\text{pH } 7.4$) in a final volume of 0.5 ml . After incubation of the assay mixtures at 37°C for 5 min , the reaction was started by the addition of the NADPH-regenerating system and stopped after 60 min by the addition of 5 ml ethyl acetate. The metabolites were measured by HPLC described above.

Immunoinhibition of 6- and 7-hydroxylation by anti-CYP antiserum

The assay mixture consisted of microsomal protein (1 mg ml^{-1}), the NADPH-regenerating system, ^{14}C -*S*- or ^{14}C -*R*-etodolac ($50 \mu\text{M}$), and 0.1 M potassium phosphate buffer containing 0.1 mM EDTA ($\text{pH } 7.4$) in a final volume of 0.5 ml . The hepatic microsomes were mixed with anti-CYP antisera (0.5 mg IgG) at room temperature 30 min before the reaction. After the incubation of the assay mixtures at 37°C for 5 min , the reaction was started by the addition of the NADPH-regenerating system and stopped after 60 min by the addition of $0.1 \text{ ml } 1 \text{ M HCl}$. The metabolites were measured by HPLC.

Inhibition of 6- and 7-hydroxylation of etodolac by the CYP2C9 inhibitor, sulfaphenazole

The assay mixture consisted of microsomal protein (1 mg ml^{-1}), the NADPH-regenerating system, ^{14}C -*S*- or ^{14}C -*R*-etodolac ($50 \mu\text{M}$), sulfaphenazole (final concentrations: $0.1\text{--}1 \mu\text{M}$), and 0.1 M potassium phosphate buffer containing 0.1 mM EDTA ($\text{pH } 7.4$) in a final volume of 0.5 ml . After the incubation of the assay mixtures at 37°C for 5 min , the reaction was started by the addition of the NADPH-regenerating system and stopped after 60 min by the addition of $0.1 \text{ ml } 1 \text{ M HCl}$. The metabolites formed were analysed by HPLC.

Etodolac glucuronidation by human liver microsomes

UGT assays using alamethicin were carried out as described by Fisher *et al.* (2000). The assay mixture consisted of human liver microsomes (1 mg ml^{-1}), UDPGA (5 mM), 20 mM potassium phosphate ($\text{pH } 7.4$) containing 0.1 mM MgCl_2 , 0.5 mM D -saccharic acid-1,4-lactone, alamethicin ($50 \mu\text{g ml}^{-1}$) and *S*- ^{14}C -etodolac or *R*- ^{14}C -etodolac ($2.5\text{--}500 \mu\text{M}$) in a final volume of 0.5 ml . After incubation of the assay mixtures at 37°C for 5 min , the reaction was started by the addition of $50 \mu\text{l}$ of an aqueous solution of UDPGA (5 mM) and stopped by the addition of $500 \mu\text{l}$ acetonitrile after 60 min . The formation rate of etodolac glucuronide was linear for incubation times up to 90 min . After centrifugation ($9000g$, 10 min), a portion of the supernatant was injected into an HPLC column (Inertsil ODS-3, $4.6 \times 150 \text{ mm}$; GL Sciences). The column temperature was 40°C . The metabolites were detected with a radiodetector (Raytest Romona). The mobile phase consisted of 35% acetonitrile in 0.1% ammonium acetate and the flow rate was 1.0 ml min^{-1} .

Etodolac glucuronidation by human recombinant UGT enzymes

The assay mixture consisted of the microsomes (0.25 mg) of baculovirus-infected insect cells expressing human UGTs, 20 mM potassium phosphate (pH 7.4) containing 0.1 mM MgCl₂, 0.5 mM D-saccharic acid-1,4-lactone, alamethicin (50 µg ml⁻¹) and *S*- or *R*-¹⁴C-etodolac (50 µM) in a final volume of 0.5 ml. After incubation of the assay mixtures at 37°C for 5 min, the reaction was started by the addition of 50 µl 5 mM UDPGA and stopped by the addition of 500 µl acetonitrile after 60 min. The metabolites were analysed by HPLC as described above.

Chemical inhibition of etodolac glucuronidation

The assay mixture consisted of human liver microsomes, chemical inhibitors (propofol, 50–1000 µM; thyroxine, 50–500 µM), 20 mM potassium phosphate (pH 7.4) containing 0.1 mM MgCl₂, 0.5 mM D-saccharic acid-1,4-lactone, alamethicin (50 µg ml⁻¹), and *S*- or *R*-¹⁴C-etodolac (50 µM) in a final volume of 0.5 ml. After incubation of the assay mixtures at 37°C for 5 min, the reaction was started by the addition of 50 µl 5 mM UDPGA and stopped by the addition of 500 µl acetonitrile after 60 min. The metabolites were analysed by HPLC as described above.

Metabolic studies with human cryopreserved hepatocytes

Thawing and resuspending of the cryopreserved human hepatocytes were carried out as described by Countouris *et al.* (1993). Cryopreserved human hepatocytes (2 × 10⁶ cells ml⁻¹) were incubated at 37°C under a 95% O₂/5% CO₂ atmosphere for 15 min before the addition of etodolac. The reaction was stopped by the addition of an equal volume of acetonitrile for protein precipitation. The unchanged drug and metabolites in the incubation mixtures were analysed by HPLC. After centrifugation of the reaction mixtures, a portion of the supernatant was injected into an HPLC chiral column (Chiralcel OD-RH, 4.6 × 150 mm; Daicel Chemical Industries). The column was operated at a flow rate of 1.0 ml min⁻¹ and a temperature of 40°C. Etodolac and its metabolites were monitored with a spectrophotometer at 255 nm. The mobile phases were acetonitrile/0.1% HClO₄; 20–40% acetonitrile for 10 min, to 45% acetonitrile in 0.1 min, 45% acetonitrile for 10 min.

Determination of protein

The protein contents in microsomes of cryopreserved hepatocytes were determined by the method of Lowry *et al.* (1951).

Results

Hydroxylation of etodolac by human liver microsomes

In vitro metabolic studies of *S*- and *R*-etodolac by human hepatic microsomes were carried out. Etodolac was metabolized to 6- and 7-OH-etodolac and there was a marked stereoselectivity in the hydroxylation of etodolac. *R*-etodolac was preferentially hydroxylated compared with *S*-etodolac in human liver microsomes. The kinetic parameter values for the 6- and 7-hydroxylation of etodolac were calculated from the double-reciprocal plots (figure 2 and table 1). The *K_m* values for the 6- and 7-hydroxylation of *R*-etodolac were smaller than those of *S*-etodolac. However, the *V_{max}* values of 6- and 7-hydroxylation of *R*-etodolac were similar to those of *S*-etodolac. The results suggest that *R*-etodolac has a higher affinity for the P450 enzymes responsible for the hydroxylation of etodolac than *S*-etodolac.

Hydroxylation of etodolac by baculovirus-infected insect cells expressing human CYP enzymes

The *in vitro* metabolism of *S*- and *R*-etodolac by baculovirus-infected insect cell microsomes expressing recombinant CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 was examined (figure 3). CYP2C9 showed the greatest activities for the 6- and 7-hydroxylation of *S*- and *R*-etodolac. Stereoselectivity was observed in the hydroxylation of etodolac by the recombinant human P450s. *R*-etodolac was metabolized more preferentially than *S*-etodolac and is consistent with the *in vitro* studies using human microsomes.

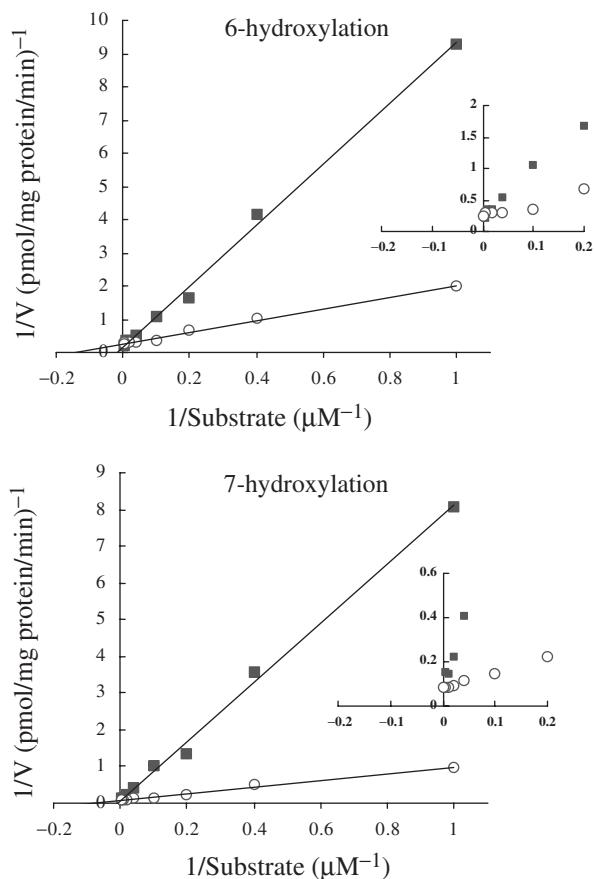


Figure 2. Double-reciprocal plot of the 6- or 7-hydroxylation of *S*- (■) and *R*- (○) etodolac by human liver microsomes. Each point is the mean of duplicate determinations.

Table 1. Kinetic parameter values of the hydroxylation of etodolac by human liver microsomes.

Reactions	Isomers	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{mg}^{-1}$)	$V_{\max}/K_m$ ($\mu\text{l min}^{-1} \text{mg}^{-1}$)
6-hydroxylation	<i>S</i> -	52.9	5.78	0.109
	<i>R</i> -	6.68	3.80	0.569
7-hydroxylation	<i>S</i> -	101	12.6	0.124
	<i>R</i> -	11.8	13.1	1.11

Values are the mean of duplicate determinations.

Effects of anti-P450 antibodies and a CYP2C9 inhibitor (sulfaphenazole) on the 6- and 7-hydroxylation of etodolac

To confirm the P450 enzymes involved in the 6- and 7-hydroxylation of *S*- and *R*-etodolac, inhibition studies were carried out using anti-CYP antibodies and a chemical inhibitor. As shown in figure 4, anti-CYP2C9 antibody markedly inhibited the formation of 6- and 7-OH-etodolac. Anti-CYP2C8 and CYP2C19 antibodies also inhibited these reactions. Sulfaphenazole, an inhibitor of CYP2C9

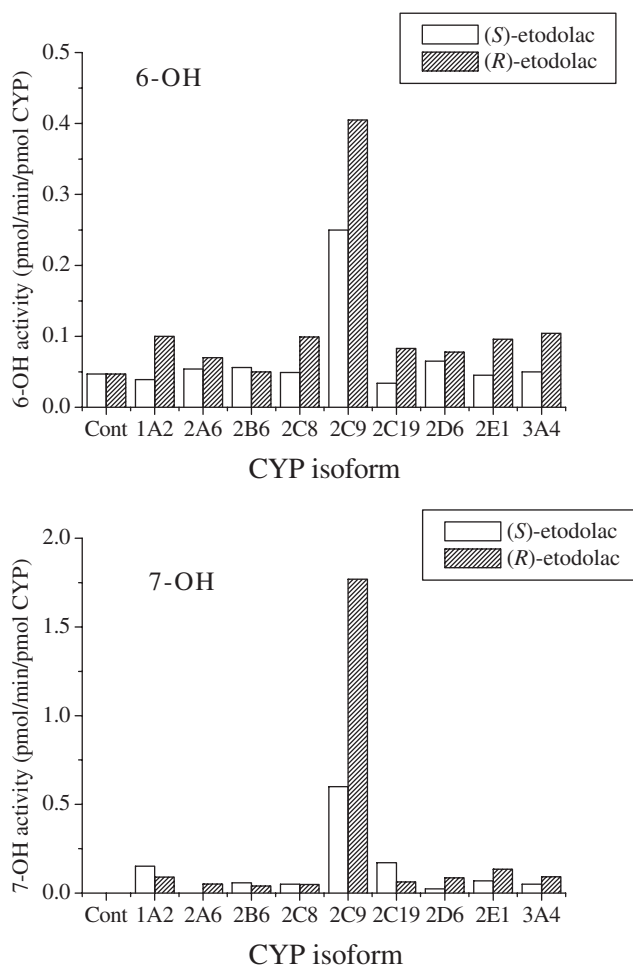


Figure 3. Hydroxylation of etodolac by microsomes from baculovirus-infected insect cells expressing P450 enzymes. Each bar is the mean of duplicate determinations.

(Baldwin *et al.* 1995), inhibited the formation of 6- and 7-OH-etodolac (figure 5). The results demonstrate that CYP2C9 mainly contributes to the hydroxylation of *S*- and *R*-etodolac.

Glucuronidation of etodolac by human liver microsomes

The *in vitro* glucuronidation of *S*- and *R*-etodolac in human hepatic microsomes was studied. Stereoselectivity was also observed in the glucuronidation of etodolac. *S*-etodolac was preferentially glucuronidated as compared with *R*-etodolac by human liver microsomes. Interestingly, the stereoselectivity of UGT was opposite to that of CYP2C9. The kinetic parameter values for the glucuronidation of *S*- and *R*-etodolac were calculated from the double-reciprocal plots (figure 6 and table 2). The K_m of the glucuronidation of *S*-etodolac was similar to that of the glucuronidation of *R*-etodolac. However, the V_{max} for the glucuronidation of *S*-etodolac was about threefold higher than that of the glucuronidation of *R*-etodolac. As a result, the V_{max}/K_m for *S*-etodolac was about threefold higher than that for *R*-etodolac.

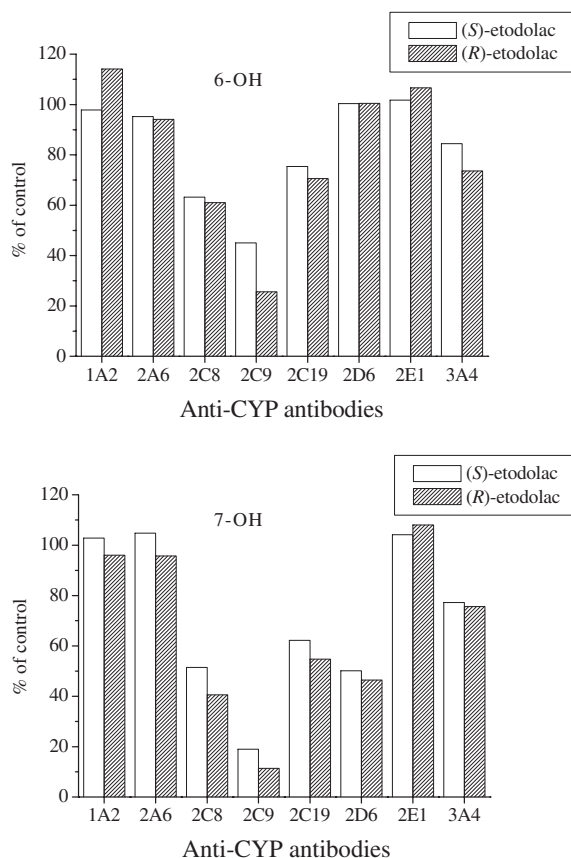


Figure 4. Effects of anti-P450 antibodies on the hydroxylation of etodolac by human liver microsomes. Each bar is the mean of duplicate determinations.

S- and R-etodolac glucuronidation by baculovirus-infected insect cells expressing human UGT enzymes

The *in vitro* glucuronidation of *S*- and *R*-etodolac by baculovirus-infected insect cell microsomes expressing recombinant UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 was examined (figure 7). UGT1A9 exhibited the greatest activity for the glucuronidation of *S*-etodolac. UGT1A10 and 2B7 also catalysed the glucuronidation of *S*-etodolac. UGT1A9 had very low activity for the glucuronidation of *R*-etodolac.

Effects of UGT1A9 substrates (propofol and thyroxine) on etodolac glucuronidation in human liver microsomes

The glucuronidation of *S*- and *R*-etodolac was inhibited by propofol (0–1000 μ M) and thyroxine (0–500 μ M), substrates of UGT1A9 (Findlay *et al.* 2000), in a concentration-dependent manner (figure 8). Thus, UGT1A9 is mainly responsible for the glucuronidation of both isomers.

Metabolism of etodolac by cryopreserved human hepatocytes

Because etodolac is metabolized by P450 as well as UGT, cryopreserved hepatocytes are a useful tool for metabolism studies; as it is possible to estimate

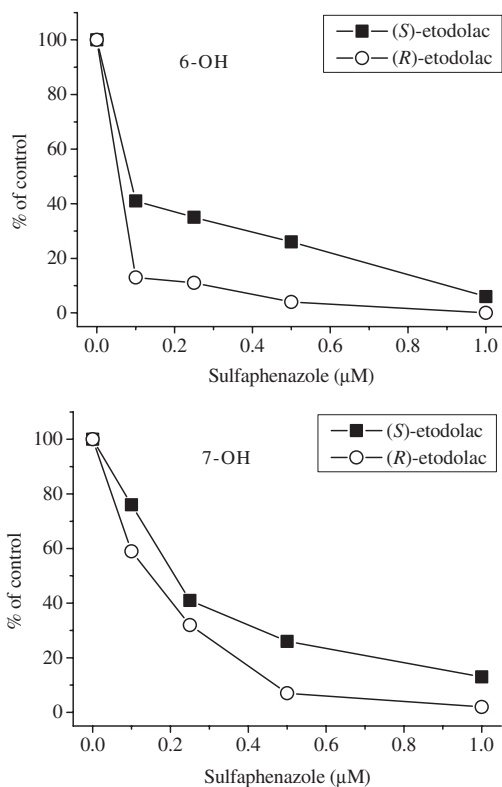


Figure 5. Effects of sulphafenazole on the hydroxylation of etodolac by human liver microsomes. Each point is the mean of duplicate determinations.

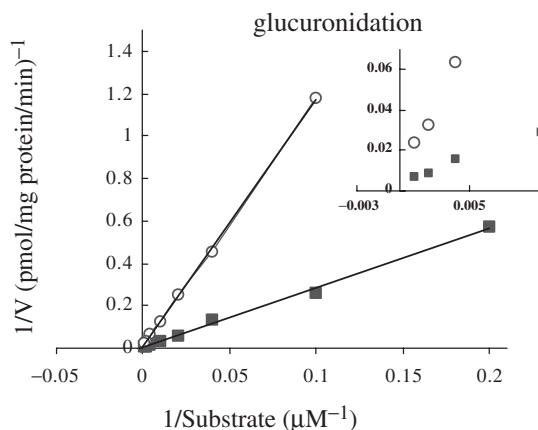


Figure 6. Double-reciprocal plot of the glucuronidation of *S*- (■) and *R*- (○) etodolac by human liver microsomes. Each point is the mean of duplicate determinations.

Phase I and II reactions of etodolac simultaneously. To evaluate the overall metabolism of *S*- and *R*-etodolac, *in vitro* metabolic studies were conducted with cryopreserved human hepatocytes (Lot No. 272, 293 and 301B). Although marked differences in the metabolism of etodolac were observed among the three preparations, *S*-etodolac was metabolized more rapidly than *R*-etodolac in all

Table 2. Kinetic parameter values of the glucuronidation of etodolac by human liver microsomes.

Isomers	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{mg}^{-1}$)	$V_{\max}/K_m$ ($\mu\text{l min}^{-1} \text{mg}^{-1}$)
S-	714	255	0.357
R-	963	82.8	0.086

Values are the mean of duplicate determinations.

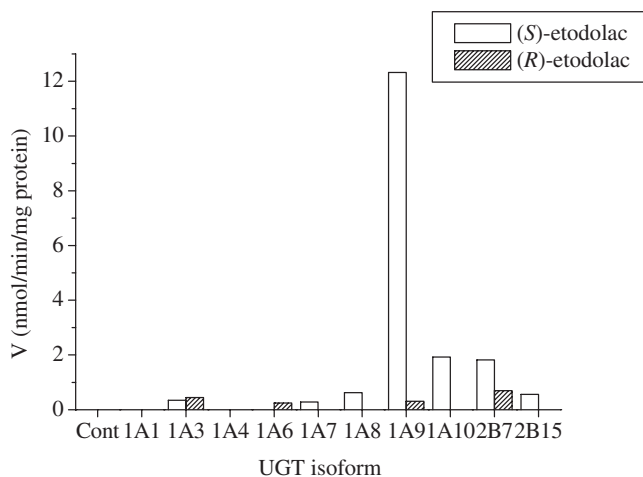


Figure 7. Glucuronidation of etodolac by microsomes from baculovirus-infected insect cells expressing UGT enzymes. Each bar is the mean of duplicate determinations.

three preparations (figure 9). In addition, the concentrations of glucuronide and 7-OH-etodolac of *S*-etodolac were higher than those of *R*-etodolac (figure 10). The results indicate that the stereoselectivity of UGT substantially influenced the overall metabolic rates of *S*- and *R*-etodolac.

Discussion

Etodolac exhibits stereoselective pharmacokinetics after administration to rats and man (Cayen *et al.* 1981, Ferdinandi *et al.* 1986, Jamali 1988, Jamali *et al.* 1988, 1989, Kramel *et al.* 1988, Brocks and Jamali 1991, Brocks *et al.* 1991, 1992, Honda *et al.* 1991). The present authors previously reported the *in vivo* stereoselective metabolism and disposition of etodolac in rats (Honda *et al.* 1991). The plasma concentrations of the unchanged drug were compared after intravenous administration (10 mg kg⁻¹) of *S*- and *R*-etodolac. There was a marked second peak in the plasma concentration of the unchanged drug after administration of *S*-etodolac, whereas the plasma concentration of *R*-etodolac decreased more rapidly without a second peak. The half-life (*T*_{1/2}) of *S*-etodolac was three times longer than that of *R*-etodolac (*S*-, 20.6 h; *R*-, 8.2 h). The results suggested that *S*-etodolac underwent enterohepatic circulation more extensively than *R*-etodolac. In fact, the biliary excretion of *S*-etodolac glucuronide after intravenous administration was two times higher than that of *R*-etodolac glucuronide (*S*-, 71.5%; *R*-, 36.3%). It was concluded that the stereoselective disposition of etodolac was

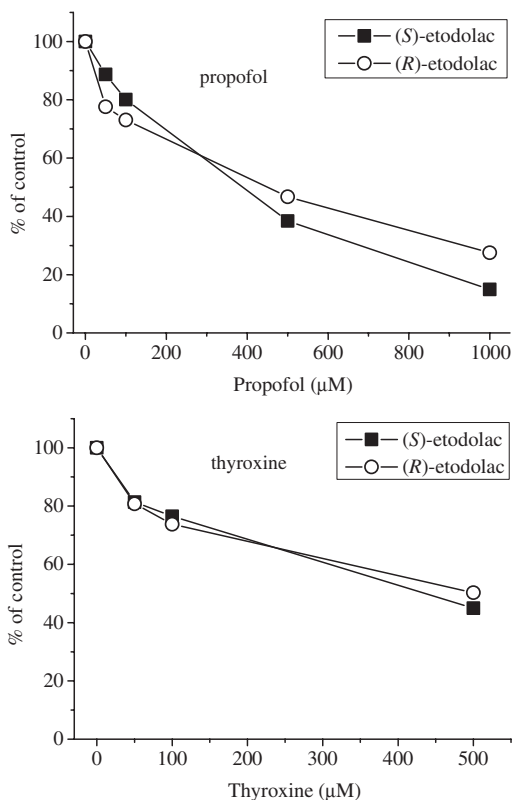


Figure 8. Effects of propofol and thyroxine on the glucuronidation of etodolac by human liver microsomes. Each point is the mean of duplicate determinations.

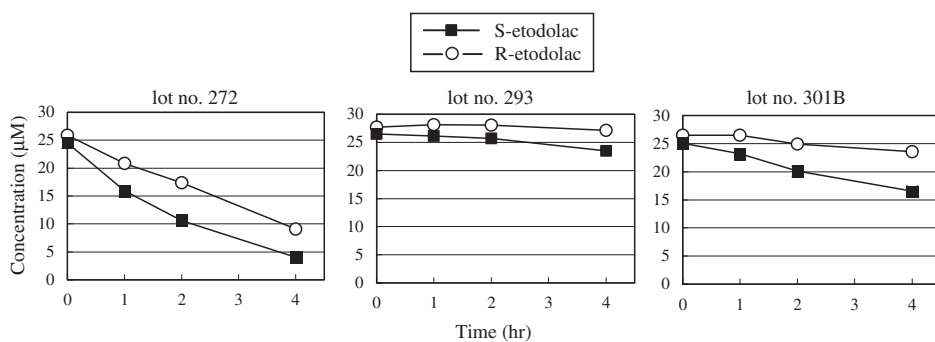


Figure 9. Disappearance of *S*- and *R*-etodolac by cryopreserved human hepatocytes. Each point is the mean of triplicate determinations.

caused by stereoselective glucuronidation. In the contrary, the plasma concentrations of *S*-etodolac decreased more rapidly than those of *R*-etodolac in man. The $T_{1/2}$ values of *S*- and *R*-etodolac were 2.4 and 4.3 h, respectively (Jamali *et al.* 1988). Because the acylglucuronides of *S*-etodolac are excreted into urine in man, *S*-etodolac undergoes no enterohepatic circulation. The results indicate that the glucuronidation of etodolac is the major determinant of the *in vivo* disposition.

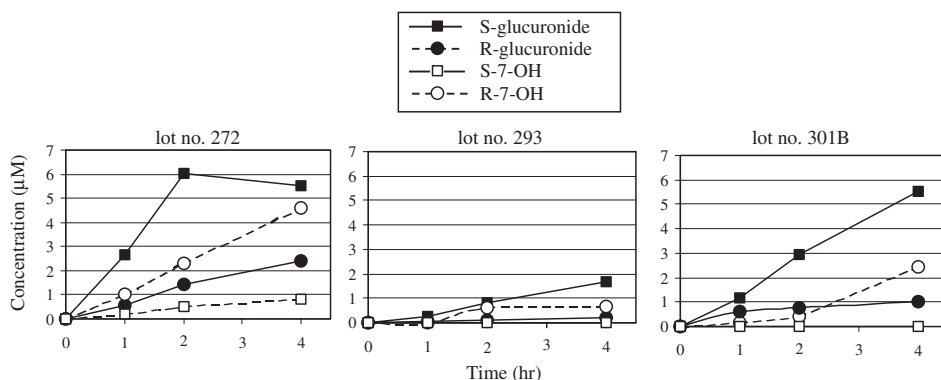


Figure 10. Formation of etodolac metabolites by cryopreserved human hepatocytes. Each point is the mean of triplicate determinations.

There are some other reports on the P450 and UGT enzymes responsible for the metabolism of NSAIDs. For example, CYP2C9 has a major role in the hydroxylation of diclofenac, indomethacin, naproxen and meloxicam (Hamman *et al.* 1997, Chesne *et al.* 1998, Nakajima *et al.* 1998). Human UGT1A3, UGT1A9 and UGT2B7 have been shown to catalyse the glucuronidation of NSAIDs (Ebner and Burchell 1993, Green and Tephy 1996, King *et al.* 2001). Particularly, UGT1A9 catalyses the glucuronidation of ibuprofen, ketoprofen and diclofenac (Jin *et al.* 1993, Pritchard *et al.* 1994). To confirm the factors responsible for this stereoselective disposition of etodolac in human, the P450 and UGT enzymes involved in the metabolism of etodolac were identified. The *in vitro* metabolic studies using human liver microsomes and recombinant enzymes showed that CYP2C9 and UGT1A9 contribute to the hydroxylation and glucuronidation of etodolac, respectively.

Brocks and Jamali (1991) reported the opposite stereoselectivities of P450 and UGT for etodolac in rat liver microsomes. The $V_{\max}$ of *S*-etodolac for the glucuronidation was 3.4-fold greater than that of *R*-etodolac, although the isomers possessed similar K_m values. In contrast, the stereoselectivity in the oxidative metabolism of etodolac by hepatic microsomes was in favour of the *R*-isomer. The present study also demonstrated that the stereoselectivities of UGT and P450 for etodolac were opposite. CYP2C9 has a marked stereoselectivity for *R*-etodolac but, in contrast, UGT1A9 has a marked stereoselectivity for *S*-etodolac. Because the main metabolic pathway of etodolac is acylglucuronidation in man (Ferdinandi *et al.* 1986), the stereoselectivity of UGT appears to influence the disposition of etodolac. In fact, *S*-etodolac was metabolized more rapidly than *R*-etodolac in human cryopreserved hepatocytes.

In conclusion, *S*- and *R*-etodolac were converted to their acylglucuronide and hydroxylated metabolites by UGT and P450 in human microsomes. The stereoselectivities of UGT and P450 for the isomers were opposite. *S*-etodolac was more preferentially glucuronidated than *R*-etodolac by UGT. In contrast, *R*-etodolac was hydroxylated more preferentially than *S*-etodolac by P450. *S*-etodolac disappeared more rapidly than *R*-etodolac and the main metabolite of etodolac is acylglucuronide in human cryopreserved hepatocytes, as is *in vivo*. UGT1A9 has a major role in the stereoselective disposition of etodolac in man.

Acknowledgement

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Role of CYP3A enzymes in the biotransformation of triazolam in rat liver

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1. Triazolam (TRZ) has been used extensively in rat to evaluate its benzodiazepine agonist central nervous system effects. However, the pharmacokinetics of TRZ in the male rat are not well understood.

2. To characterize further TRZ biotransformation across species, the NADPH-dependent biotransformation of TRZ was examined in rat and human liver microsomes. The role of specific cytochrome P450s (CYPs) in the biotransformation of TRZ in the rat were also determined using both rat cDNA-expressed CYPs and chemical and antibody inhibition techniques.

3. The formation of TRZ's primary hydroxylated products, α -OH- and 4-OH-TRZ, was consistent with a single-enzyme Michaelis–Menten model in humans.

4. Although 4-OH-TRZ formation in the male rat liver was also approximated by a single-enzyme model, a second low-affinity component was identified as contributing to α -OH-TRZ formation in the rat.

5. The K_m values for the primary metabolic pathway differed between the two species. However, the net intrinsic clearances were similar for the rat and human.

6. As observed previously for humans, chemical and antibody inhibition studies suggested that CYP3A enzymes contribute significantly to TRZ hydroxylation in the rat. This finding was further supported by the use of rat cDNA-expressed CYPs.

7. The male rat might serve as a useful model for evaluating mechanisms regulating TRZ metabolism *in vivo*.

Introduction

The triazolobenzodiazepine triazolam (TRZ) is widely prescribed for use as a hypnotic agent (Greenblatt *et al.* 1998). The formation of its two major metabolites, α -OH- and 4-OH-TRZ, is largely mediated by cytochrome P4503A (CYP3A) enzymes in humans (Kronbach *et al.* 1989, Von Moltke *et al.* 1996, Perloff *et al.* 2000). In a number of human *in vitro* studies, triazolam has been used as a marker for CYP3A activity (von Moltke *et al.* 1996, 2001, Voorman *et al.* 1998).

The rat model has been used frequently to study the central nervous system effects of triazolam and other benzodiazepine agonists (Griebel *et al.* 1998, Aburawi *et al.* 2001, Kotegawa *et al.* 2002, Laurijssens and Greenblatt 2002). However, the pharmacokinetics and pharmacodynamics of TRZ in animals have only been studied to a limited extent (Gaudreault *et al.* 1996, Nakashima *et al.* 2002). A direct comparison of the metabolism of triazolam across species has

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received even less attention. To the present authors' knowledge, the specific CYPs that contribute to the biotransformation of TRZ in the rat liver have not been fully characterized.

With the advent of more specific tools such as immuno-inhibitory antibodies and rat cDNA-expressed CYPs, a direct characterization of TRZ biotransformation in the rat is now possible. Using these latter techniques, recent studies have identified differences in the metabolic pathways between the rodents and humans for commonly used substrates (Warrington *et al.* 2000, 2002, Kobayashi *et al.* 2002, Kotegawa *et al.* 2002, Perloff *et al.* 2000, 2003). These findings highlight the importance of a more complete characterization of triazolam biotransformation across species. Thus, TRZ biotransformation was examined in human and rat liver and the contribution of specific CYPs to TRZ hydroxylation was investigated in the male rat.

Materials and methods

Materials

Human liver samples from three Caucasian male donors (20–40 years old) with no known liver disease were obtained from the International Institute for the Advancement of Medicine (Exton, PA, USA), the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN, USA) or the National Disease Research Interchange (Philadelphia, PA, USA). Fischer 344 male rats (300–360g) were acquired from the National Institutes of Aging (aged 1–2 months; held at Harlan Sprague Dawley, Inc.). Rats were then housed at the Tufts University Animal Facilities for an additional 1–2 months before sacrifice. Livers were procured immediately after sacrifice and stored at -80°C , as described (Warrington *et al.* 2002).

Triazolam was purchased from Sigma Laboratories and its metabolites were kindly provided by The Upjohn Co. (Kalamazoo, MI, USA). All other reagents were purchased from commercial sources.

Microsomal preparations

Hepatic microsomes were prepared by differential centrifugation, as described (Lake 1987, von Moltke *et al.* 1996, Warrington *et al.* 2000). Briefly, liver sections were homogenized in a 50-mM potassium phosphate buffer containing 0.15 M KCl and 0.25 M sucrose. Using a Sorvall RC-5B centrifuge with a SS-34 rotor (Dupont Instruments, Wilmington, DE, USA), samples were centrifuged at 11 960g for 22 min at 4°C . Supernatants were then centrifuged for an additional 70 min using a Beckman L8-M ultracentrifuge with a 70Ti rotor (Beckman, Fullerton, CA, USA) at 111 884g at 4°C and pellets were homogenized in 0.1 M potassium phosphate buffer containing 20% glycerol. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, USA) with albumin as the protein standard. Samples were stored at -80°C until use.

Triazolam hydroxylation using rat and human liver microsomes

Increasing concentrations of TRZ (0–750 μM) were incubated with either rat ($n=10$) or human ($n=3$) liver microsomes (0.25 mg ml^{-1}) in the presence of an NADPH-regenerating cofactor system (6.3 mM MgCl_2 , 0.5 U isocitric dehydrogenase, 3.8 mM isocitric acid, 0.5 mM NADP^+ in 50 mM potassium phosphate, pH 7.4). After 20 min at 37°C , incubations (250 μl) were terminated by the addition of acetonitrile (100 μl) and exposure to ice. Phenacetin (5.6 nmol) was added as an internal standard and samples were centrifuged in a Eppendorf microcentrifuge (Model 5415C; Eppendorf, Westbury, New York, USA) at 15 996g for 10 min to remove the protein. The supernatants were transferred to autosampling vials and analysed by high-performance liquid chromatography (HPLC). Both microsomal concentrations and incubation times were within a linear range for both rat and human (data not shown). All incubations were performed in duplicate.

Triazolam hydroxylation using rat cDNA-expressed CYPs

Rat cDNA-expressed CYPs were used to evaluate the role of specific CYPs in the formation of $\alpha\text{-OH}$ and 4-OH-TRZ, as described for other substrates (Kotegawa *et al.* 2002, Warrington *et al.* 2002). Briefly, TRZ (250 μM) was incubated for 20 min at 37°C with either one of 10 recombinantly expressed CYPs (1A1, 1A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1 or 3A2; Gentest, Woburn, MA, USA) or a vector control (1 mg ml^{-1} ; from Hi5 insect cell line). CYP2E1 (at 1 mg ml^{-1}) was expressed in an AHH-1 TK+/- human lymphoblastoid cell line and was compared with its own vector control

(1 mg ml⁻¹). CYPs expressed in the Hi5 insect cell line (Supersomes; Gentest) were incubated at 50 pmol P450 ml⁻¹, as recommended by the products' manufacturer. Both positive controls (containing either human or rat liver microsomes) and negative controls (in which the substrate, the cofactor system or microsomes were omitted) were incubated in parallel.

Immuno-inhibition of triazolam hydroxylation in rat liver microsomes

A variety of antirat polyclonal immuno-inhibitory CYP antibodies (1A1, 2B1/2, 2C11, 2E1 and 3A2; Gentest) were used to examine further the role of specific CYPs in the formation of α -OH and 4-OH-TRZ in the rat. The antibodies' manufacturer has identified the following antibody reactivity: antirat CYP1A2 antibody inhibits rat CYP1A1 and 1A2 activity; antirat CYP2B1 antibody inhibits rat CYP2B1 and 2B2 activity; antirat CYP2C11 antibody inhibits rat CYP2C11 activity; antirat CYP2E1 antibody inhibits rat CYP2E1 activity; and antirat CYP3A2 inhibits CYP3A1/2 activity. Rat liver microsomes were pooled ($n=10$) and samples containing pooled microsomes were performed in triplicate. Microsomes (10 μ g), TRZ (250 μ M) and either a CYP antibody (100 μ g), a vector control (goat or rabbit; 100 μ g) or 50 mM potassium phosphate buffer were incubated for 30 min at 37°C to a reaction volume of 30 μ l. An NADPH-regenerating cofactor system was added such that the final reaction volume was 250 μ l. Samples were incubated for an additional 20 min and subjected to the same conditions as described above. Samples were then analysed by HPLC.

Ketoconazole inhibition of triazolam hydroxylation in rat liver microsomes

Triazolam (250 μ M) was incubated with rat liver microsomes ($n=10$) and the human CYP3A inhibitor ketoconazole (2.5 μ M) (Venkatakrishnan *et al.* 2000). Using human liver microsomes, ketoconazole has been shown to be relatively selective for CYP3A enzymes at concentrations < 5 μ M (Stresser *et al.* 2004).

Incubations were performed as described above. Samples were performed in duplicate and analysed by HPLC.

HPLC analysis

Samples were analysed by HPLC, as described (von Moltke *et al.* 1996, Perloff *et al.* 2000). The mobile phase contained 70% 10 mM potassium phosphate buffer, 20% acetonitrile and 10% methanol. After passage through a stainless steel 15 cm $\times$ 3.9 mm reverse-phase C₁₈ NovaPak column (Waters Associates, Milford, MA, USA) at a flow rate of 1.4 ml min⁻¹, the effluent was monitored at a wavelength of 220 nm.

Michaelis–Menten kinetic models

The chromatographic peak heights of each metabolite were compared with those of the internal standard to generate chromatographic peak height ratios. Reaction velocities were calculated based on calibration curves of known concentrations of metabolites subjected to the same processing procedures.

Formation of both metabolites in human, and of 4-OH-TRZ in rat, was consistent with a single-enzyme Michaelis–Menten kinetic model. The following equation was fitted to data points by non-linear regression:

$$V = \frac{V_{\max} S}{K_m + S},$$

where V is the rate of metabolite formation, S is the concentration of the substrate, triazolam, $V_{\max}$ is the maximal reaction velocity and K_m is the substrate concentration at which 50% of $V_{\max}$ is achieved.

In rats, formation of α -OH-TRZ was consistent with a two-enzyme model, which is composed of a single-enzyme Michaelis–Menten model together with a second low-affinity component that was approximated by a linear function. Data points for α -OH-TRZ formation in rats were thus fitted to the following equation by non-linear regression:

$$V = \frac{V_{\max} S}{K_m + S} + zS,$$

where V , S , $V_{\max}$ and K_m are as described above, and z is the slope of the linear function used to approximate the low-affinity enzymatic component.

Statistical analysis

Kinetic parameters for rat and human TRZ biotransformation were compared using Student's t -tests or, if a normal distribution was not evident, Mann–Whitney Rank Sum tests. For immuno-inhibition studies, t -tests were used to compare the chromatographic peak height ratios with those of the appropriate serum controls.

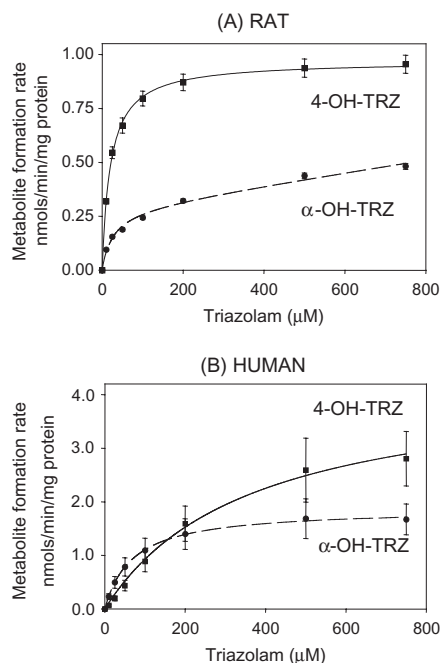


Figure 1. TRZ biotransformation using human and rat liver microsomes: increasing concentrations of TRZ (0–750 μM) were incubated with rat (A; $n = 10$) or human (B; $n = 3$) liver microsomes (0.25 mg mL^{-1}). Formation of α -OH-TRZ (dashed lines and circles) and 4-OH-TRZ (solid lines and squares) was approximated by non-linear regression using Michaelis–Menten kinetic models. Values are the mean $\pm$ SE.

Results

Triazolam's two primary hydroxylated products, α -OH- and 4-OH-TRZ, were produced by both rat and human liver microsomes (figure 1). Whereas the production of both metabolites conformed to a single-enzyme kinetic model in the human samples, α -OH-TRZ formation in the rat was best approximated using a single-enzyme kinetic model with a second, linear component. In the rats, the formation of 4-OH-TRZ was the primary metabolic pathway; on the other hand, the α -OH-TRZ pathway represents the predominant pathway in humans.

The net intrinsic clearances for the rat were apparently greater than that observed for humans, although statistical significance was not achieved between these two parameters. This small, but statistically non-significant, difference in the net intrinsic clearances between the rat and human was largely attributable to differences in K_m values for the primary metabolic pathways (table 1).

Studies using rat cDNA-expressed CYPs demonstrated that both CYP2C6 and CYP3A enzymes contribute to the formation of both hydroxylated metabolites (figure 2). However, while CYP2C6 appears to contribute significantly to α -OH-TRZ formation, CYP3A2 accounts for a greater proportion of 4-OH-TRZ production. CYP2C11, 1A2 and 2C12 also contributed to a lesser extent to the production of these metabolites.

In addition, the relative contribution of CYP3A1 and CYP3A2 differed for the two metabolic pathways in these assays. Whereas approximately 75% of the CYP3A-mediated production of 4-OH-TRZ was attributable to CYP3A2 activity,

Table 1. Biotransformation of triazolam using human and rat liver microsomes.

Kinetic parameter	Human (<i>n</i> = 3)	Rat (<i>n</i> = 10)
α -OH-TRZ:		
$V_{\max}^{**}$	1.9 ± 0.2	0.3 ± 0.01
K_m^*	63.1 ± 2.7	27.4 ± 2.4
Cl_{int}^*	31.7 ± 4.5	11.4 ± 1.0
Percent of net Cl_{int}	72.9	19.4
4-OH-TRZ:		
$V_{\max}^{**}$	4.3 ± 0.4	1.0 ± 0.04
K_m^{**}	376.6 ± 16.6	21.1 ± 1.4
Cl_{int}^*	11.9 ± 1.6	47.3 ± 2.6
Per cent of net Cl_{int}	27.1	80.6
Net Cl_{int}	43.5 ± 10.5	58.7 ± 3.3

Values are the mean $\pm$ SE.

Cl_{int} (intrinsic clearance ($1000 V_{\max}/K_m$)) is expressed as $\text{ml min}^{-1} \text{mg}^{-1}$ protein; $V_{\max}$ is expressed in $\text{nmol min}^{-1} \text{mg}^{-1}$ protein; K_m is expressed as μM .

Percentages represent a ratio of the mean Cl_{int} for each metabolite pathway divided by the net Cl_{int} .

*Difference between rat and human parameters (Student's *t*-test, $p < 0.001$).

**Difference between rat and human parameters (Mann-Whitney Sum test, $p < 0.05$).

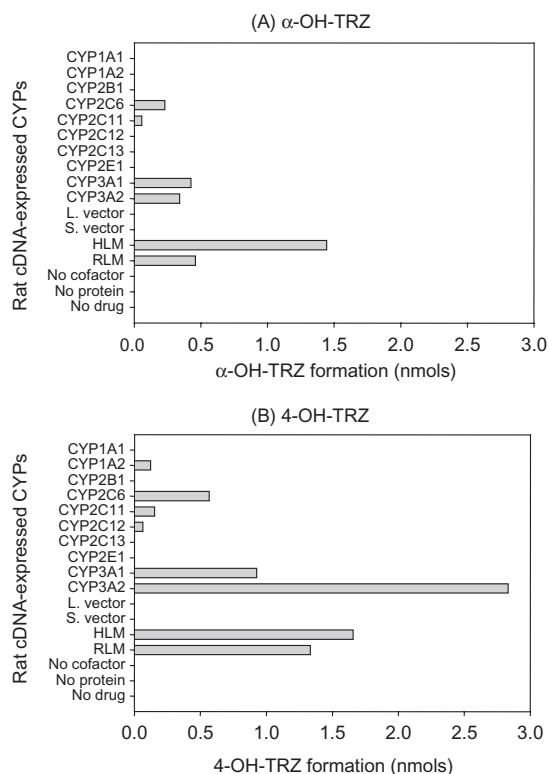


Figure 2. Triazolam biotransformation using rat cDNA-expressed CYPs: triazolam ($250 \mu\text{M}$) was incubated with a variety of CYPs (1A1, 1A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1 or 3A2). The formation of α -OH-TRZ (A) and 4-OH-TRZ (B) was observed and expressed as the average of duplicate values (nmol formed in 20 min). As negative controls, lymphoblastoid vector (L vector), supersomes vector (S vector) and samples containing no cofactor, no substrate or no protein were included. Rat liver microsomes (RLM) and human liver microsomes (HLM) served as positive controls.

both CYP3A1 and CYP3A2 contributed equally to the production of α -OH-TRZ in this screen.

Since the use of rat cDNA-expressed enzymes does not adjust for the relative abundance of specific enzymes in the rat liver, chemical and antibody inhibitors were used to evaluate the role of specific CYPs in TRZ biotransformation further. Using a variety of antirat CYP antibodies, only the antirat CYP3A2 antibody inhibited both α -OH- and 4-OH-TRZ production (figure 3). However, for both pathways, the CYP3A2 antibody did not completely inhibit metabolite formation. The degree of inhibition was unchanged when antibody concentrations were increased by fivefold (500 μ g; data not shown). In addition, the CYP2C11 antibody resulted in an unexplained activation of the production of both metabolites, as observed by Perloff *et al.* (2000) and Warrington *et al.* (2000).

Ketoconazole (2.5 μ M), a known human CYP3A inhibitor, inhibited both α -OH- and 4-OH-TRZ production (figure 4). Chemical inhibition with ketoconazole was similar to that observed for the immuno-inhibition assays.

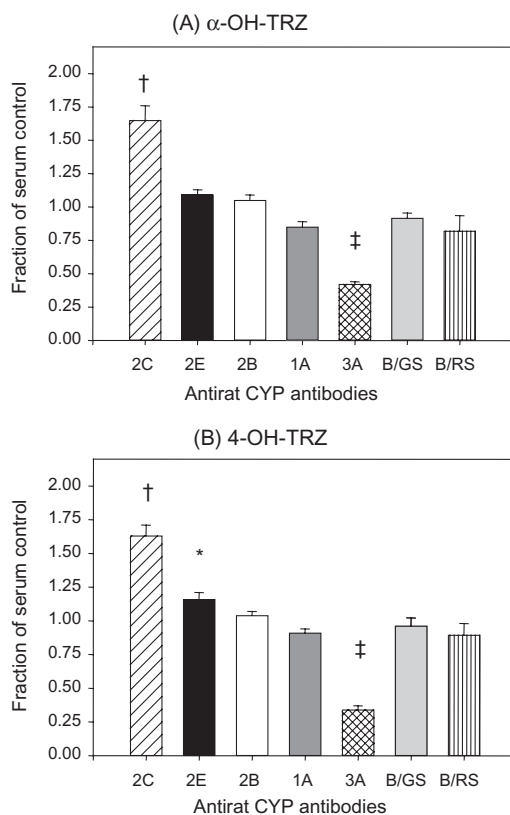


Figure 3. Immuno-inhibition of TRZ biotransformation in rat liver microsomes: immuno-inhibition of hepatic TRZ biotransformation with a variety of antirat CYP antibodies. A screen of five immuno-inhibitory antibodies (antirat CYP3A2, 2E1, 1A1/2, 2B1/2 and 2C11 at 1:10) and a 50-mM potassium phosphate buffer control (B/GS or B/RS) were used to inhibit α -OH-TRZ (A) and 4-OH-TRZ (B) formation. Samples were pooled from young rats ($n = 10$) and performed in triplicate. Histograms are expressed as a fraction of the serum control (mean $\pm$ SE). Buffer controls were expressed as a fraction of goat (B/GS) or rabbit (B/RS) sera. Student's t -tests were used to evaluate the difference from unity; * $p < 0.05$, [†] $p < 0.01$, [‡] $p < 0.001$.

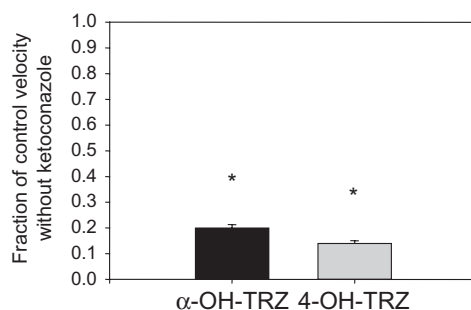


Figure 4. Inhibition of TRZ biotransformation in rat liver microsomes by ketoconazole: formation of α -OH-TRZ (black bar; $n=10$) and 4-OH-TRZ (grey bar; $n=10$) from TRZ (250 μ M) was inhibited by ketoconazole (2.5 μ M). Histograms are expressed as a fraction of the control velocity without an inhibitor and represent the mean $\pm$ SE. Mann-Whitney Rank Sum tests were used to evaluate differences in metabolite formation of the samples containing ketoconazole in comparison with controls without an inhibitor; * $p < 0.001$.

Discussion

Whereas 4-OH-TRZ was the major metabolite in rats, the α -OH-TRZ pathway represented the more predominant pathway in humans. The present findings differed from a previous study in the present authors' laboratory in which the two metabolic pathways contributed equally to the net intrinsic clearance in humans (von Moltke *et al.* 1996). The difference between these latter two studies was largely attributable to the lower estimation of $V_{\max}$ values for the 4-OH-TRZ pathway in the current study. This $V_{\max}$ estimation might have been affected by the use of a more restricted range of substrate concentrations (0–750 μ M) since the 4-OH-TRZ pathway represents a low-affinity pathway. This hypothesis is consistent with data presented by Perloff *et al.* (2000), who used a similar concentration range as used in the present study to examine TRZ biotransformation in humans. In each of these three human studies, the K_m values differed for the two metabolites. In rat, on the other hand, the relative affinities of the two metabolic pathways were similar. Although significant differences were identified between the rat and human in the two metabolic pathways, the overall net intrinsic clearances were not statistically different between the two species.

It was found that TRZ biotransformation was largely mediated by CYP3As in the male rat, as observed in humans (Kronbach *et al.* 1989, von Moltke *et al.* 1996, Perloff *et al.* 2000). Nonetheless, in rats, CYP2C enzymes can contribute to a non-CYP3A-mediated component of TRZ biotransformation. Using a variety of rat cDNA-expressed CYPs, we demonstrated that CYP2C6 can contribute to the formation of both α -OH- and 4-OH-TRZ. While these assays do not account for the relative abundance of CYP enzymes in the rat liver, both CYP3A and 2C enzymes are likely to be highly expressed in the male rat liver (Guengerich *et al.* 1982, Imaoka *et al.* 1990). Further evaluation of the role of CYP2C enzymes in TRZ hydroxylation in the rat liver is warranted.

It is also possible that CYP enzymes that are not readily available through heterologous expression systems might contribute to TRZ hydroxylation, as the CYPs studied in the current study do not represent all CYP enzymes expressed in the male rat liver. However, as the currently available cDNA-expressed CYP enzymes represent the major hepatic CYP enzymes, any additional involvement of

specific CYP enzymes is likely to be secondary to CYPs that are of low expression in the male rat liver.

The current study is limited by a lack of estimates on the relative abundance of specific CYP enzymes in the male rat liver. In previous attempts to immun quantify the amount of specific CYPs, the sum of the estimated expression levels of these CYPs have exceeded the spectrally determined total CYP content in the liver by approximately 50% (Guengerich *et al.* 1982, Imaoka *et al.* 1990), suggesting a possible limitation in antibody specificity. In the absence of relative abundance factors, an approach using multiple techniques in parallel (such as chemical and antibody inhibition) is necessary.

The role of CYP3A and possibly CYP2C enzymes in TRZ hydroxylation is similar to that observed previously for midazolam (MDZ), a structurally related benzodiazepine. Kotegawa *et al.* (2002) found that MDZ hydroxylation to its two primary metabolites, α -OH-MDZ and 4-OH-MDZ, was largely mediated by CYP3A enzymes. However, as observed with TRZ, CYP2C enzymes might also contribute to the formation of MDZ's hydroxylated products to a minor degree (Perloff *et al.* 2003). Moreover, for both TRZ and MDZ, the formation of the α -OH metabolite might represent a combined effect of multiple CYP enzymes and formation of this metabolite may be mediated, in part, by CYP2Cs. This might be consistent with the finding that α -OH-TRZ formation was best fit to a single-enzyme Michaelis–Menten model with a second low-affinity component approximated by a linear function. A similar role of CYP2C enzymes in the formation of α -OH-MDZ and α -OH-TRZ has been previously observed in mice (Perloff *et al.* 2000, Warrington *et al.* 2000).

Whereas the relative production of TRZ's two hydroxylated products may differ between the rat and human, the overall net intrinsic clearance was similar between rat and man. In addition, for both species, the formation of both hydroxylated products was largely mediated by CYP3A enzymes. Further evaluation of species differences in TRZ hydroxylation may serve to highlight important mechanisms that regulate the expression and function of CYP3A enzymes in humans.

Acknowledgements

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Influence of different recombinant systems on the cooperativity exhibited by cytochrome P4503A4

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1. The *in vitro* cooperativity exhibited by cytochrome P450 (CYP) 3A4 is influenced by the nature of the recombinant system in which the phenomenon is studied. Diclofenac, piroxicam and *R*-warfarin were used as model substrates, and quinidine was the effector.

2. The 5-, 5'- and 10-hydroxylation of diclofenac, piroxicam and *R*-warfarin, respectively, were enhanced five- to sevenfold by quinidine in human liver microsomal incubations. Whereas these cooperative drug interactions were apparent in incubations with CYP3A4 expressed in human lymphoblast cells, similar phenomena were not observed with the enzyme expressed in insect cells.

3. Insect cell microsomes were treated with a detergent and CYP3A4 was solubilized into a buffer medium. In incubations with CYP3A4 'freed' from its host membrane, the 5-hydroxylation of diclofenac increased with increasing quinidine concentrations, reaching a maximal eightfold elevation relative to controls. The metabolism of piroxicam and warfarin was similarly enhanced by quinidine.

4. Kinetically, enhancement by quinidine of the 5-hydroxylation of diclofenac in incubations with solubilized CYP3A4 was characterized by increases in the rate of metabolism with little change in the substrate-binding affinity. Conversely, the 3-hydroxylation of quinidine was not affected by diclofenac.

5. The data suggest that certain properties of CYP3A4 are masked by expression of the protein in insect cells and reinforce the concept that the enzyme possesses multiple binding domains. The absence of cooperative drug interactions with quinidine when CYP3A4 was expressed in insect cells might be due to an absence of enzyme conformation changes on quinidine binding, or the inability of quinidine to gain access to a putative effector-binding domain.

6. Caution should be exercised when comparing models for CYP3A4 cooperativity derived from different recombinant preparations of the enzyme.

Introduction

Cytochrome P450 (CYP) 3A4 is the most abundant CYP enzyme in the human liver and intestine and is involved in the metabolism of approximately 50% of therapeutic agents (Wrighton and Stevens 1992, Guengerich 1997). In certain situations, it has been shown to exhibit positive cooperativity, which might be either homo- or heterotropic in nature (Domanski *et al.* 2000, Tang and Stearns 2001). For instance, a sigmoidal pattern of increases in metabolite formation as a

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function of substrate concentration is associated with the 10,11-epoxidation of carbamazepine and the 3-hydroxylation of diazepam, which was taken to reflect homotropic cooperativity (Kerr *et al.* 1994, Shou *et al.* 1999). Examples for heterotropic cooperativity include enhancement by 7,8-benzoflavone of the 6 β -hydroxylation of progesterone and the 1'-hydroxylation of midazolam (Harlow and Halpert 1998, Wang *et al.* 2000). Similarly, the rates of 5-hydroxylation of diclofenac and 10-hydroxylation of *R*-warfarin are elevated several fold in the presence of quinidine (Ngui *et al.* 2000, 2001). In these cases, quinidine and 7,8-benzoflavone serve as effectors because of their stimulatory effect on CYP activities. Although the majority of examples of cooperativity involving CYP3A4 have derived from *in vitro* studies, it can be speculated that enzyme behaviour of this type *in vivo* could lead to pharmacological or toxicological consequences similar to those elicited by enzyme induction. In this regard, hepatic clearance of diclofenac in monkeys was enhanced by co-administration of quinidine, and stimulation of aflatoxin B1 metabolism by 7,8-benzoflavone was associated with an increased mutagenic response in TA98/TA1000 of *Salmonella typhimurium* (Buening *et al.* 1981, Tang *et al.* 1999a). A decrease in plasma concentrations of carbamazepine in epileptic patients following addition of felbamate to the initial monotherapy also was suggested to result from drug interactions mediated by CYP3A4 cooperativity (Egnell *et al.* 2003).

Because of the important role of CYP enzymes in biotransformation processes, several recombinant systems have been developed for the heterologous expression of these enzymes. These systems include bacteria, yeast, insect cells and mammalian cells, each with its own advantages and disadvantages (Gonzalez and Korzekwa 1995, Crespi and Miller 1999). With respect to the enzyme cooperativity exhibited by CYP3A4, several models have been developed to explain the phenomenon based on data generated with various recombinant systems (Korzekwa *et al.* 1998, Galetin *et al.* 2002). However, it is possible that due to differences in the compositions of host membranes, recombinant CYP3A4 preparations can respond differently to effectors as compared with the corresponding native enzyme in human liver. Preliminary data generated in the present authors' laboratories indicated that the previously observed enhancement by quinidine of *R*-warfarin metabolism was not evident in incubations with CYP3A4 expressed in insect cells. The present paper demonstrates that CYP3A4 cooperativity is indeed influenced by the nature of the recombinant system. Diclofenac, piroxicam and *R*-warfarin were employed as model substrates, and quinidine served as the effector (figure 1). Experiments were conducted to compare human liver microsomes with CYP3A4 expressed either in human lymphoblast cells or in insect cells for the potential of these *in vitro* systems to exhibit cooperative drug interactions.

Materials and methods

Materials

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), cinchonine, diclofenac, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glutathione, glycerol, isoxicam, ketoconazole, mefenamic acid, β -nicotinamide adenine dinucleotide phosphate (NADPH), piroxicam, quinidine and sulfaphenazole were from Sigma Chemical Co. (St Louis, MO, USA). 3-Hydroxyquinidine, *R*-warfarin, 10-hydroxywarfarin and [phenyl-²H₅]7-hydroxywarfarin were from BD Biosciences (Bedford, MA, USA). The Oasis MCX extraction plates were from Waters Co. (Milford, MA, USA), and the SnakeSkin pleated dialysis tubing (MWCO 10,000) was from Pierce Chemical Co. (Rockford, IL, USA).

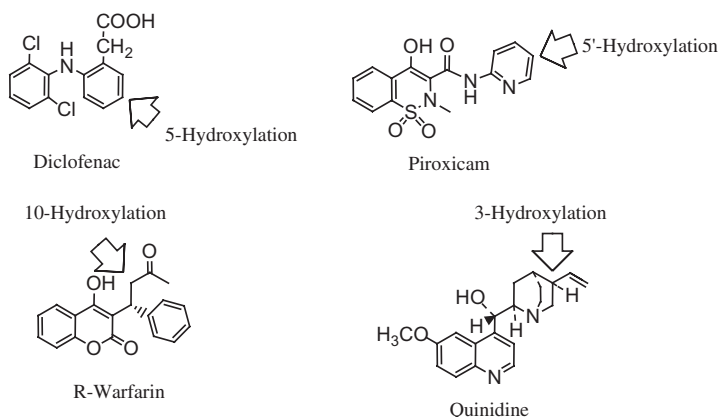


Figure 1. Structures of diclofenac, piroxicam, *R*-warfarin and quinidine. Sites of hydroxylation are denoted by arrows.

Cytochrome-b₅ and NADPH-CYP oxidoreductase (OR) were from PanVera Co. (Madison, WI, USA). Recombinant CYP3A4 co-expressed with OR in human lymphoblast cells (3A4-Lym) was a product of BD Biosciences, and CYP3A4 expressed in baculovirus infected insect cells (CYP3A4-Bac) was obtained from BD Biosciences and from Merck Research Laboratories (Shou *et al.* 1999). The two preparations of CYP3A4-Bac were indistinguishable with respect to their responsiveness to CYP3A4 cooperativity elicited by quinidine.

Solubilized CYP3A4 was prepared by treatment of CYP3A4-Bac with CHAPS (Xue *et al.* 2001). Briefly, recombinant CYP3A4-Bac (2 nmol CYP ml⁻¹) was suspended in phosphate buffer (100 mM, pH 7.4) containing glycerol (20%, v/v) and EDTA (0.1 mM), and aqueous CHAPS was added dropwise (final concentration, 1%, w/v). The suspension was stirred at 4°C for 1.5 h and subsequently centrifuged at 100 000g for 30 min. The supernatant was dialysed in SnakeSkin pleated dialysis tubing at 4°C against 1000 vols phosphate buffer (20 mM, pH 7.4) containing glycerol (7.5%, v/v), EDTA (0.1 mM) and DTT (0.05 mM). The dialysis was performed three times (4, 4 and 16 h), and the resulting solubilized CYP3A4 was stored at -70°C. The yield of enzyme was approximately 85% based on measurements of the reduced carbon monoxide-difference spectra (Omura and Sato 1964).

Human liver samples were obtained from the Pennsylvania Regional Tissue Bank (Exton, PA, USA). Liver microsomes were prepared from individual livers by differential centrifugation, and aliquots of each preparation were pooled based on equivalent protein concentrations (Raucy and Lasker 1991).

5-Hydroxydiclofenac (97% purity) and quinidine *N*-oxide (99% purity) were prepared by synthesis (Tang *et al.* 1999b, Ngui *et al.* 2000). 5'-Hydroxypiroxicam was synthesized by coupling methyl 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide with 2-amino-5-nitropyridine to afford 5'-nitropiroxicam, followed by reduction of the nitro group and diazotization and hydrolysis of the resulting 5'-aminopiroxicam (Lombardino 1981). ¹H-NMR (²H₄-methanol): δ 8.10 (1H, s), 8.09 (2H, d, J = 1 Hz), 8.07 (1H, d, J = 1 Hz), 8.00 and 7.97 (1H, dd, J_1 = 15 Hz, J_2 = 1 Hz), 7.99 (1H, d, J = 1 Hz), 7.96 and 7.93 (1H, dd, J_1 = 15 Hz, J_2 = 1 Hz), 7.94 (1H, d, J = 1 Hz), 3.22 (3H, s).

Instrumentation

Liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) was carried out on a Perkin-Elmer SCIEX API 3000 tandem mass spectrometer (Toronto, Ontario, Canada), interfaced to a Perkin-Elmer HPLC system (Norwalk, CT, USA) consisting of a Series 200 quaternary pump and auto-sampler. Samples were delivered at a flow rate of 1 ml min⁻¹ and the metabolites of interest were quantified using either a heated nebulizer interface or an ionspray interface operated in the positive-ion detection mode. With the heated nebulizer interface, the source temperature was set at 400°C, the declustering potential at 21 V, the focusing potential at 160 V, the entrance potential at -10 V and the collision energy was 27 eV. With the ionspray interface, the source temperature was set at 150°C, the ion spray voltage at 5 kV, the focusing potential at 200 V, the entrance potential at -10 V and the collision energy was 26 eV. The collision gas in both cases was nitrogen.

Incubations with human liver microsomes or recombinant CYP3A4

Diclofenac, piroxicam, *R*-warfarin or quinidine in methanol was added to human liver microsomes (0.5 nmol CYP ml⁻¹) or recombinant CYP3A4 (20 pmol CYP ml⁻¹) suspended in phosphate buffer

(0.1 M, pH 7.4) containing EDTA (1 mM) and glutathione (5 mM). With solubilized CYP3A4 (100 pmol CYP ml⁻¹), cytochrome-b₅ (100 pmol ml⁻¹), OR (200 pmol ml⁻¹), DLPC (20 µg ml⁻¹) and CHAPS (0.04%, w/v) were also added to the incubations. The concentration of substrates (diclofenac, piroxicam or *R*-warfarin) ranged from 0 to 500 µM, and the concentration of quinidine was from 0 to 200 µM. Control incubations contained no quinidine, but the same amount of methanol (0.1%, v/v). In each case, the mixture was incubated at 37°C for 5 min, following which NADPH in phosphate buffer was added (1 mg ml⁻¹) to reach a final incubation volume of 0.25 ml. After incubation for an additional 20 min, aliquots (100 µl) of the reaction mixture were removed and added to solutions of aqueous urea (8 M, 1.5 ml) containing internal standards for LC-MS/MS analysis (see below).

Extraction and quantification of metabolites

The internal standards used were mefenamic acid, [phenyl-²H₅]7-hydroxywarfarin, cinchonine (75 ng, each) and isoxicam (15 ng) for quantification of 5-hydroxydiclofenac, 10-hydroxywarfarin, 3-hydroxyquinidine and 5'-hydroxypiroxicam, respectively. The samples were applied to an Oasis MCX extraction plate, which was prewashed with methanol and water. The cartridge was washed consecutively with water (500 µl) and methanol (300 µl), and aliquots of the resulting eluates (75 µl) were injected into the LC-MS/MS system.

For analyses of 5-hydroxydiclofenac, LC-MS/MS was conducted using the heated nebulizer interface with multiple reaction monitoring. Transitions monitored were *m/z* 312.2 → 230.3 (5-hydroxydiclofenac) and 242.3 → 208.8 (mefenamic acid). Chromatography was performed on a Keystone Scientific (Wilmington, DE, USA) Betasil C8 column (4.6 × 50 mm, 5 µm). The mobile phase consisted of aqueous acetonitrile (90%, v/v) containing ammonium acetate (1 mM) and trifluoroacetic acid (0.1%, v/v).

For analyses of 10-hydroxywarfarin, LC-MS/MS was conducted using the heated nebulizer interface with multiple reaction monitoring. Transitions monitored were *m/z* 325.1 → 251.0 (10-hydroxywarfarin) and 330.1 → 188.9 ([phenyl-²H₅]7-hydroxywarfarin). Chromatography was performed on an Agilent (Palo Alto, CA, USA) Zorbax RX C8 column (4.6 × 250 mm, 5 µm). The mobile phase consisted of aqueous acetonitrile (40%, v/v) containing methanol (5%, v/v) and trifluoroacetic acid (0.05%, v/v).

For analyses of 5'-hydroxypiroxicam, LC-MS/MS was conducted using the ionspray interface with multiple reaction monitoring. Transitions monitored were *m/z* 348.3 → 111.1 (5'-hydroxypiroxicam) and 336.2 → 111.1 (isoxicam). Chromatography was performed on a Waters YMC-AQ C18 column (4.6 × 150 mm, 5 µm). The mobile phase consisted of aqueous acetonitrile containing ammonium acetate (5 mM); a linear gradient was employed in which the acetonitrile content increased from 14 to 23% over 15 min.

For analyses of 3-hydroxyquinidine and quinidine *N*-oxide, LC-MS/MS was conducted using the ionspray interface with multiple reaction monitoring. Transitions monitored were *m/z* 341.2 → 160.2 (quinidine *N*-oxide and 3-hydroxyquinidine) and 295.0 → 166.0 (cinchonine). Chromatography was performed on the Waters YMC-AQ C18 column. The mobile phase consisted of aqueous acetonitrile (25%, v/v) containing ammonium acetate (5 mM).

All standard curves were generated over a concentration range of 10–10 000 ng ml⁻¹.

Kinetic calculations

The kinetics of diclofenac and quinidine metabolism were analysed based on a model consisting of two distinct binding domains in the active site of CYP3A4 (figure 2) (Ngui *et al.* 2000). The velocity equations derived from this model, assuming rapid equilibrium between the enzyme and substrates, were as follows:

$$v_{\text{Qin}} = \frac{V_{\text{maxQ}}[S_1](K_{S2} + \alpha[S_2])}{K_{S1}K_{S2} + K_{S2}[S_1] + K_{S1}[S_2] + [S_1][S_2]} \quad (1)$$

$$v_{\text{Dic}} = \frac{V_{\text{maxD}}[S_2](K_{S1} + \beta[S_1])}{K_{S1}K_{S2} + K_{S2}[S_1] + K_{S1}[S_2] + [S_1][S_2]} \quad (2)$$

$$V_{\text{maxQ}} = [E_t]k_q \quad V_{\text{maxD}} = [E_t]k_d \quad [E_t] = [E] + [ES_1] + [ES_2] + [S_2ES_1], \quad (3)$$

where v_{Qin} and v_{Dic} are the initial velocities, V_{maxQ} and V_{maxD} are the maximum velocities, and k_q and k_d are the rate constants for the formation of 3-hydroxyquinidine and 5-hydroxydiclofenac, respectively; $[S_1]$ and $[S_2]$ are the concentrations of quinidine and diclofenac, respectively; K_{S1} and K_{S2} are the dissociation constants for $E \rightleftharpoons ES_1$ and $E \rightleftharpoons ES_2$, respectively; α and β are the factors by which V_{max} values increase (or decrease) when the second substrate interacts with the enzyme (figure 2). The kinetic parameters were generated with the Marquardt–Levenberg non-linear least-squares algorithm (Marquardt 1963).

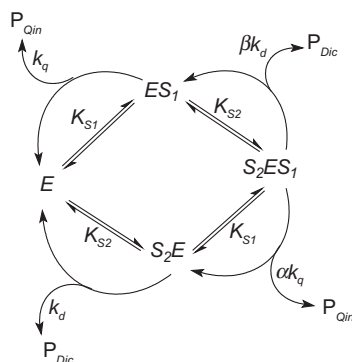


Figure 2. Kinetic model consisting of two substrate-binding domains in the active site of CYP3A4. E, enzyme; S_1 and S_2 , quinidine and diclofenac, respectively; P, product(s).

Results

Metabolism of diclofenac, piroxicam and R-warfarin

The metabolites of diclofenac, piroxicam and *R*-warfarin were identified in incubations with human liver microsomes or recombinant CYP3A4 based on comparison of HPLC retention times and product-ion spectra with those of the reference corresponding standards. The formation of 5-hydroxydiclofenac, 5'-hydroxypiroxicam and 10-hydroxywarfarin in these incubations was linear over 30 min, and therefore all experimental data were collected following a 20-min incubation.

Cooperative drug interactions in human liver microsomal incubations

The 5-hydroxylation of diclofenac and 10-hydroxylation of *R*-warfarin are catalysed primarily by CYP3A4 in human liver microsomal incubations. This conclusion was based on data derived from studies with selective chemical inhibitors and monoclonal inhibitory antibodies (Ngui *et al.* 2000, 2001). The 5'-hydroxylation of piroxicam in human liver microsomal incubations was inhibited by approximately 70% by sulfaphenazole and by 40% by ketoconazole over a substrate concentration range of 5–50 μM , suggesting that this metabolic reaction was catalysed by both CYP2C9 and CYP3A4 (figure 3). Piroxicam was identified previously as a substrate of CYP2C9 and its metabolism *in vitro* was reported to exhibit cooperativity in the presence of dapsone (Zhao *et al.* 1992, Hutzler *et al.* 2001).

The rate of formation of 5-hydroxydiclofenac in human liver microsomes was estimated to be $245 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein at 50 μM diclofenac. The rate of this metabolic reaction increased in the presence of quinidine, the increase being dependent on effector concentration (figure 4, A). The augmentation of diclofenac metabolism in incubations containing 100 μM quinidine was approximately sixfold relative to controls lacking the effector. The rate of formation of 10-hydroxywarfarin in human liver microsomes was $9 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein at 50 μM *R*-warfarin. Similar to diclofenac metabolism, the 10-hydroxylation of *R*-warfarin was enhanced by quinidine in a concentration-dependent manner. The greatest increase in the metabolic turnover of *R*-warfarin was fivefold of control values (figure 4, B). These data are consistent with those reported previously, confirming the enhancement by quinidine of diclofenac and *R*-warfarin metabolism attributed to CYP3A4 cooperativity (Ngui *et al.* 2000, 2001).

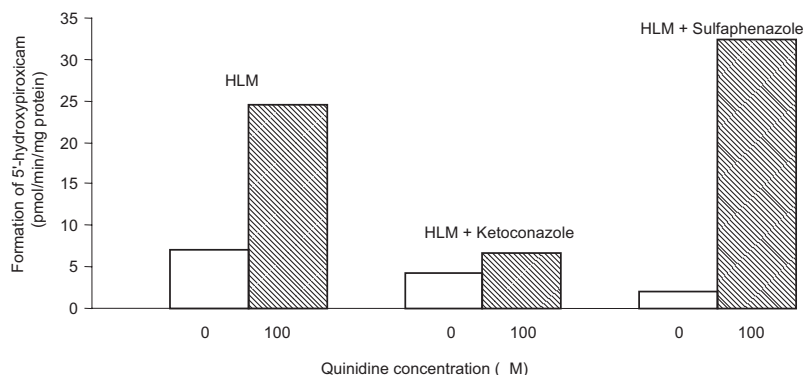


Figure 3. Effects of quinidine, ketoconazole and sulfaphenazole on the 5'-hydroxylation of piroxicam in incubations with human liver microsomes. The concentration of piroxicam was 50 μ M; experiments were performed in duplicate.

The rate of formation of 5'-hydroxypiroxicam in human liver microsomes was 7 $\text{pmol min}^{-1} \text{mg}^{-1}$ protein at 50 μ M piroxicam. A 3.5-fold increase in the metabolic turnover of piroxicam was associated with incubations containing 100 μ M quinidine (figures 3 and 4C). This enhancement by quinidine of the 5'-hydroxylation of piroxicam decreased to 1.5-fold of control values following pretreatment of human liver microsomes with ketoconazole. Conversely, the degree of enhancement of piroxicam metabolism was 15-fold that of controls after human liver microsomes were pretreated with sulfaphenazole (figure 3). Ketoconazole and sulfaphenazole are selective inhibitors of CYP3A4 and CYP2C9, respectively, at the concentrations used in the present study (Newton *et al.* 1995). These data together suggest that the CYP3A4 cooperativity elicited by quinidine is responsible for the observed augmentation of piroxicam metabolism in human liver microsomal incubations.

Cooperative drug interactions in recombinant CYP systems

The rates of the 5-, 5'- and 10-hydroxylation of diclofenac, piroxicam and *R*-warfarin, respectively, in incubations with CYP3A4-Lym were 2, 8 and 6 $\text{pmol min}^{-1} \text{pmol}^{-1}$. The substrate concentration in these cases was 50 μ M. Metabolic turnover of diclofenac, piroxicam and *R*-warfarin increased when incubations were performed in the presence of quinidine, in a manner similar to that observed with human liver microsomes. Thus, the augmentation at 100 μ M quinidine was approximately fivefold for the formation of 5-hydroxydiclofenac, 15-fold for the formation of 5'-hydroxypiroxicam and sixfold for the formation of 10-hydroxywarfarin, relative to respective controls (figures 3 and 4). These data further support the concept that enhancement of diclofenac, piroxicam and *R*-warfarin metabolism is due to CYP3A4 cooperativity mediated by quinidine.

The rates of the 5-, 5'- and 10-hydroxylation of diclofenac, piroxicam and *R*-warfarin, respectively, in incubations with CYP3A4-Bac were 3, 18 and 7 $\text{pmol min}^{-1} \text{pmol}^{-1}$. The substrate concentration was 50 μ M. In contrast to CYP3A4-Lym, metabolic turnover of the three substrates in incubations with CYP3A4-Bac was not affected by quinidine (figure 4). In other words, neither enzyme cooperativity nor inhibition was observed with CYP3A4-Bac in the presence of quinidine (figure 4). Experiments with diclofenac concentrations

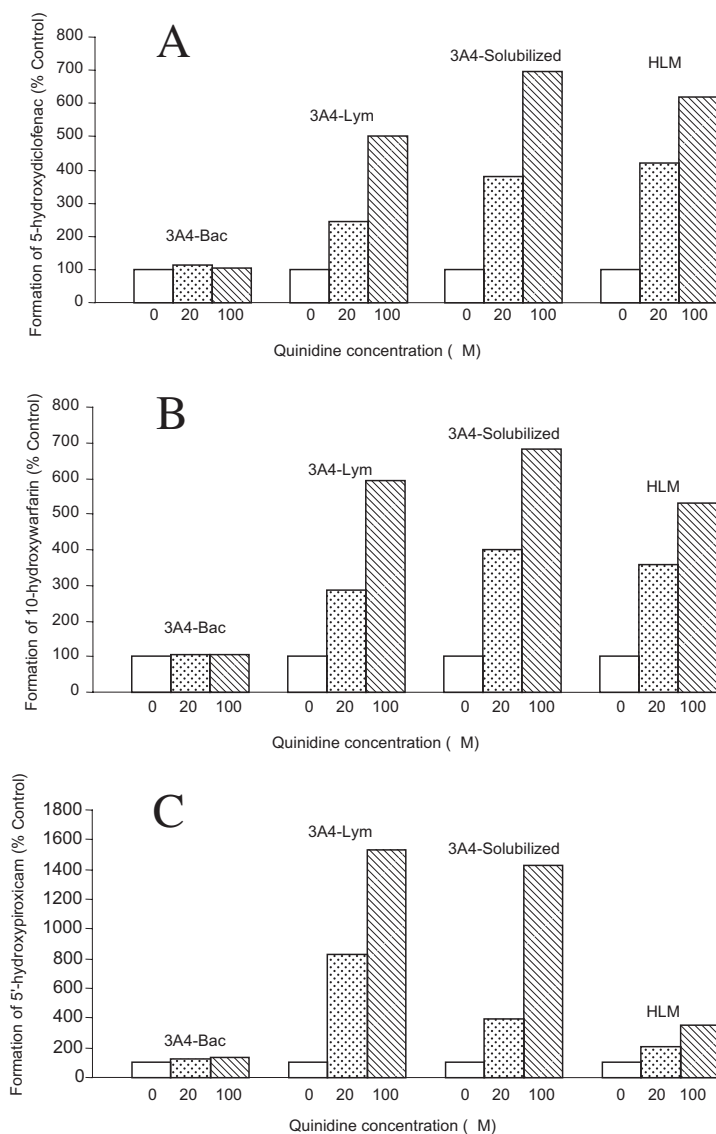


Figure 4. Effects of quinidine on diclofenac (A), *R*-warfarin (B) and piroxicam (C) metabolism in incubations with human liver microsomes or recombinant CYP3A4. The concentration of the substrates was $50 \mu\text{M}$. Activity, expressed as the percentage of control, was based on the formation of 5-hydroxydiclofenac, 10-hydroxywarfarin and 5'-hydroxypiroxicam in test incubations relative to the corresponding values in control experiments that lacked quinidine. 3A4-Bac, CYP3A4 expressed in baculovirus infected insect cells; 3A4-Lym, CYP3A4 expressed in human lymphoblast cells; 3A4 (solubilized), CYP3A4-Bac solubilized by CHAPS; HLM, human liver microsomes. The rates of the 5-hydroxylation of diclofenac were $245 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein in human liver microsomal incubations, and 3, 2 and $6 \text{ pmol min}^{-1} \text{ pmol}^{-1}$, respectively, in incubations with 3A4-Bac, 3A4-Lym and 3A4 solubilized. The rates of the 10-hydroxylation of *R*-warfarin were $9 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein in human liver microsomal incubations, and 7, 6 and $0.3 \text{ pmol min}^{-1} \text{ pmol}^{-1}$, respectively, in incubations with 3A4-Bac, 3A4-Lym and 3A4 solubilized. The rates of the 5'-hydroxylation of piroxicam were $7 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein in incubations with human liver microsomes, and 18, 8 and $0.7 \text{ pmol min}^{-1} \text{ pmol}^{-1}$, respectively, in incubations with 3A4-Bac, 3A4-Lym and 3A4 solubilized. Experiments were performed in duplicate.

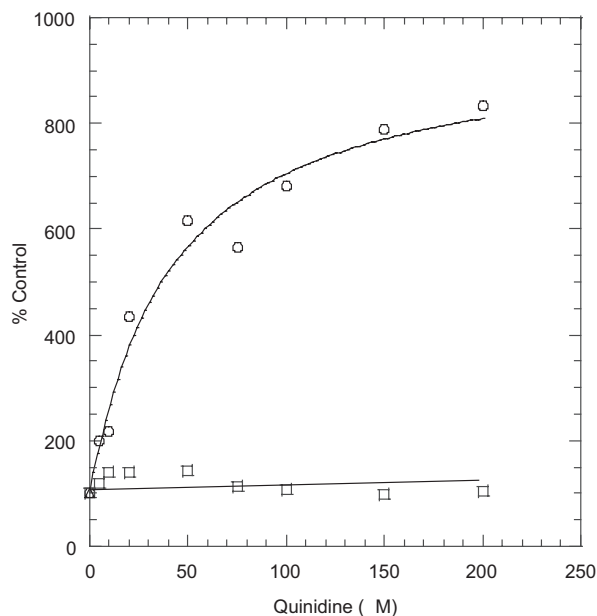


Figure 5. Enhancement by quinidine of the 5-hydroxylation of diclofenac in incubations with solubilized CYP3A4 (○). Data derived from CYP3A4-Bac are included for comparison (□). The concentration of diclofenac was 50 μM . The reaction rate in control incubations (absence of quinidine) was 6 $\text{pmol min}^{-1} \text{pmol}^{-1}$ for solubilized CYP3A4 and 3 $\text{pmol min}^{-1} \text{pmol}^{-1}$ for CYP-Bac. Experiments were performed in duplicate.

varying from 1 to 200 μM resulted in the same conclusion (data not shown). Quinidine is a substrate of CYP3A4-Bac. The rates of the 3-hydroxylation and *N*-oxidation of quinidine in incubations with CYP3A4-Bac remained unchanged in the presence of 0–200 μM diclofenac (data not shown).

Subsequently, recombinant CYP3A4-Bac was treated with CHAPS, and the resulting solubilized enzyme was employed for further studies. The rate of formation of 5-hydroxydiclofenac in this preparation was 6 $\text{pmol min}^{-1} \text{pmol}^{-1}$ at 50 μM substrate. Enhancement by quinidine of diclofenac metabolism was apparent in that the formation of 5-hydroxydiclofenac increased with increasing quinidine concentrations, reaching a maximum of eightfold relative to the control value (figures 4 and 5). The rates of the 5'- and 10-hydroxylation of piroxicam and *R*-warfarin, respectively, were 0.7 and 0.3 $\text{pmol min}^{-1} \text{pmol}^{-1}$ in incubations with solubilized CYP3A4. The concentration of the substrates was 50 μM . Both metabolic reactions were enhanced by quinidine and the magnitude of the augmentation was comparable with that observed with human liver microsomes or CYP3A4-Lym (figure 4).

Cytochrome- b_5 was examined for its effect on CYP3A4 cooperativity. Enhancement by quinidine of the hydroxylation of the three substrates was equivalent, regardless of whether incubations with solubilized CYP3A4 were performed in the presence or absence of cytochrome- b_5 (data not shown).

Kinetic characteristics of diclofenac and quinidine interactions mediated by solubilized CYP3A4

The 5-hydroxylation of diclofenac and 3-hydroxylation of quinidine in incubations with solubilized CYP3A4 were described by a kinetic model based

Table 1. Kinetic parameters for the interaction of diclofenac and quinidine with CYP3A4.^a

Reaction	K_{S1}^b (μM)	K_{S2}^b (μM)	$V_{\max}^b$ ($\text{pmol min}^{-1} \text{pmol}^{-1}$)	Factor ^b	Equation ^b
Quinidine 3-hydroxylation	27 ± 2	114 ± 15	26.7 ± 0.9 ($V_{\max Q}$)	1.1 ± 0.1 (α)	1
Diclofenac 5-hydroxylation	28 ± 3	116 ± 9	18.9 ± 3.1 ($V_{\max D}$)	10.1 ± 1.7 (β)	2

^a Kinetic model is shown in figure 2; equations are described in the Materials and methods.

^b $V_{\max Q}$ and $V_{\max D}$, maximum velocities for the formation of 3-hydroxyquinidine and 5-hydroxydiclofenac, respectively; K_{S1} , dissociation constant for $E \rightleftharpoons ES_1$ and K_{S2} for $E \rightleftharpoons ES_2$ (S_1 = quinidine, S_2 = diclofenac); α and β , factors by which $V_{\max}$ values increase (or decrease) when the second substrate is included in the interaction with the enzyme. Data are the mean $\pm$ standard error.

on assumptions of rapid equilibria between substrate and substrate–enzyme complexes, fast release of products, and the presence of independent substrate-binding domains (Ngui *et al.* 2000). Experimental data were fit to velocity equations derived from the model. The resulting dissociation constants were about 115 and about 27 μM for the diclofenac–CYP3A4 and quinidine–CYP3A4 complexes, respectively, and these constants remained essentially unchanged regardless of the binding of a second substrate (or an effector) to the enzyme (table 1). The rate of formation of 5-hydroxydiclofenac from the diclofenac–CYP3A4–quinidine complex increased 10-fold compared with that from the diclofenac–CYP3A4 complex, whereas the rate of formation of 3-hydroxyquinidine from the quinidine–CYP3A4–diclofenac complex was similar to that from the quinidine–CYP3A4 complex (figure 6 and table 1).

Discussion

The 5-hydroxylation of diclofenac and 10-hydroxylation of *R*-warfarin in incubations with human liver microsomes were shown previously to be enhanced by quinidine (Ngui *et al.* 2000, 2001). These *in vitro* drug interactions appeared to result from alteration of CYP3A4 activities, as increases in the formation of diclofenac and *R*-warfarin metabolites were diminished following pretreatment of human liver microsomes with a monoclonal antibody against the enzyme (Ngui *et al.* 2000, 2001). Consistent with this conclusion, enhancement by quinidine of the 5- and 10-hydroxylation of diclofenac and *R*-warfarin, respectively, was observed in the present study with recombinant CYP3A4-Lym. Piroxicam and quinidine also were subject to a drug interaction that could be attributed to CYP3A4 cooperativity, in which the rate of 5'-hydroxylation of piroxicam was increased several-fold in human liver microsomal incubations containing quinidine, while increases in piroxicam metabolism were absent once liver microsomes were treated with ketoconazole, a selective inhibitor of CYP3A4. In addition, enhancement by quinidine of the 5'-hydroxylation of piroxicam was apparent with recombinant CYP3A4-Lym. This was in agreement with a previous report that the metabolism of meloxicam is stimulated by quinidine, since piroxicam and meloxicam are close structural analogues of one another (Ludwig *et al.* 1999).

It is clear that CYP cooperativity is not merely a microsomal artefact; drug interactions similar to those described above have also been demonstrated in hepatocyte systems and *in vivo* in animals (Tang *et al.* 1999a, Ngui *et al.* 2000). More recently, CYP3A4 cooperativity has been implicated as a possible

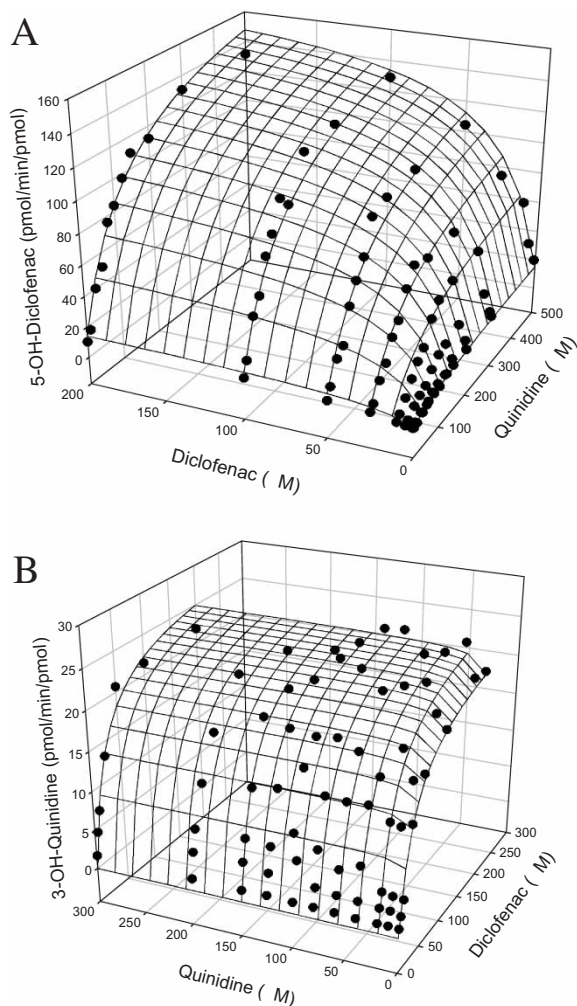


Figure 6. Formation of 5-hydroxydiclofenac (5-OH-diclofenac; A) and 3-hydroxyquinidine (3-OH-quinidine; B) in incubations with solubilized CYP3A4. The surfaces are the theoretical fits to equations (1) and (2). Experiments were performed in duplicate.

mechanism for the drug interaction between felbamate and carbamazepine in human subjects (Egnell *et al.* 2003). Thus, the differences between recombinant CYP3A4 expressed in human lymphoblast cells versus that expressed in insect cells were initially puzzling with respect to enzyme cooperativity. Enhancement by quinidine of the 5-, 5'- and 10-hydroxylation of diclofenac, piroxicam and *R*-warfarin, respectively, was detected with the former recombinant system but not with the latter. A plausible explanation for these findings was that CYP3A4 cooperativity was masked by expression in insect cells via a mechanism yet to be identified. If this was the case, then 'liberation' of the enzyme from its host membrane should result in restoration of the cooperativity. To test this hypothesis, CYP3A4-Bac was treated with CHAPS and the microsomal membrane-embedded enzyme was solubilized in buffer. Incubations then were performed with solubilized CYP3A4 and enhancement of diclofenac, piroxicam and *R*-warfarin

metabolism immediately became apparent in the presence of quinidine. The magnitude of increases in metabolite formation was comparable with that observed in studies with human liver microsomes or with recombinant CYP3A4-Lym. Cytochrome-b₅ did not appear to play a role in this case, since addition of this protein did not influence the enhancement by quinidine of the activity of solubilized CYP3A4. These data are consistent with the hypothesis that cooperativity is an intrinsic property of human hepatic CYP3A4.

While the underlying mechanisms of CYP heterotropic cooperativity remain obscure, several preliminary models have been postulated, one of which attributes the increases in CYP activities to the presence of multiple substrate-binding domains in the active site of the enzyme (Harlow and Halpert 1998, Korzekwa *et al.* 1998, Hosea *et al.* 2000). With respect to CYP3A4 cooperativity, a kinetic model has been developed to account for the stimulation of diclofenac metabolism by quinidine in human liver microsomal incubations (Ngui *et al.* 2000). A similar model was used in the present study to illustrate the cooperative interactions between diclofenac and quinidine in incubations with solubilized CYP3A4. In this model, the rate of diclofenac metabolism increased in the presence of quinidine, whereas quinidine metabolism was unaffected by diclofenac, implying simultaneous access to the ferrihaem–oxygen complex by the two substrates. The binding affinity of diclofenac to the enzyme, assessed by dissociation constants, remained little changed upon binding of quinidine, and vice versa. The results can be interpreted to indicate the existence of two distinct binding domains in the active site of CYP3A4, one for the substrate and the other for the effector, which also is a substrate. The binding of either substrate is independent of the other. It is also possible that a third binding domain exists, occupation of which by the effector could elicit changes in enzyme conformation leading to cooperativity (Koley *et al.* 1997, Ueng *et al.* 1997, Atkins *et al.* 2001).

Microsomal CYP enzymes are a group of membrane proteins whose catalytic domains are exposed to the cytoplasm with a loose association with the transmembrane helices (Johnson 2003). The catalytic efficiency and substrate specificity of a CYP, however, have been suggested to depend highly on membrane components such as phospholipids (Ingelman-Sundberg 1986). It is conceivable that CYP cooperativity also is influenced by host membranes, since (1) cooperativity in nifedipine metabolism, measured by changes in the Hill coefficient, increased with human liver microsomal incubations containing 7,8-benzoflavone, whereas similar phenomena were not observed with human intestinal microsomes; and (2) stimulation by testosterone of nifedipine oxidase occurred in incubations with human liver microsomes but not with the recombinant enzyme (Emoto *et al.* 2001, Niwa *et al.* 2003). In the present study, CYP3A4-Bac also failed to exhibit the positive cooperativity evident with human liver microsomes, suggesting that certain properties of the native protein were altered by expression in insect cells. An interesting observation with CYP3A4-Bac was that despite the absence of cooperative drug interactions, diclofenac metabolism was not inhibited by quinidine, and vice versa, implying retention of simultaneous access by the two substrates to the active site of the membrane-embedded enzyme. In other words, two substrate-binding domains were available in CYP3A4-Bac: one for diclofenac, the other for quinidine (figure 7). The same seemed also to hold true for the combinations of piroxicam–quinidine and warfarin–quinidine. Subsequent solubilization of CYP3A4-Bac from insect cell microsomes was performed with

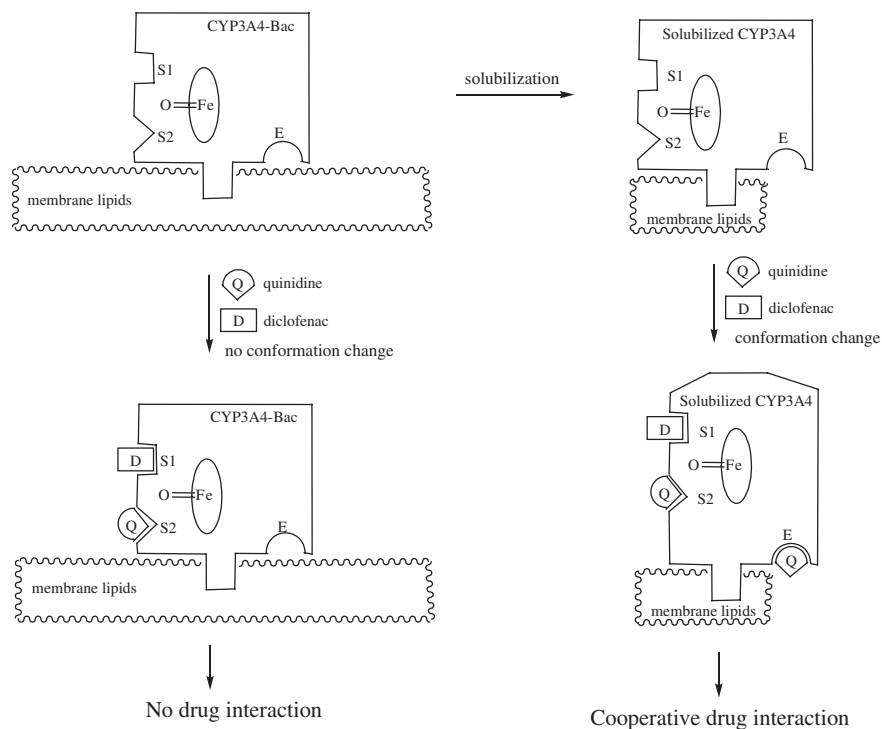


Figure 7. Interactions of diclofenac and quinidine with CYP3A4 expressed in insect cells before and after solubilization. In cases where the quinidine substrate-binding domain (S2) also functioned as an effector-binding domain, this model would be identical to the kinetic model shown in figure 2.

CHAPS, a zwitterionic detergent widely used during reconstitution of CYP monooxygenase systems. The process is believed to involve removal by the detergent of a large amount of membrane lipids surrounding the enzyme (Le Maire *et al.* 2000). Thus, restoration of the cooperativity with solubilized CYP3A4 can be taken as a reflection of changes in enzyme conformation on quinidine binding, such that the new conformation is optimal for diclofenac metabolism. It is also conceivable that a third effector-binding domain, which is inaccessible to quinidine in CYP3A4-Bac, becomes available for binding following solubilization (figure 7). A recent study of the crystal structure of CYP2C9 indicated the presence of a warfarin-binding domain that was too distant from the haem prosthetic group for hydroxylation to occur, but which might play a role in mediating allosteric effects (Williams *et al.* 2003).

In summary, the present study has demonstrated a distinction between CYP3A4 expressed in insect cells versus that expressed in human lymphoblast cells, with the former failing to exhibit enzyme cooperativity elicited by quinidine. Restoration of CYP3A4 cooperativity by 'freeing' the enzyme from insect cell microsomes indicates that some of the intrinsic properties of a CYP are altered during expression in recombinant systems. This is an important finding, since the development of kinetic models to explain the cooperativity exhibited by CYP3A4 and related enzymes depends largely on experimental data derived from recombinant enzyme preparations. Although preliminary in nature, the studies reported herein highlight further the complex catalytic behaviour of CYP3A4 and provide

additional evidence for the existence of multiple binding domains within the active site of the enzyme.

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Disposition and pharmacokinetics of temozolomide in rat

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1. Temozolomide, an imidazotetrazine derivative, is a cytotoxic alkylating agent of broad-spectrum antitumour activity. The absorption, metabolism, distribution and excretion of temozolomide have been investigated in male and female Sprague–Dawley and Long–Evans rats following single oral or intravenous dose administration of 200 mg m⁻² non-radiolabelled or ¹⁴C-radiolabelled temozolomide. The distribution of ¹⁴C-temozolomide was also evaluated by whole-body autoradiography in male Sprague–Dawley rats. Plasma concentrations of temozolomide and its active metabolite 3-methyl-(triazene-1-yl)imidazole-4-carboxamide (MTIC) were determined by high-performance liquid chromatography (HPLC) with ultraviolet detection. Plasma, urine and faeces were profiled by HPLC with radiochemical detection.

2. Temozolomide was rapidly and extensively (>90%) absorbed and widely distributed in tissues. The distribution pattern of radioactivity was gender independent. Penetration into the brain following oral or intravenous administration was 35–39% based on the brain/plasma AUC ratio.

3. Following intravenous or oral administration, temozolomide was primarily eliminated renally (75–85% of the dose) as either unchanged drug, a carboxylic acid analogue, AIC (a degradation product) and a highly polar unidentified peak. Biliary excretion was minimal (1.4–1.6%). The pharmacokinetics (oral versus intravenous) were similar and gender independent. The absolute oral availability was 96–100%. Temozolomide was rapidly eliminated ($t_{1/2}$ = 1.2 h) and converted to MTIC.

4. Systemic exposure to MTIC was about 2% that of temozolomide. Overall, the disposition of temozolomide in rats was similar to that observed in humans.

Introduction

Temozolomide, an imidazotetrazine derivative, is a cytotoxic alkylating agent that undergoes spontaneous hydrolysis at physiological pH to its active metabolite 3-methyl-(triazene-1-yl)imidazole-4-carboxamide (MTIC), which in turn degrades to a reactive methyl-diazonium cation and 5(4)-aminoimidazole-4(5)-carboxamide (AIC) (figure 1) (Slack *et al.* 1986, Stevens *et al.* 1987, Tsang *et al.* 1991, Wheelhouse and Stevens 1993, Denny *et al.* 1994).

The cytotoxicity of MTIC is thought to be primarily due to alkylation of DNA at the O⁶ and N⁷ positions of guanine (Denny *et al.* 1994). MTIC is also believed to be the active metabolite of dacarbazine, which is widely used for the treatment of malignant melanoma (Tsang *et al.* 1991). The final degradation product, AIC, is an intermediate on the biosynthetic pathway to purines and ultimately to nucleic acids.

Preclinical studies indicated that temozolomide possessed antitumour activity when administered intraperitoneally against haematological (L1210 and P388

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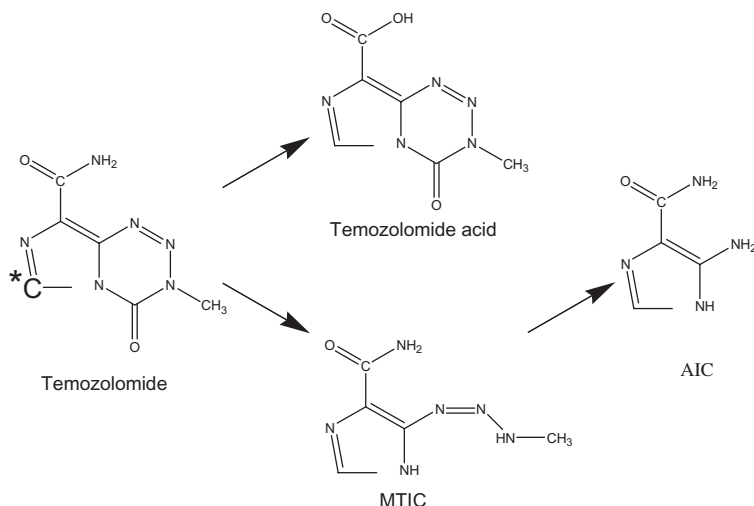


Figure 1. Chemical structure of temozolomide [8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4 (3H)-one], temozolomide acid metabolite, MTIC (3-methyl-(triazen-1-yl)imidazole-4-carboxamide), and AIC. *Site of the ^{14}C label.

leukaemias) and solid murine tumour models (e.g. M5076 sarcoma, ADJ/PC6A plasmacytoma, B16 melanoma, Lewis lung sarcoma) (Stevens *et al.* 1987). The antitumour activity of temozolomide was schedule dependent, with multiple-dose administration being more effective than a single bolus dose. Temozolomide is currently approved for the treatment of recurrent glioma and metastatic malignant melanoma. Temozolomide has also been demonstrated to be cytotoxic in human melanoma, ovarian and colon tumour xenografts (Carter *et al.* 1994).

The objectives of the current study were to assess the absorption, distribution, metabolism, excretion of temozolomide and pharmacokinetics of temozolomide and MTIC following oral and intravenous (i.v.) administration to Sprague–Dawley and Long–Evans male and female rats.

Materials and methods

Formulation

The structure of temozolomide and position of the ^{14}C label are shown in figure 1. Temozolomide was from Schering–Plough Research Institute (Kenilworth, NJ, USA). [^{14}C]-radiolabelled (radiochemical purity >99%, specific activity $10.1 \mu\text{Ci mg}^{-1}$) or non-radiolabelled (purity 98.7%) temozolomide was formulated as a 0.4% (w/v) aqueous methylcellulose suspension for oral administration and as a solution with dimethyl sulfoxide in 0.9% saline (20% v/v) for i.v. administration.

Animals

Male and female Sprague–Dawley and Long–Evans rats were obtained from Charles River Laboratories, Inc. (Wilmington, WA, USA) and weighed 183–373 g on the day of dosing. Within a study, individual body weights were within ± 2 standard deviations from the mean body weight of each sex, and group mean body weights for each group of each sex were not statistically different at the 0.5% probability level. For the autoradiography study, male Sprague–Dawley rats weighing 116–140 g were used. Animals were housed in stainless-steel cages before dosing and provided with certified rodent diet #5002 (PMI[®], Inc., Madison, WI, USA) or Teklad Premier Laboratory diet and water *ad libitum*. For the excretion study, the rats were housed in Nalgene[®] metabolism cages (Nalge Co., Rochester, New York, USA) or glass metabolism cages (Crown Glass Co., Somerville, NJ, USA).

To collect bile, each rat was anaesthetized with Ketaset® (ketamine HCl; Fort Dodge Laboratories, Fort Dodge, IA, USA) and a midline abdominal incision along the linea alba was made on the day of dosing. The common bile duct was exposed, cannulated with PE-50 tubing and exteriorized. Following surgery, the rats were fitted with rodent jackets and tether lines, and placed in individual metabolism cages. The rats were allowed to recover fully from anaesthesia before dosing.

Dosing

Rats were fasted overnight before dose administration and approximately 4 h post-dose. The target dose of 200 mg m⁻² was administered either i.v. via the lateral tail vein or orally by gavage using a ball-tipped needle. All doses were determined based on body surface area using the following calculation (Freireich *et al.* 1996):

$$S = \frac{K \cdot W^{2/3}}{10000}$$

where S is the surface area (m²), W is the body weight taken on the day of dosing (g) and $K=9.0$ (constant for estimating surface area). All doses were administered within 4 h of dose preparation.

Blood collection

Blood samples (approximately 1 ml) were collected from four rats/gender/time point by cardiac puncture under carbon dioxide-induced anaesthesia at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h post-dose. Blood was collected into prechilled syringes and pooled at each time point/gender into prechilled heparinized Vacutainer® tubes. Plasma was collected and separate aliquots were removed for temozolomide and MTIC analysis.

In vitro stability of temozolomide is pH sensitive. Temozolomide is stable at pH < 5 and labile at pH > 7. In aqueous buffer (pH 7.0 or 7.4), temozolomide *in vitro* hydrolysis $t_{1/2}$ ranges from 1.24 to 1.83 h. The $t_{1/2}$ for the *in vitro* degradation of temozolomide in plasma at 37°C is shorter than that reported in buffered solutions and ranges from 15 to 35 min (Slack *et al.* 1986, Stevens *et al.* 1987). The shorter $t_{1/2}$ values are probably the result of increases in plasma pH during incubation at 37°C (Welch *et al.* 1995). Therefore, plasma samples designated for temozolomide analysis were stabilized by adding 8.5% phosphoric acid (H₃PO₄) (3 µl/100 µl plasma).

MTIC is unstable below pH 7, but somewhat more stable at alkaline pH. *In vitro* degradation $t_{1/2}$ values for MTIC range from 2 to 8 min (Denny *et al.* 1994). To prevent decomposition, plasma samples designated for MTIC analysis were collected on ice, immediately centrifuged at 4°C and flash-frozen on dry ice. The sample procurement time for MTIC was kept under 20 min. All plasma samples were stored below -65°C before HPLC analyses.

AIC has no pharmacological activity and is not considered a major metabolite of temozolomide. Therefore, plasma AIC concentrations were not determined.

Distribution in the brain

After each blood collection, the animals were euthanized with carbon dioxide and the brain was collected, weighed, homogenized, preserved in acidic media using saline and 0.3% H₃PO₄ (20% w/v) and stored at -70°C until HPLC analyses.

Pharmacokinetic analysis

Mean plasma and brain concentration-time data were analysed by non-compartmental methods (Gibaldi and Perrier 1982). Plasma concentration-time profiles for total radioactivity were also analysed by non-compartmental methods. The maximum plasma concentration ($C_{\max}$) and time of maximum plasma concentration ($T_{\max}$) were the observed values. The terminal phase rate constant (K) was calculated as the negative of the slope of the log-linear terminal portion of the plasma concentration-time curve using linear regression. The terminal phase half-life ($t_{1/2}$) was calculated as $0.693/K$. The area under the plasma concentration-time curve from time 0 to the time of the final quantifiable sample [$\text{AUC}(t_f)$] was calculated using the linear trapezoidal method and extrapolated to infinity (inf) as follows:

$$\text{AUC}(\text{inf}) = \text{AUC}(t_f) + C_{\text{est}}(t_f)/K,$$

where $C_{\text{est}}(t_f)$ is the estimated concentration determined from the linear regression at time t_f . The absolute bioavailability (F) was estimated from the plasma $\text{AUC}(\text{inf})$ data of unchanged temozolomide after parenteral and oral treatments.

Mass balance/excretion

Urine was collected into glass containers surrounded by dry ice, each containing 10% H₃PO₄ (about 1 ml) at 0-4, >4-24, >24-48, >48-72, >72-96, >96-120, >120-144 and >144-168 h post-dose.

Faeces were collected in the same manner at intervals of 0–24, >24–48, >48–72, >72–96, >96–120, >120–144 and >144–168 h post-dose.

Following each daily excreta collection (at 24-h intervals), cages were rinsed with approximately 10 ml deionized water into plastic containers each containing about 3 ml 10% H_3PO_4 . After the last excreta collection, cages were washed with about 400 ml 1% trisodium phosphate and wiped with gauze pads. The wash was collected into plastic containers, each of which contained about 12 ml 10% H_3PO_4 . Expired air was passed through activated charcoal traps to recover volatile organic compounds, then through carbon dioxide trapping solutions (each containing about 400 ml 2-ethoxyethanol:ethanolamine, 1:1) to recover radiolabelled carbon. These samples were collected at 24-h post-dose intervals up to 168 h post-dose.

At 168 h post-dose, each rat was killed via overexposure to halothane anaesthesia. The carcasses were also retained for radioanalysis. All urine, faeces and carcass samples were stored frozen at approximately -70°C before analysis.

Tissue distribution

Female Long-Evans rats were administered a single 200 mg m^{-2} oral dose of temozolomide. Animals were killed at 0.25, 2, 6, 12, 24 and 72 h post-dose and selected tissues were excised, rinsed with 10% H_3PO_4 , blotted dry, weighed and stored at -20°C until analysed.

Instrumentation

Sample combustions were performed in a Model 306 or 307 Sample Oxidizer (Packard Instrument Co., Meriden, CT, USA) or a Model OX-300 biological Oxidizer (R. J. Harvey Instrument Co., Hillsdale, NJ, USA). The resulting $^{14}\text{CO}_2$ was trapped in Carbo-Sorb[®], and the radioactivity was quantitated by liquid scintillation spectrometry (LSS). Plasma, urine and faeces were assayed for total radioactivity using a Packard Liquid Scintillation Counter, Model 2550TR/LL, 1900TR, 1500 or 4640 for at least 5 min or until 100 000 counts were accumulated.

A Leica Cryomacrot[®] (Heidelberg, Germany) microtome (Serial No. 00441249) was used to section rat tissues.

Determination of radioactivity

Plasma and urine were separately mixed with appropriate volumes of Ultima Gold[®] Liquid Scintillation Cocktail (Packard, Meriden, CT, USA) and analysed by LSS. Scintillation counting data (cpm) were automatically corrected for counting efficiency using the external standardization technique and an instrument-stored quench curve was generated from a series of sealed quenched standards or Ultima-Gold[®] or Permafluor[®] E+ scintillation cocktail (Meriden, CT, USA).

Faeces were homogenized with water (1:3) using a Stomacher[®] mixer (Stomacher Lab blender Model 3500; Seaward, London, UK), then oxidized using a Packard TRI-CARB[®] Oxidizer. Residues were mixed with Permafluor[®] Liquid Scintillation Cocktail and analysed by LSS.

Gastrointestinal tissues were digested using Unisol[®] (Isolabs, Inc., Akron, OH, USA). Carcasses were digested by submerging the entire rat in 500 ml 6N potassium hydroxide solution. Aliquots (0.5 ml) of either each rat carcass homogenate or gastrointestinal tissues were mixed with 0.5 ml methanol to prevent precipitation and 10 ml Unisol-ComplementTM scintillation cocktail, then analysed by LSS.

Autoradiography

Two rats/time point at 0.25, 1, 3, 6, 24 and 168 h after dose administration were euthanized by carbon dioxide inhalation and the carcasses frozen in hexane-dry ice bath (-70°C). The frozen carcasses were embedded and frozen in 1.5% (w/v) aqueous carboxymethylcellulose blocks at -40°C until sectioned. Sagittal sections (30 μm thick) were harvested on 3M transparent tape #810 using a cryo-microtome. Sections were allowed to dry frozen for 48 h or until completely desiccated within the cryo-microtome chamber. The dried rat tissue sections were attached to a cardboard backing, covered with Saran[®] plastic wrap and placed against Fuji BAS III imaging plates. After a 24-h exposure in a lead-lined box, imaging plates were scanned using a Fuji BAS 2000 Image Analysis System (Tokyo, Japan) with AIS software (Ontario, Canada) and the resulting autoradiographs were visually evaluated for distribution of radioactivity.

Metabolite profiles

Blood/plasma was collected at 0, 0.08 (i.v. only), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, 72 and 120 h post-dose. Urine and faeces were collected in block intervals of 0–4, >4–8, >8–24, >24–48, >48–72, >72–96, >96–120, >120–144 and >144–168 h post-dose. Plasma, urine and faecal samples were pooled to obtain sufficient amount of radioactivity at each time point, stabilized by the addition of $30\text{ }\mu\text{l ml}^{-1}$ 10% H_3PO_4 and extracted with 5 ml ethyl acetate using an Eberbach shaker for 30–45 min. The mixture was separated by centrifugation at 2500 rpm for 10 min and the supernatant was removed.

Table 1. High-performance liquid chromatography (HPLC)/ultraviolet light assay validation parameters for temozolomide and MTIC.

Matrix	Linear range ($\mu\text{g ml}^{-1}$ or g)	Correlation coefficient	LLOQ ($\mu\text{g ml}^{-1}$ or g)	Precision (%CV)	Accuracy (%CV)
Temozolomide:					
Brain	0.1–20	≥ 0.9977	0.1	1.1–3.7	0.1–7.2
Plasma	0.1–20	≥ 0.9977	0.1	1.0–7.7	4–6
Urine	2–200	≥ 0.9973	2	1.4–4.3	–5.7 to 0.4
MTIC:					
Plasma	0.010–0.50	> 0.98	0.01	9.11–10.4	–0.25 to –10.5

CV, coefficient of variation.

LLOQ, lower limit of quantitation.

The aqueous portion was further extracted by repeating the ethyl acetate separation and centrifugation steps. The ethyl acetate layers were combined and evaporated to dryness in conical borosilicate tubes using a Turbo-vap evaporator under a constant stream of nitrogen at 30°C. Before injection, 20 μl dimethyl sulfoxide standard solution (containing 40 μg each of temozolomide, temozolomide acid metabolite and AIC) and 230 μl hexane sulfonic acid solution were added to each sample. The final samples were analysed for total radioactivity (20 μl) and 200 μl were injected onto the HPLC system.

Chromatography

All plasma, brain, urine and faecal homogenate samples were assayed by HPLC using the following equipment and analytical conditions. Plasma (0.1 ml), to which the internal standard (ethazolastone) had been added, was extracted with ethyl acetate. The organic phase was evaporated to dryness at 45°C. The residue was dissolved in 0.2 ml mobile phase and 20 μl of the supernatant were injected onto the HPLC system equipped with a C18 column and ultraviolet light detector at 316 nm.

Gradient solvent programme: methanol/10 mM hexane sulfonic acid-acetic acid (linear gradient, Waters Expert-Ease gradient #6, stepwise from 100% hexane sulfonic acid for 5 min to 3% methanol for 15 min and finally 10% methanol for 10 min) with a continuous flow rate of 1.0 ml/min.

The HPLC system consisted of a Waters Chromatography Model 510 solvent delivery pump (Milford, MA, USA), a Waters Model 712 Autoinjector, a Supelco LC-DB C-18 (25 cm) HPLC Column (Bellefonte, PA, USA), a Waters Model 490E Variable Wavelength Ultraviolet Detector (254 nm) and a Packard Instruments Radiomatic Model 525A Detector (Packard Flo-Scint V Liquid Scintillation Cocktail: eluent (3:1 ratio)).

The HPLC/ultraviolet light method was sensitive, accurate and precise for the measurement of temozolomide and MTIC in rat plasma, urine and brain (table 1).

Results

Plasma pharmacokinetics

Temozolomide was rapidly absorbed following oral administration to Long-Evans and Sprague-Dawley rats (figures 2 and 3). The mean C_{max} values for temozolomide ranged from 21.5 to 40.9 $\mu\text{g ml}^{-1}$ and were comparable with those observed for total radioactivity (31.8–45.9 $\mu\text{g eq. ml}^{-1}$) (table 2). The pharmacokinetics of temozolomide were gender independent and similar in Sprague-Dawley and Long-Evans rats. Exposures (AUC) were 55.2 and 56.4 $\mu\text{g h ml}^{-1}$ in male and female Sprague-Dawley rats, respectively. Both oral and i.v. routes of administration resulted in similar exposures to temozolomide (about 55 $\mu\text{g h ml}^{-1}$), indicating 100% bioavailability. Temozolomide was rapidly eliminated with a mean terminal phase half-life of about 1.2 h.

In plasma, temozolomide was rapidly converted to MTIC, its reactive metabolite (figure 1). Mean C_{max} values for MTIC were similar in male and female Sprague-Dawley rats (0.329 and 0.385 $\mu\text{g ml}^{-1}$) and were reached at 0.5 and 1.0 h, post-temozolomide administration, respectively. Mean AUCs were 1.04 and

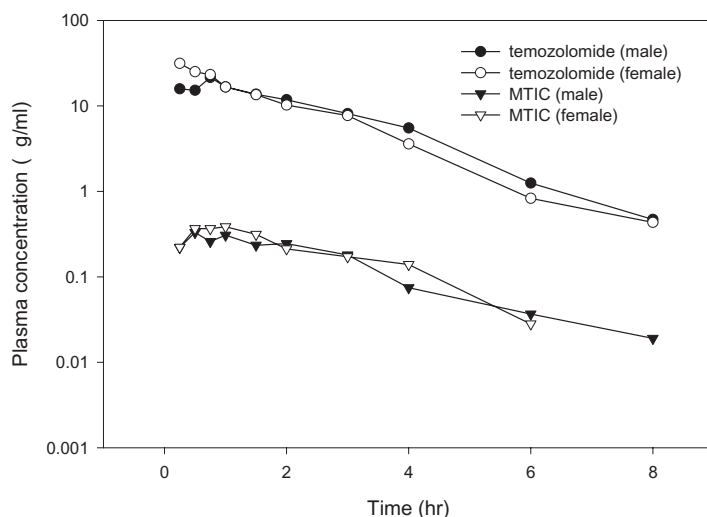


Figure 2. Mean plasma concentration-time profiles of temozolomide and MTIC following a single 200 mg m^{-2} oral temozolomide administration to Sprague-Dawley rats ($n = 3$ rats/time point).

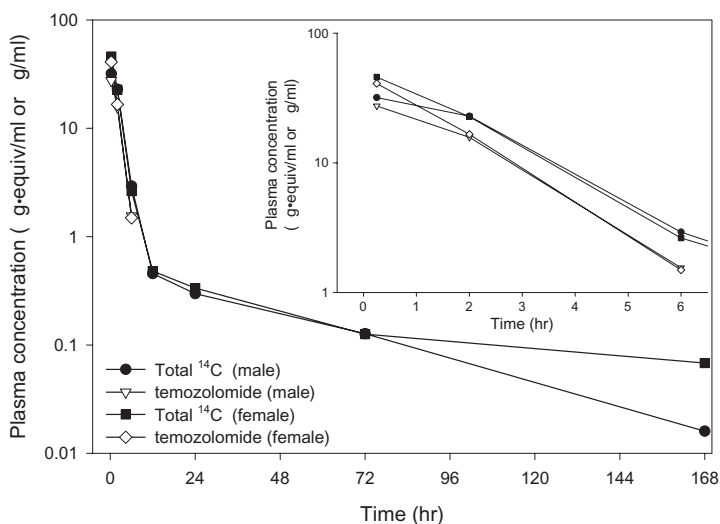


Figure 3. Mean plasma concentration-time profiles of temozolomide and total radioactivity following oral (200 mg m^{-2}) administration to Long-Evans rats. Data are the mean of five rats/time point.

$1.17 \mu\text{g h ml}^{-1}$ in male and female rats, respectively. MTIC was rapidly eliminated with a mean terminal phase half-life of 1.4–1.6 h.

Distribution in the brain

Maximum brain concentrations of temozolomide were $7.9 \mu\text{g ml}^{-1}$ in male and $8.3 \mu\text{g ml}^{-1}$ in female Sprague-Dawley rats following oral administration (figure 4 and table 3). These concentrations were observed at 0.5 and 1 h post-dose for the females and males, respectively, suggesting rapid penetration of circulating temozolomide into brain tissue and rapid conversion to MTIC. The AUCs

Table 2. Plasma temozolomide pharmacokinetic parameters following a single 200 mg m⁻² oral or intravenous dose of temozolomide to rats.

	Gender	$C_{\max}$ ($\mu\text{g ml}^{-1}$)	$T_{\max}$ (h)	AUC(inf) ($\mu\text{g h ml}^{-1}$)	$t_{1/2}$ (h)
Oral:					
Sprague–Dawley ^a :					
Temozolomide	male	21.5	0.75	55.2	1.17
	female	31.4	0.25	56.4	1.22
MTIC	male	0.329	0.5	1.04	1.64
	female	0.385	1.0	1.17	1.35
Long–Evans ^b :					
Temozolomide	male	27.5	0.25	78.8	1.4
	female	40.9	0.25	91.6	1.2
Total radioactivity	male	31.8	0.25	136	–
	female	45.9	0.25	151	–
Intravenous:					
Sprague–Dawley:					
Temozolomide	male	34.7	0.08	55.6	1.2
	female	35.3	0.08	50.6	1.1

^a $n = 3$ rats/gender/time point.^b $n = 5$ rats/gender/time point.Table 3. Brain temozolomide pharmacokinetic parameters following a single 200 mg m⁻² oral or intravenous dose of temozolomide to Sprague–Dawley rats.

	$C_{\max}$ ($\mu\text{g g}^{-1}$)	$T_{\max}$ (h)	AUC(inf) ($\mu\text{g h g}^{-1}$)	$t_{1/2}$ (h)
Oral:				
Male	7.9	1.0	19.8	1.3
Female	8.3	0.5	17.5	1.1
Intravenous:				
Male	11.4	0.25	21.8	1.2
Female	11.1	0.08	18.5	1.1

Data are the mean derived from four rats/time point.

were similar for males (19.8 $\mu\text{g h ml}^{-1}$) and females (17.5 $\mu\text{g h ml}^{-1}$) following oral administration. The terminal phase half-life (1.1–1.3 h) was also gender independent.

Following i.v. administration, temozolomide concentrations in the brain increased rapidly in males and females; a mean $C_{\max}$ of 11.4 $\mu\text{g g}^{-1}$ was achieved at 0.25 h in males, the corresponding $C_{\max}$ was 11.1 $\mu\text{g g}^{-1}$ at 0.08 h in females. AUCs were also comparable in males and females (21.8 and 18.5 $\mu\text{g h ml}$). The terminal phase half-life of 1.1–1.2 h indicated rapid elimination of the drug from brain tissue, similar to that observed in plasma. Based on the AUC ratio of brain/plasma, exposure to temozolomide in the brain ranged from 35 to 39% of that in plasma.

Tissue distribution

The distribution pattern of total drug-derived radioactivity was similar across genders and strains of rats (Long–Evans versus Sprague–Dawley). Figure 5 shows the concentration of total radioactivity in selected tissues in Long–Evans female rats after oral administration.

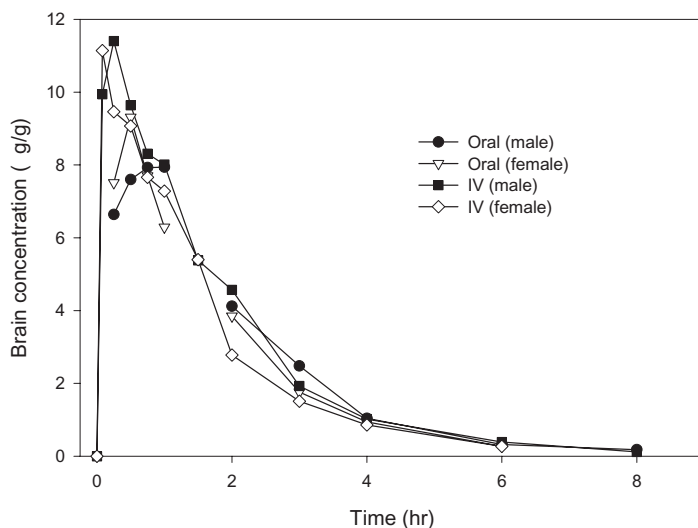


Figure 4. Mean brain concentration-time profiles following a single temozolomide administration to Sprague-Dawley rats ($n=4$ rats/time point).

Mean $C_{\max}$ values in many tissues were reached 0.25 h post-dose, indicating rapid penetration of drug-derived radioactivity into tissues. At 0.25 h post-dose, the tissues with the highest concentrations of radioactivity (excluding the gastrointestinal tract and contents) were the kidney, liver, bone marrow, spleen and heart with mean concentrations of 50.6, 37.2, 35.5, 34.5 and 32.7 $\mu\text{g eq. g}^{-1}$, respectively. Maximum concentrations in these tissues were higher than those found in plasma. All other tissues, including fat, which had the lowest maximum concentration, contained radioactivity lower than those seen in plasma. Radioactivity in tissues declined to less than 15 $\mu\text{g eq. g}^{-1}$ at 168 h post-dose, except for kidney, lung and liver, which had concentrations of 22.7, 16.7 and 15.1 $\mu\text{g eq. g}^{-1}$, respectively. The lowest radioactivity concentrations were observed in the reproductive fat (1.42–6.18 $\mu\text{g eq. g}^{-1}$) at all time points. Concentrations of radioactivity in the muscle also declined to low levels after 2 h post-dose (2.23 to 5.57 $\mu\text{g eq. g}^{-1}$).

Whole-body autoradiography

A representative whole-body autoradiography obtained at 1 h following oral administration to male Sprague-Dawley rats is shown in figure 5. Autoradiographical data suggest that ^{14}C -temozolomide was rapidly absorbed after oral dosing and extensively distributed into the tissues, including the brain. Radioactivity in the kidney peaked at 3 h, which was consistent with renal elimination of drug-derived radioactivity in the rat. The liver, testis, bone marrow, skeletal muscle, thymus and small intestine contents maintained relatively high levels of radioactivity. At 6 h post-dose, the liver, kidney, spleen, testis, bone marrow, thymus, salivary gland and wall of the small intestine retained relatively high concentrations of radioactivity whereas levels in the brain, heart and skeletal muscle were lower. At 24 h, the drug-derived radioactivity distributed throughout the rat was lower than that at 6 h and distribution was essentially limited to the liver and large intestinal wall. The salivary gland, bone marrow and Harderian

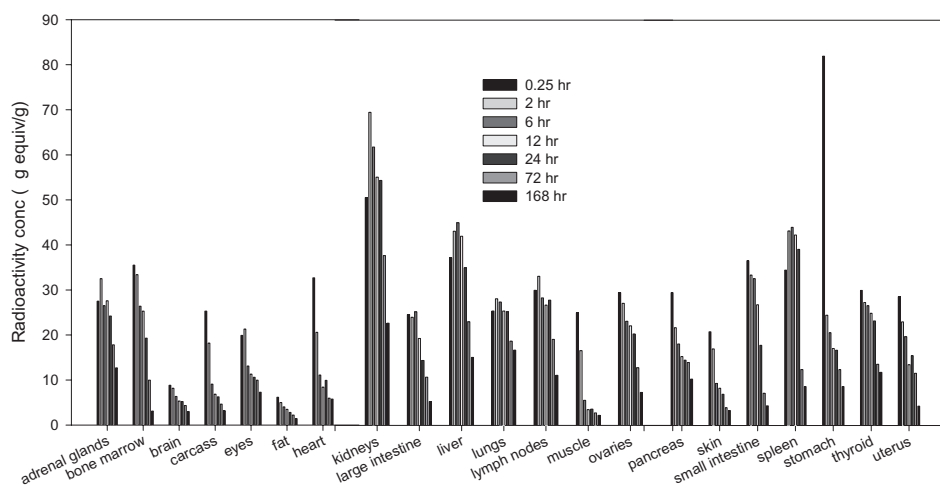


Figure 5. Mean concentrations of radioactivity in tissue following oral administration of 200 mg ^{14}C -temozolomide m^{-2} to female Long-Evans rats. Data are the mean of five rats/time point.

Table 4. Mean (%CV) percentage cumulative recovery of total radioactivity over 168 h post-dose following oral and intravenous administration of [^{14}C]-temozolomide to rats.

	Mean per cent radioactive dose			
	Sprague-Dawley ^c		Long-Evans	
	Male		Male	Female
	Intravenous	Oral	Oral	
Urine ^a	81.4 (3)	74.9 (3)	84.7	81.1
Faeces	3.50 (23)	6.44 (27)	2.84	2.85
Carbon dioxide	1.58 (20)	2.81 (27)	—	—
Carcass	8.75 (19)	13.6 (7)	9.86	9.83
Tissues ^b	—	—	5.34	8.40
Bile ^c	1.57 (50)	1.35 (41)	—	—
Gastrointestinal tract ^c	3.27 (57)	16.0 (116)	—	—
Total	95.2	97.7	103	102

^aIncludes cage rinse.

^bIncludes plasma.

^cSamples collected over 48 h post-dose (not included in the calculation of total recovered dose).

gland retained radioactivity at 24 h; however, levels in the testis and brain had diminished. At 168 h, autoradiographical data indicated marked clearance of drug-derived radioactivity from the rat, although some radioactivity remained throughout the entire section with the highest levels in the liver and kidney, testis and salivary gland. Overall, the results of the whole-body autoradiography showed similar distribution of radioactivity in various tissues as determined by liquid scintillation counting.

Excretion

The main route of elimination following a single i.v. or oral dose of ^{14}C -drug derived radioactivity was via renal excretion. On average, 81% (range 75–85%)

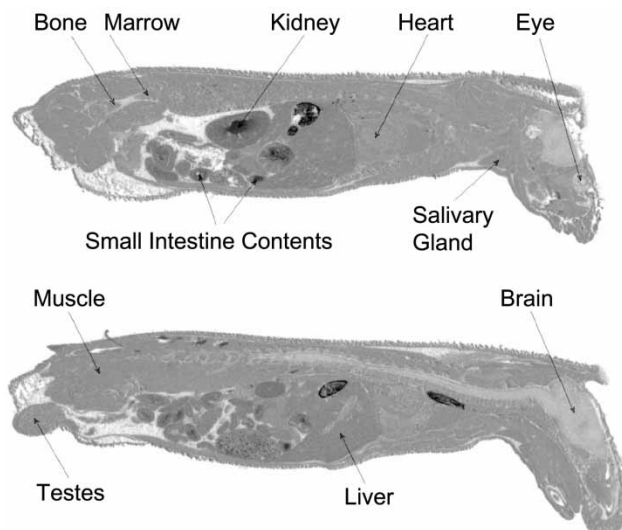


Figure 6. Representative whole-body autoradiographs of radioactivity in a male Sprague-Dawley rat 1 h after oral administration of 200 mg ^{14}C -temozolomide m^{-2} .

of the dose were excreted in the urine over 168 h after i.v. and oral dosing (table 4). Comparison of urinary excretion following i.v. and oral administration demonstrated that ^{14}C -temozolomide was well absorbed (>90%) following oral administration.

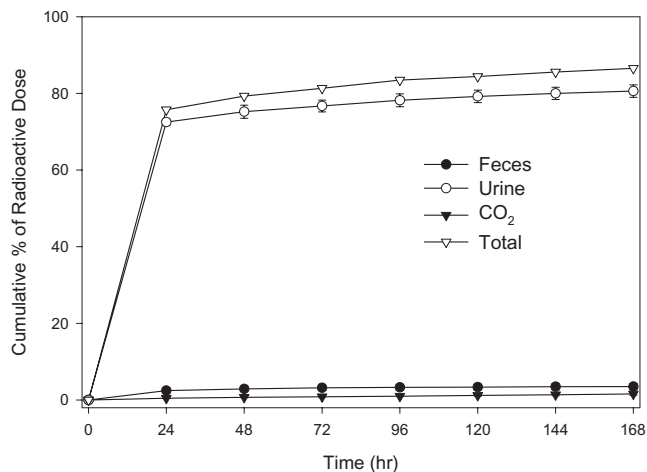
The initial rate of excretion of drug-derived radioactivity was rapid after either i.v. or oral administration (figure 7). Within 24 h post-dose, 75.2% of the i.v. dose and 66.2% of the oral dose was excreted in the urine (including cage rinse) and faeces. Thereafter, the rate of elimination was slower. The carcass also contained a moderate amount of radioactivity at 168 h post-dose; means of 8.8 and 9.8–13.6% of the radiocarbon dose were recovered from the i.v. and orally dosed rats, respectively, at 168 h. Expired $^{14}\text{CO}_2$ represented means of 1.58 and 2.81% of the dose in the i.v. and oral dosed rats, respectively. Less than 0.01% of the dose was volatilized in the expired air. Excretion of radioactivity in the faeces was low, with approximately 3.5 and 2.8–6.4% of the dose recovered following i.v. and oral dosing, respectively. The mean cumulative biliary excretion of radioactivity was 1.1 (1.5)% and 1.4 (1.6)% of the dose 24 and 48 h after oral (i.v.) dose, respectively. The total recovery range of the radiocarbon was 95–103%.

Metabolite profiles of plasma and urine

A representative metabolite profile of drug-derived radioactivity in plasma following i.v. administration to Sprague-Dawley rats is shown in figure 8. Most of the extracted plasma radioactivity was associated with temozolomide following i.v. administration. In the 0.083–6-h plasma samples, 91% of the radioactivity was associated with temozolomide. No significant plasma metabolites were observed at any time point after i.v. administration.

Essentially all of the extracted radioactivity was associated with the temozolomide standard; 93% of the analysed radioactivity was coincident with temozolomide standard in the 0.25–6-h samples. No significant metabolites were observed in the plasma of orally dosed rats at any time point. These data suggest

IV



Oral

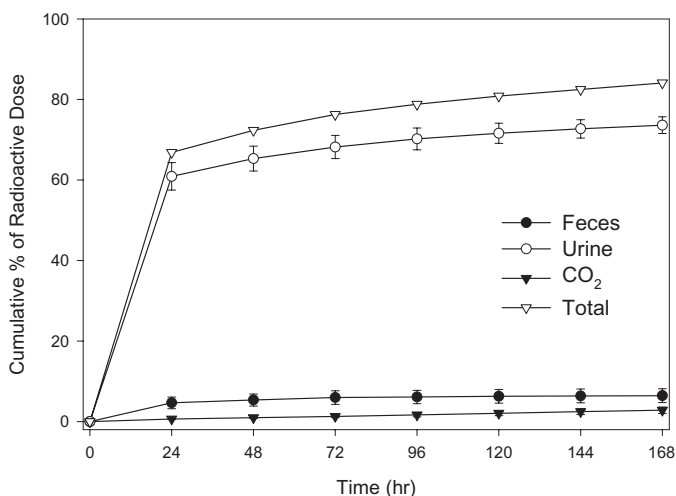


Figure 7. Cumulative temozolomide radioactivity concentration–time profiles following intravenous (top) or oral (bottom) administration to rats. Data are the mean of five rats/time point.

no, if any, first-pass metabolism following oral administration of temozolomide to rats.

The majority of radioactivity in the urine following either i.v. or oral administration was associated with temozolomide (59%), temozolomide acid (3%) and AIC (24%). Additionally, a highly polar peak (12%) was observed in the solvent front of the analytical run. The 4–8- and 8–24-h urine profiles showed decreasing concentrations of temozolomide, temozolomide acid and AIC with increasing concentrations of the very polar metabolite(s) peak in the solvent front. The 24–48-h urine profile showed only small amounts (about 3%) of temozolomide, temozolomide acid (about 1%) and AIC-related (about 4%) radioactivity; most of the radioactivity was associated with the metabolite(s) present in the solvent front of the analytical run.

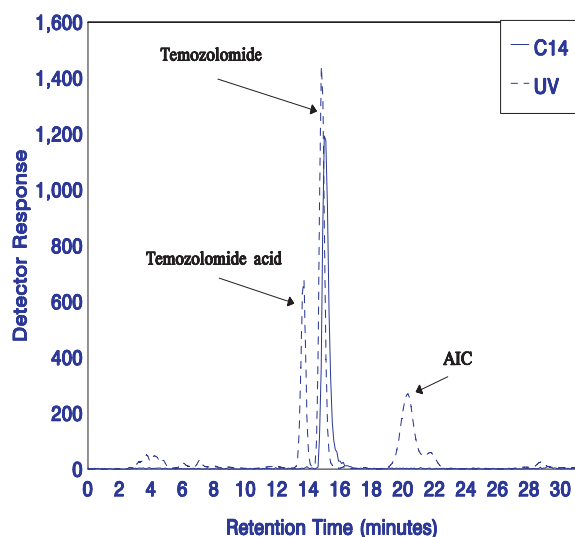


Figure 8. Representative metabolite profile of drug-derived radioactivity in plasma following intravenous administration of ^{14}C -temozolomide to Sprague-Dawley rats.

Faecal metabolite profiles were not determined due to insufficient radioactivity concentrations after both i.v. and oral administration.

Discussion

The present results demonstrate that temozolomide is rapidly and extensively ($>90\%$) absorbed and widely distributed following oral and i.v. administration to Sprague-Dawley and Long-Evans rats. The absolute oral bioavailability (F) of temozolomide is 96–100%. Temozolomide is rapidly converted to MTIC, its reactive metabolite. There are no apparent gender-related differences in either temozolomide or MTIC plasma concentration-time profiles. Concentrations of MTIC are lower than those of temozolomide (figure 2) and represent approximately 2% that of temozolomide. Disappearance of MTIC ($t_{1/2} = 1.35 - 1.64$ h) from plasma parallels that of temozolomide ($t_{1/2} = 1.17 - 1.22$ h), suggesting formation-rate limited elimination of the metabolite.

Temozolomide is a lipophilic alkylating agent that crosses the blood-brain barrier. Maximum temozolomide concentrations in the brain are observed shortly after i.v. administration (0.25 h post-dose). Based on the plasma/brain AUC ratio, the penetration of temozolomide in the brain represents between 35 and 39% following either oral or i.v. dosing, respectively. The extent of temozolomide penetration into the brain in the rat is consistent with that observed in humans and is crucial for the treatment of brain tumours (Agarwala *et al.* 1998, Stupp *et al.* 2001). Thus, rat represents a good preclinical model predictive of temozolomide penetration into the brain in the clinic.

The distribution pattern of drug-derived radioactivity is gender independent. Maximum radioactivity concentrations are observed at the first sampling time point (0.25 h) in kidney, liver, bone marrow, spleen and heart and are higher than those found in plasma. The kidney contains the highest concentrations of radioactivity at 2 h post-dose and the fat contains the lowest. Concentrations at 2 h are

3 and 0.2 times those found in plasma, respectively. The decline of radioactivity is slow in tissues and rapid in plasma. The terminal phase half-life of total radioactivity is 35 h in plasma and is estimated to range from 37 to 276 h in tissues. In contrast, plasma $t_{1/2}$ for temozolomide is about 1 h.

Based on the whole-body autoradiography, the radioactivity in the liver, kidney, testis and salivary gland remain prominent at 168 h after dosing. This observation is also consistent with the tissue distribution data, which showed retention (up to 14% of the administered dose) of radioactivity in the carcass of male rats for up to 1 week (168 h) post-dose. Temozolomide degrades via pH-dependent hydrolysis to MTIC and then AIC, the latter being known to be incorporated into purine metabolism pathways in tissues (Schulman and Buchanan 1952, Horgan and Tisdale 1985). Thus, these data suggest possible incorporation of radiolabel into purine metabolism intermediates via its degradation to AIC.

It was postulated (Horgan and Tisdale 1985) that decomposition products of mitozolomide (a compound analogous to temozolomide) are incorporated into the purine bases of nucleic acids via the ribotide formed because of salvage by adenosine phosphoribosyl transferase. Furthermore, it was shown that radioactivity from ring-labelled mitozolomide was associated solely with adenine and guanine and not with any modified bases. Thus, it can be concluded that incorporation of radiolabel into macromolecules does not arise as a result of a covalent adduct formation between mitozolomide and nucleic acids. Due to the structural similarity between mitozolomide and temozolomide, the retention of radioactivity in the carcass 168 h after dosing with temozolomide might be due to the incorporation of the ^{14}C label into purine metabolism intermediates.

The exposure to total plasma radioactivity is almost twice that of the parent compound. The apparently slower elimination of total radioactivity indicates that the conversion products of temozolomide persist in the body longer than the parent compound.

^{14}C -drug-derived radioactivity is eliminated mostly renally as either unchanged drug, a carboxylic acid analogue, AIC (a degradation product) or a highly polar unidentified peak in the solvent form. The majority of the ^{14}C -dose (75%) is excreted in the urine and faeces within 24 h of dosing. Faecal metabolite profiles show that temozolomide is metabolized to temozolomide acid and to a smaller extent further degrade to AIC. ^{14}C -temozolomide is the only drug-derived radioactivity observed in the plasma after i.v. and oral administration to Sprague–Dawley rats. Biliary excretion is 1.4 and 1.6%, following oral and i.v. administration, respectively. Therefore, assessment of enterohepatic circulation is not considered warranted.

In summary, temozolomide is rapidly absorbed, widely distributed to most tissues and organs, degraded to MTIC and AIC, and excreted primarily via the renal route in rats. Temozolomide metabolic profiles in rats are consistent with those observed in man (Baker *et al.* 1999). The brain penetration data in rats are similar with those observed subsequently in humans, making the rat a good pre-clinical species to assess the disposition of temozolomide and further supporting the use of temozolomide in the treatment of brain tumours.

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Quantitative structure-activity relationships within a homologous series of 7-alkoxyresorufins exhibiting activity towards CYP1A and CYP2B enzymes: molecular modelling studies on key members of the resorufin series with CYP2C5-derived models of human CYP1A1, CYP1A2, CYP2B6 and CYP3A4

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1. The results of quantitative structure–activity relationships (QSARs) within a homologous series of 7-*n*-alkoxyresorufins are reported. They are consistent with homology modelling of the relevant P450s involved in their metabolism.
2. QSARs were generated for activities involving CYP1A and CYP2B enzymes with structural descriptors relating to compound planarity and other shape parameters, together with certain features of the *n*-alkoxyresorufin electronic structure, especially electron densities and superdelocalizabilities.
3. A quadratic relationship between compound lipophilicity and binding to CYP2B enzymes was apparent, and which indicated maximal interaction for 7-pentoxyresorufin. Such indications help to explain enzyme selectivity in terms of optimal alkyl chain length for fitting within the relevant active site region.
4. Calculation of the binding affinities for methoxy-, ethoxy-, pentoxy- and benzyloxy-resorufins towards either CYP1A2 or CYP2B6 enzymes, depending on the 7-alkoxyresorufin agree favourably with experimental values obtained from K_m determinations.

Introduction

The cytochromes P450 (CYP) are a superfamily of haem-thiolate enzymes, of which over 2700 individual members have been sequenced, involved in the oxidative metabolism primarily of a vast number and range of organic chemicals, both endogenous and exogenous (Lewis 2001, Rendic 2002). (CYP is the standard abbreviation when referring to the particular gene, enzyme family, subfamily or individual enzyme. However, it is used in reference to P450s in general.) The *n*-alkoxyresorufins have been used as substrate probes for CYP1, CYP2B and CYP3A activities due to the high level of selectivity afforded by 7-ethoxyresorufin and 7-methoxyresorufin for CYP1A1 and CYP1A2, respectively, and by 7-pentoxyresorufin for CYP2B, together with 7-benzyloxyresorufin, which is fairly selective towards both CYP2B and CYP3A enzymes (Burke and Mayer 1983, Burke *et al.* 1985, 1994, Lum *et al.* 1986, Lubet *et al.* 1990, Mayer *et al.*

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1990, Nerurkar *et al.* 1993, Nims and Lubet 1996, Stresser *et al.* 2002). However, a recent publication has indicated that 7-pentoxoresorufin can bind avidly to human CYP1A1 (Liu *et al.* 2003).

In 1995, the present authors published a QSAR study on a homologous series of *n*-alkoxyresorufins based on the biological data of Burke and Mayer (1983) together with other data generated in house (Lum 1987) that involved P450 models based on the CYP102 crystallographic template (Lewis *et al.* 1995). Since then, we have undertaken further QSAR investigations on these compound datasets that involve the consideration of other structural descriptors that were not looked at previously, including the lipophilicity parameter $\log P$, where P is the octanol-water partition coefficient. Furthermore, with the advent of the first mammalian P450 crystal structure, namely that of the rabbit enzyme CYP2C5 (Williams *et al.* 2000), there has been an opportunity to construct new homology models of human and other mammalian P450 enzymes, especially those within the CYP2 family (Lewis 2002a). In addition, enzymes of the CYP1 family have also been constructed from the CYP2C5 template (Lewis 2002b, Lewis *et al.* 2002, 2003a, b) and from the substrate-bound CYP102 structure (Lewis *et al.* 1999).

The current work relates to human P450 models generated from CYP2C5 (pdb code: 1dt6) that affords greater primary sequence homology with both CYP1 and CYP2 family enzymes than that exhibited by CYP102. Moreover, the new models agree well with information reported elsewhere from site-directed mutagenesis studies (reviewed in Lewis 2002a). Using these enzymes models, it has been possible to estimate substrate binding affinities based on first principles and by employing partition coefficients for calculating partition energies related to substrate binding-dependent desolvation of the active site (Lewis 2002a).

Materials and methods

Molecular modelling

Based on previously reported homology models of CYP1A2 and CYP2B6 using the CYP2C5 crystallographic template (Lewis 2002a, b, Lewis *et al.* 2002, 2003a), individual congeners of the *n*-alkoxyresorufin series were docked interactively within the putative active sites of the relevant enzymes. All molecular modelling was carried out using Sybyl Software (Tripos Associates, St Louis, MO, USA) and used a dynamic hydrogen bond feature in Sybyl to facilitate optimal interaction between resorufin substrates and the putative active site regions. The known site of substrate oxidation was also taken into consideration when orientating substrates relative to the haem, together with previously reported evidence from site-directed mutagenesis.

QSAR analysis

Using the biological activities previously reported using induced liver microsomes (Burke and Mayer 1983, Burke *et al.* 1985) as the dependent y variables, a dataset of molecular and structural descriptors was generated as independent x variables for the homologous series of *n*-alkoxyresorufins (namely methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl and octyl, but not benzyl) using molecular modelling techniques. These included molecular orbital (MO) calculations and evaluation of the congeners' molecular size and shape, such as planarity (area/depth² ratio) and rectangularity (length/width ratio), which have been identified as important descriptors for P450 selectivity (Lewis *et al.* 1986, 1987). Lipophilicities of the *n*-alkoxyresorufins were also calculated from the original data reported by Burke and Mayer (1983) and by Burke *et al.* (1985). Furthermore, $\log P$ values were estimated using Pallas software (CompuDrug Ltd, Budapest, Hungary) as experimental values for the $\log P$ of *n*-alkoxyresorufins have not been reported in the literature. However, as shown below, there is a good correlation ($R=0.97$) between experimental $\log R_m$ data and $\log P$ values obtained via calculation using the Pallas system or from Clog P software (BioByte Corporation, Pomona, CA, USA).

Results and discussion

From the TLC-derived retention factor, R_f , it is possible to derive (Mannhold *et al.* 1996) R_m via the following expression:

$$R_m = \log\left(\frac{1 - R_f}{R_f}\right),$$

where R_m is the linear physicochemical TLC descriptor (Mannhold *et al.* 1996), which is essentially a lipophilicity parameter. For a fairly large number of compounds, there is an approximately linear relationship between R_m and $\log P$ (Mannhold *et al.* 1996), which is referred to as a Collander relationship.

Consequently, it can be expected that such a relation exists for the *n*-alkoxyresorufins presented here. In fact, calculated $\log P$ values derived from either ClogP or the Pallas software systems exhibit good linear correlations with R_m for the 7-alkoxyresorufins (eight compounds) and for a slightly larger dataset, which also includes 7-benzoyloxyresorufin and the parent compound phenoxazone (10 compounds) where the R_f values have been reported (Burke and Mayer 1983).

Tables 1 and 2 represent the relevant data for the homologous series of *n*-alkoxyresorufins compiled for the present study and which were used to generate quantitative structure–activity relationships (QSARs) with this series of compounds. It is apparent that the biological activities indicate a change in P450 enzyme selectivity as the homologous series is ascended. For example, the peak of CYP2B selectivity arises at the N_c =five congener, where N_c is the alkyl chain length, whereas peak activity towards CYP1A lies at N_c =2, although it is known that

Table 1. Dataset for 7-alkoxyresorufin series lipophilicity.

Compound	$\log P^a$	R_m^b	$\log PB^c$	
1. Phenoxazone	3.08	−0.1943	N/A	
2. 7-Methoxyresorufin	3.15	−0.1047	0.0	
3. 7-Ethoxyresorufin	3.61	0.0174	0.0	
4. 7-Propoxyresorufin	4.12	0.0871	0.3010	
5. 7-Butoxyresorufin	4.63	0.3680	0.6021	
6. 7-Pentoxyresorufin	5.14	0.6021	2.0043	
7. 7-Hexoxyresorufin	5.65	0.6297	0.8451	
8. 7-Heptoxyresorufin	6.16	1.0607	0.6990	
9. 7-Octoxyresorufin	6.67	1.3802	0.3010	
10. 7-Benzoyloxyresorufin	4.75	0.1761	N/A	
QSAR expression	<i>n</i>	<i>s</i>	<i>R</i>	<i>F</i>
(1) $R_m = 0.412 \log P - 1.509$ (±0.035)	8	0.1160	0.9785	135.05
(2) $R_m = 0.405 \log P - 1.500$ (±0.037)	10	0.1372	0.9680	119.03
(3) $\log PB = 0.465 N_c - 0.042 N_c^2 - 0.593$ (±0.139) (±0.015)	7	0.1777	0.901	17.25
(4) $\log PB = 0.113 + 1.872 R_m - 1.251 R_m^2$ (±0.229) (±0.175)	7	0.938	0.973	71.09

n, Number of observations; *s*, standard error; *R*, correlation coefficient; *F*, variance ratio; PB, relative reaction rate for dealkylation by phenobarbital-induced microsomes in the mouse.

QSAR equation (1) relates only to the eight 7-alkoxyresorufins.

^aCalculated using the Pallas System (CompuDrug Ltd, Budapest, Hungary),

^b $R_m = \log((1 - R_f)/R_f)$.

^cData from Burke and Mayer (1983).

Table 2. Biological data and structural descriptors for eight alkoxyresorufins.

Congener	S_N	Q_O	Q_C	N_C	π	Area/depth ²	β NF	log β NF
Methoxy	22.56	-0.4087	0.3681	1	0.00	5.21	0.75	-0.1249
Ethoxy	23.49	-0.4200	0.3724	2	0.44	4.82	8.00	0.9031
Propoxy	25.16	-0.4211	0.3697	3	0.99	4.32	7.00	0.8451
Butoxy	26.76	-0.4213	0.3685	4	1.58	4.44	1.83	0.2625
Pentoxo	28.38	-0.4215	0.3685	5	2.25	4.81	0.16	-0.7959
Hexoxy	29.14	-0.4214	0.3686	6	2.77	3.85	0.12	-0.9208
Heptoxy	31.68	-0.4215	0.3687	7	3.36	3.74	0.015	-1.8239
Octoxy	33.34	-0.4215	0.3687	8	3.81	3.85	0.006	-2.2218

S_N , sum of nucleophilic superdelocalizability for all atoms in the molecule; Q_O , net atomic charge on the alkoxy oxygen; Q_C , net atomic charge on the α -carbon of the alkyl group; N_C , number of carbons in the alkyl group; π , hydrophobic substituent constant for the alkyl group based on experimental log P values for the corresponding primary alcohols (Sangster 1989); area/depth², ratio of molecular area to the square of depth; β NF, CYP1-mediated metabolism (expressed as pmol min⁻¹ mg⁻¹ protein) for rats pretreated with β -naphthoflavone as reported by Burke *et al.* (1985).

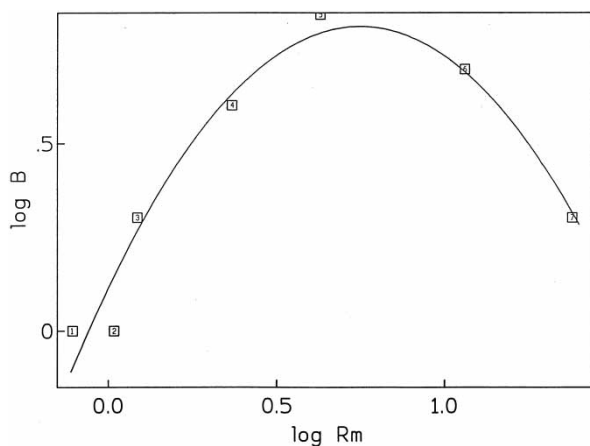


Figure 1. Plot of the parabolic relationship between log PB activity and R_m for seven n -alkoxyresorufins using the data presented in table 1.

CYP1A2 exhibits a slight preference towards 7-methoxyresorufin compared with 7-ethoxyresorufin; the reverse is true for CYP1A1 selectivity (Kawajiri and Hayashi 1996, Nims and Lubet 1996). Quantitative structure–activity analysis of the biological data for the resorufin homologous series indicates that the variation in enzyme selectivities can be explained by a number of different factors (Lewis 2003a). For example, there is a parabolic relationship (table 1) between CYP2B selectivity and either alkyl chain length or R_M , which relates to the log P via the relationship:

$$R_M = 0.405 \log P - 1.50, \text{ where } n = 10, s = 0.1372, R = 0.968 \text{ and } F = 119.0, (\pm 0.037)$$

where n is number of observations (i.e. this comprises all of the compounds in the series, including the eight n -alkoxyresorufins, benzyloxyresorufin and phenoxazone, the parent compound), s is the standard error, R is the correlation coefficient and F is the variance ratio. Figure 1 shows a plot of the parabolic relationship mentioned above and using the data presented in table 1, including equation (4).

Moreover, in the case of CYP1A selectivity, the variation in activity can be explained by a three-variable QSAR expression involving the molecular planarity (area/depth²), the partial electronic charge on the alkoxy oxygen atom (Q_O) and the number of carbon atoms in the alkyl group (N_C) as follows:

$$\log \beta\text{NF} = -0.68N_C - 148.59Q_O - 0.50a/d^2 - 57.60,$$

(± 0.06) (± 21.11) (± 0.26)

where $n = 8$, $s = 0.1816$, $R = 0.99$ and $F = 94.49$,

where a/d^2 relates to the overall planarity of the alkoxyresorufin molecule based on Van der Waals radii, and βNF activity is from β -naphthoflavone-induced rat liver microsomes (Lewis *et al.* 1995). However, the number of independent variables used is rather high compared with the number of observations. Other factors also appear to be important for the description of CYP1A selectivity within this series, including the total nucleophilic superdelocalizability (S_N), which is a quantum-mechanical descriptor relating to the ability of the molecule to act as an electron acceptor. In fact, a combination of this variable with the partial electronic charge on the ether oxygen (Q_O) exhibits a good correlation with $\log \beta\text{NF}$ as follows:

$$\log \beta\text{NF} = -0.37 S_N - 137.19Q_O - 47.87,$$

(± 0.03) (± 23.31)

where $n = 8$, $s = 0.2190$, $R = 0.99$ and $F = 96.32$.

S_N is related to molecular size as well as representing an indicator of overall electron acceptor ability. It is apparent that molecular size has a role in the variation of βNF activity displayed by these compounds, which diminishes as the homologous series is ascended, so it is likely that S_N is reflecting this structural aspect. As the ether oxygen is thought to represent a potential hydrogen bond acceptor atom, and there is evidence from molecular modelling to support this view (Lewis *et al.* 2003a), it is probable that the partial atomic charge on the oxygen has an effect on the hydrogen bond acceptance ability, with a change in the electronic inductive effect of the alkyl residue being apparent with increase in chain length.

Another structural descriptor of some relevance is the partial electronic charge, Q_C , on the α carbon atom of the alkyl group. This is, in fact, the site of metabolism, as oxidation at this atom is a prelude to dealkylation of the alkoxyresorufin itself. Although there is only a small increase in Q_C for the ethoxy congener, in combination with the hydrophobic parameter, π , there is a good correlation (table 2) with the raw βNF data as follows:

$$\beta\text{NF} = 1734.8Q_C - 0.81\pi - 636.6,$$

(± 475.75) (± 0.48)

where $n = 8$, $s = 1.6092$, $R = 0.91$ and $F = 12.37$.

However, this correlation is probably not as statistically significant as the previous expressions due to the low degree of variation in Q_C and the magnitude of its coefficient in the equation presented, together with the high error in the π term coefficient. Nevertheless, the slightly higher Q_C for the ethoxy congener is interesting in view of the increased CYP1A activity for this compound.

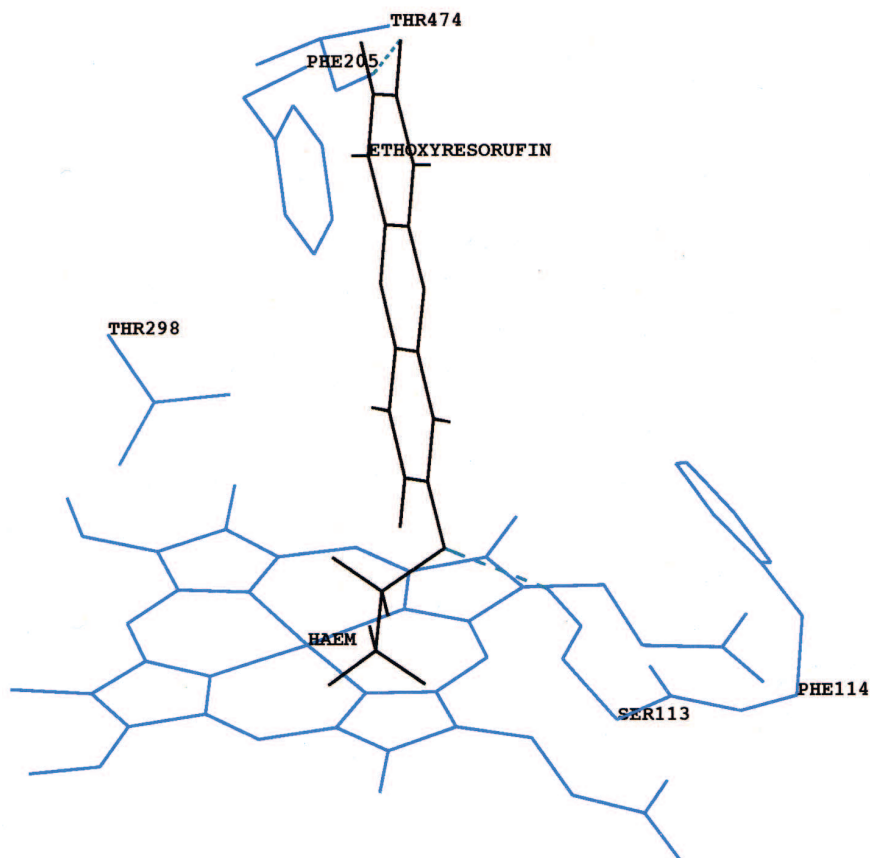


Figure 2. 7-Ethoxyresorufin positioned within the putative active site of human CYP1A1 for *O*-deethylation. Hydrogen bonds are shown as dashed lines. Amino acid residues are shown, including the conserved distal threonine.

Molecular modelling of n-alkoxyresorufins

CYP1A1 model. A possible mode of binding for 7-ethoxyresorufin within the putative active site of human CYP1A1 is shown in figure 2. It is apparent that a combination of π - π stacking and hydrogen bond interactions with complementary amino acid residues within the haem environment orientates the substrate for *O*-deethylation, as this grouping lies close to the haem iron for oxidation at the α -carbon. Some residue positions have been probed using site-directed mutagenesis or via photo-affinity labelling in related CYP1A enzymes and, consequently, there is a general agreement between the molecular modelling and experimental studies (Lewis *et al.* 1995).

CYP1A2 model. In figure 3, 7-methoxyresorufin is shown orientated in the putative active site of human CYP1A2 for *O*-demethylation. In this case, the contacts with amino acid residues in the haem pocket is similar to those exhibited by 7-ethoxyresorufin in CYP1A1, described above. Furthermore, the 7-methoxy group is positioned directly above the haem iron in agreement with the known course of metabolism. The methoxy oxygen atom forms a hydrogen bond with

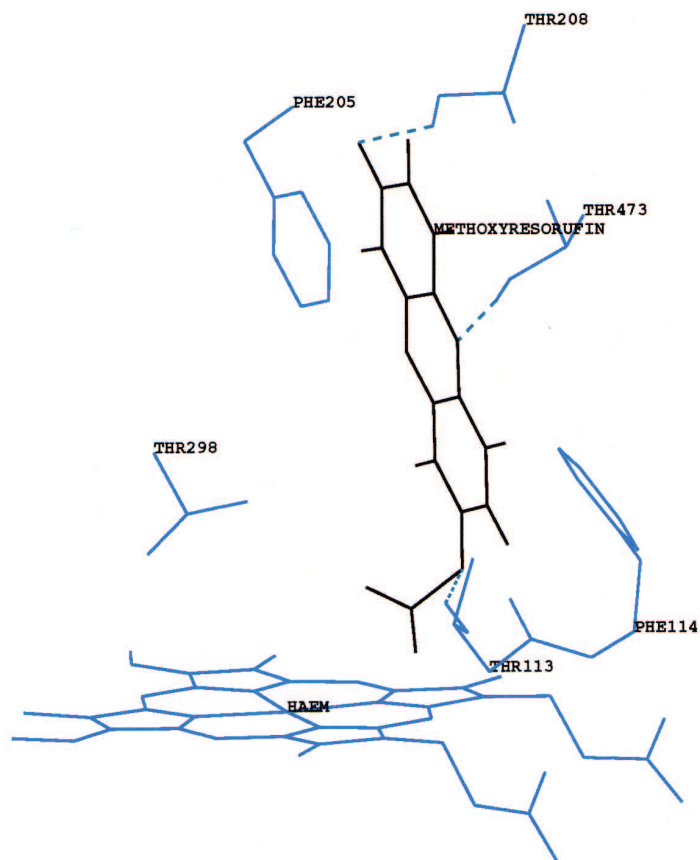


Figure 3. 7-Methoxyresorufin orientated within the putative active site of human CYP1A2 for *O*-demethylation. Hydrogen bonds are shown as dashed lines. Amino acid residues are shown, including the conserved distal threonine.

a threonine residue close to the haem, and a π - π stacking interaction with phenylalanine-205 is consistent with the results of site-directed mutagenesis experiments conducted on human CYP1A2, albeit with other substrates of this enzyme (Lewis *et al.* 2003a).

CYP2B6 model. 7-Pentoxoresorufin is shown (figure 4) positioned within the putative active site of CYP2B6 (Lewis *et al.* 2002) for *O*-depentylation. The substrate is held in place by a combination of hydrogen bonded and π - π stacking interactions with complementary amino acid residues lining the haem pocket. There is a degree of structural similarity between this substrate and 7-benzoyloxyresorufin, which is also a substrate for CYP2B enzymes (Nims and Lubet 1996) in addition to undergoing *O*-debenzylation mediated by CYP3A4, as discussed below. This may be a result of some degree of chain folding in the pentoxy congener which could thus represent a structural mimic for the benzyloxy case, and it is likely that their hydrophobic interactions are similar within the CYP2B6 active site.

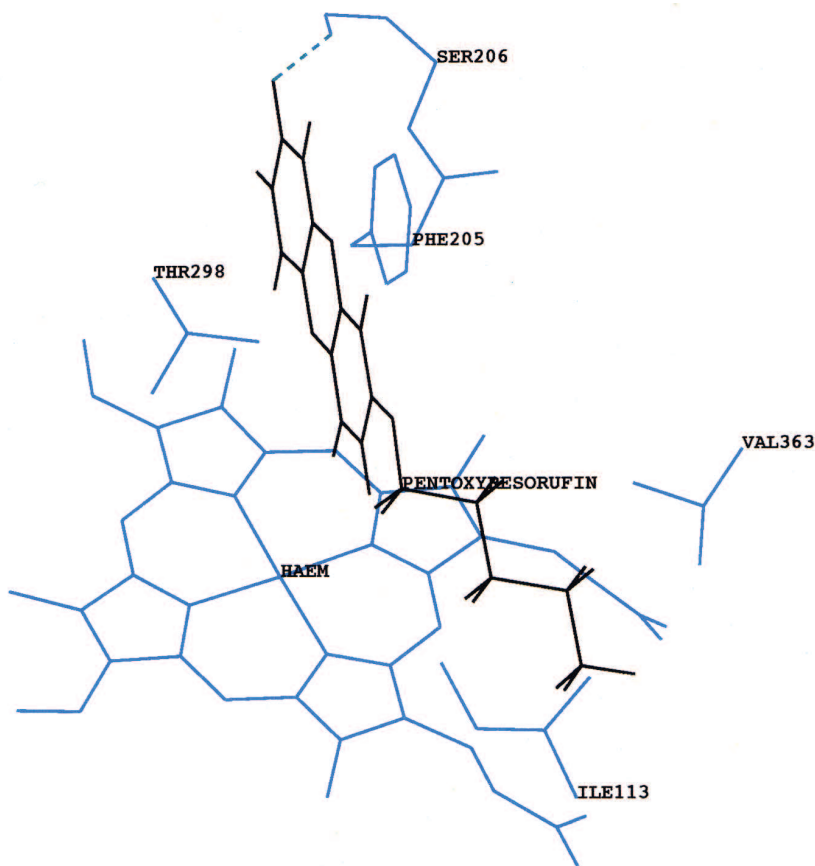


Figure 4. 7-Pentoxoresorufin positioned within the putative active site of CYP2B6 for *O*-depentylation. Hydrogen bonds are shown as dashed lines. Amino acid residues are shown, including the conserved distal threonine.

CYP3A4 model. A likely mode of binding for 7-benzyloxyresorufin in the putative active site of CYP3A4 is presented on figure 5. Although the active site topography is different from that of CYP2B6, mentioned above, there are complementary contacts with amino acid residues, including π - π stacking and hydrogen bonding, some of which have been the subject of site-directed mutagenesis experiments in CYP3A4 (reviewed in Domanski and Halpert 2001). Consequently, there is a good agreement between molecular modelling and experimental studies in this enzyme.

Overall, the differences and similarities in enzyme selectivities for certain 7-alkoxy-resorufins can be rationalized by molecular modelling of the relevant congeners within the putative active sites of the P450s concerned and table 3 summarizes the substrate recognition site (SRS) regions in each case (Gotoh 1992). In addition, the differences in selectivity for CYP1A1 and CYP1A2 towards 7-ethoxyresorufin and 7-methoxyresorufin may also be explained from a consideration of the specific active site interactions, as there are several minor, but potentially important, differences between the amino acid residues lining the haem pocket in the two enzymes. In particular, the SRS2 region shows several striking changes for CYP1A1 relative to CYP1A2 which possibly relate to

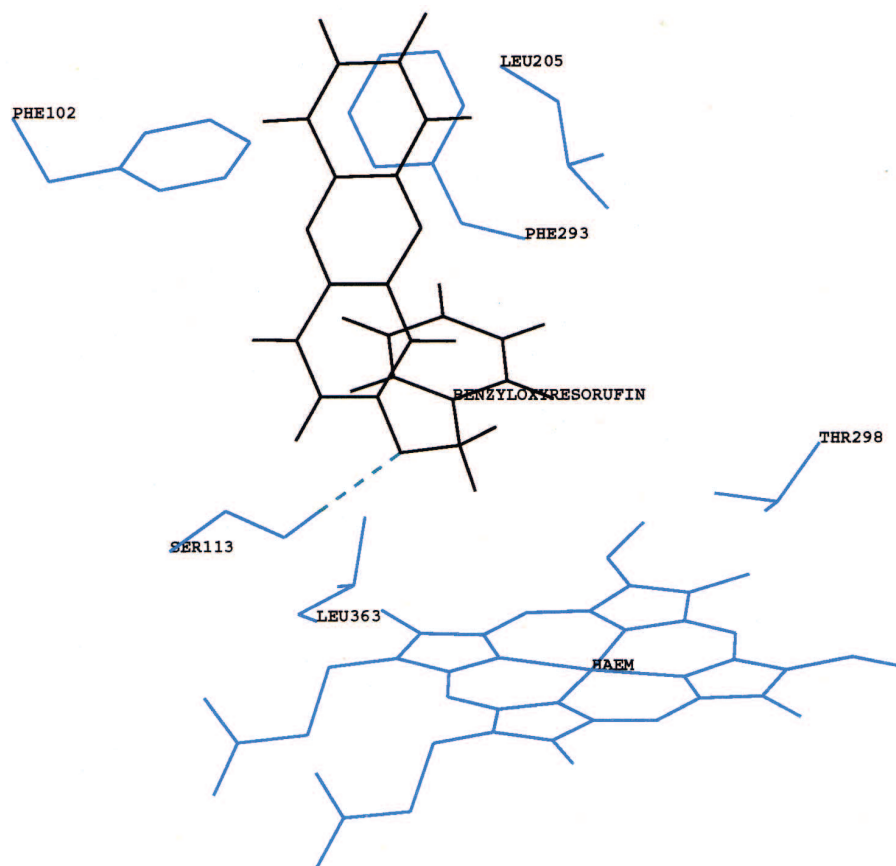


Figure 5. 7-Benzyloxyresorufin orientated within the putative active site of CYP3A4 for *O*-debenzylation. Hydrogen bonds are shown as dashed lines. Amino acid residues are shown, including the conserved distal threonine.

substrate interactions in the vicinity of the key conserved phenylalanine residue at alignment position 205. Of these, it is probable that the V208T change between CYP1A1 and CYP1A2 rationalizes the small but important selectivity differences towards ethoxy- and methoxyresorufin congeners, respectively. Furthermore, there is a conservative change, S114T in the SRS1 region where a serine in CYP1A1 becomes threonine in CYP1A2, such that the difference corresponds to a methylene group. However, this may explain the preference for 7-ethoxyresorufin in CYP1A1 as opposed to CYP1A2, together with the opposite selectivity for 7-methoxy-resorufin where, in this case, it is CYP1A2 that exhibits a preference relative to CYP1A1. Interestingly, the only difference between these two substrates is a methylene group, and this appears to be reflected in the S114T change for CYP1A1 and CYP1A2.

Using the results from molecular modelling, it has been possible to make estimates of the binding affinity towards CYP1A1, CYP1A2, CYP2B6 and CYP3A4 for some of the resorufins described above. Table 4 shows that a combination of desolvation, as calculated from the log *P* value, hydrogen bonding and π - π stacking energies, together with loss in bond rotation energy, can give a satisfactory agreement with experimental data from K_m values. However, as there

Table 3. Similarities and differences between SRS regions in human P450 sequences.

Alignment position																
SRS 1																
CYP	100			↓			↓				110			↓	↓	
1A1	L	Y	T	F	T	L	I	S	N	G	Q	S	M	S	F	S
1A2	L	Y	T	S	T	L	I	T	D	G	Q	S	L	T	F	S
2B6	I	A	M	V	D	P	F	F	R	G	Y	G	V	I	F	A
3A4	R	P	F	G	P	V	G	F	M	K	S	A	I	S	I	A
SRS 2																
CYP	200					↓	↓				210					
1A1	N	L	N	N	N	F	G	E	V	V	G					
1A2	K	N	T	H	E	F	V	E	T	A	S					
2B6	L	F	Y	Q	T	F	S	L	I	S	S					
3A4	N	T	K	K	L	L	R	F	D	F	L					
SRS 4																
CYP	290				↓	*	*			*	300					
1A1	L	D	L	F	G	A	G	F	D	T	V	T				
1A2	N	D	I	F	G	A	G	F	D	T	V	T				
2B6	L	S	L	F	F	A	G	T	E	T	T	S				
3A4	I	I	F	I	F	A	G	Y	E	T	T	S				
SRS 5																
CYP			↓		360			↓								
1A1	I	D	L	L	P	F	T	I	P	H						
1A2	S	S	F	L	P	F	T	I	P	H						
2B6	S	D	L	L	P	M	G	V	P	H						
3A4	F	P	I	A	—	M	R	L	E	R						
SRS 6																
CYP					470			↓								
1A1	M	T	P	I	Y	G	L	T	M	K						
1A2	L	T	P	I	Y	G	L	T	M	K						
2B6	L	T	P	Q	E	C	G	V	G	K						
3A4	L	K	L	S	L	G	G	L	L	Q						

*Conserved residues; ↓, potential substrate contact residue (as encountered in CYP2C5 crystal structure); SRS, substrate recognition site, a term coined by Gotoh (1992) in the analysis of CYP2 family sequences.

Residues that have been probed using site-directed mutagenesis are emboldened (those of CYP2B6 correspond to those that have been probed for the rat orthologue, CYP2B1 or the rabbit form CYP2B4).

Sources: Lewis (2001), Domanski *et al.* (2001), Hadjokas *et al.* (2002), Domanski and Halpert (2001).

Table 4. Dataset for estimation of substrate binding affinity for selected resorufins.

Compound	CYP	log <i>P</i>	ΔG_{part}	ΔG_{hb}	$\Delta G_{\pi-\pi}$	ΔG_{rot}	$\Delta G_{\text{bind}}^{\text{calc}}$	$\Delta G_{\text{bind}}^{\text{expt}}$	<i>K</i> _m (μM)
7-Ethoxyresorufin	1A1	3.61	−5.1207	−4.0	−0.9	0.6	−9.4207	−9.3174	0.27
7-Methoxyresorufin	1A2	3.15	−4.4682	−4.0	−0.9	0.0	−9.3682	−9.4577	0.21
7-Benzyloxyresorufin	2B6	4.75	−6.7377	−2.0	−0.9	1.2	−8.4377	−8.3587	1.28
7-Benzyloxyresorufin	3A4	4.75	−6.7377	−2.0	−0.9	1.2	−8.4377	−7.8340	3.00

Agreement between experimental and calculated values for ΔG_{bind} is quite satisfactory indicating that the overall approach used to estimate binding affinity has some degree of validity.

ΔG_{hb} , sum of the hydrogen bond energies (−2.0 kcal mol^{−1} per hydrogen bond); $\Delta G_{\pi-\pi}$, $\pi-\pi$ stacking energy (−0.9 kcal mol^{−1} per $\pi-\pi$ stack); ΔG_{rot} , loss of rotational bond energy (+0.6 kcal mol^{−1} per rotatable bond); $\Delta G_{\text{bind}}^{\text{calc}} = \Delta G_{\text{part}} + \Delta G_{\text{hb}} + \Delta G_{\pi-\pi} + \Delta G_{\text{rot}}$; $\Delta G_{\text{bind}}^{\text{expt}} = RT \ln K_{\text{m}}$, where *R* is the gas constant and *T* is the absolute temperature (taken as 310 K); $\Delta G_{\text{part}} = RT \ln P$, where *P* is the octanol-water partition coefficient calculated using the Pallas System (CompuDrug Ltd, Budapest, Hungary).

References to *K*_m data include: Kenworthy *et al.* (1999), Turesky *et al.* (1998), Schwarz *et al.* (2000), and Ekins and Wrighton (1999), although other values have been reported in the literature, e.g. Stresser *et al.* (2000) for 7-benzyloxyresorufin.

are only four data points employed in this exercise, it cannot be considered as statistically significant. In addition, it is recognized that P450-mediated reactions are multistage processes and this form of analysis focuses entirely on substrate binding to the enzyme. Nevertheless, it can be shown that the methodology correlates well with experimental data in a larger group of human P450 substrates (Lewis 2003b).

Conclusions

The overall pattern of variation in activity for the *n*-alkoxyresorufins series is one of a peak activity for one congener followed by a general diminution with further increase in alkyl chain length. The optimal point for selectivity in the homologous series depends on the P450 enzyme concerned. For CYP1A enzymes, this is at the ethyl case, whereas for CYP2B isoforms it is the pentyl congener. In the CYP1A subfamily, there appears to be preference for methyl as opposed to ethyl for CYP1A2 enzymes, whereas the position is reversed for CYP1A1. With CYP3A enzymes, a similarity with the situation encountered for CYP2B is apparent and it is interesting that 7-benzyloxyresorufin is a substrate for both CYP2B6 and CYP3A4, for example. Although the biological data for the QSAR studies are for rodent liver microsomes, there is a good concordance with evaluations conducted using human enzymes (Burke *et al.* 1994).

Essentially, there are two opposing factors that determine the net binding affinity of the *n*-alkoxyresorufin series. These are an increase in the lipophilicity as the homologous series is ascended, which has a positive effect on binding to the relevant P450, counter-balanced by a decrease in affinity brought about by the increased loss in bond rotational energy of the alkyl chain as the number of methylene groups increases. However, the hydrophobic effect is greater than the loss in rotational freedom although there is also a decrease in translational energy upon binding. Second, the topography of the P450 active site plays a role in the selection process and this is composed of a combination of size, shape and hydrophobic character. Probably the size of a particular hydrophobic pocket within the active site region determines the extent of this selectivity towards individual congeners within the *n*-alkoxyresorufin series. Consequently, the hydrophobic cleft in CYP1A enzymes is undoubtedly smaller than that of both CYP2B and CYP3A enzymes although, in the latter, the entire active site is relatively large. More subtle distinctions arise when the location of hydrogen bond donor residues is considered, and their orientation with respect to a hydrophobic region likely to be involved in binding the alkyl group. Indeed, such considerations can even rationalize the distinction between methoxy and ethoxy congeners towards CYP1A1 and CYP1A2.

The combination of homology modelling and P450 active site interactions with QSAR analysis enables one to gain some insights into the enzyme selectivity of alkoxyresorufins, which are important substrate probes of CYP1A, CYP2B and CYP3A activities in several mammalian species. In this way, it is possible to derive a degree of understanding of the underlying molecular mechanisms of P450 substrate selectivity and modes of interaction with the enzymes' active sites for a given series of structurally related congeners. Furthermore, it is thought that homology modelling of the drug-metabolizing P450s may also help to explain steroid hormone hydroxylase selectivities in the human enzymes (Waxman *et al.* 1991).

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Molecular modelling of CYP2A enzymes: application to metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone (NNK)

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1. Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a lung carcinogen in a variety of animal models and a putative human lung carcinogen. Its tumorigenic potential is unmasked via cytochrome P450 (CYP)-mediated hydroxylation of the carbon atoms adjacent to the nitroso moiety (i.e. α -hydroxylation). Therefore, elucidation of enzyme–substrate interactions that facilitate α -hydroxylation is important to gain insight into the tumorigenic mechanism of NNK and to develop potent inhibitors of this detrimental reaction.

2. Molecular models of CYP2A enzymes from mice, rats and humans that are catalysts of NNK bioactivation were constructed and used, in conjunction with docking experiments, to identify active-site residues that make important substrate contacts.

3. Docking studies revealed that hydrophobic residues at positions 117, 209, 365 and 481, among others, play critical roles in orienting NNK in the active site to effect α -hydroxylation. These molecular models were then used to rationalize the stereo- and regioselectivity, as well as the efficiency, of CYP2A-mediated NNK metabolism.

Introduction

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) exhibits remarkable potency and selectivity for the induction of lung tumours in a variety of animal models (Hecht 1998). The presence of NNK in cigarette smoke and tobacco products, coupled with extensive data demonstrating the uptake and metabolism of NNK in humans (Hecht 1998, 1999, 2002, 2003), warrants classification of NNK as a probable human carcinogen and necessitates investigation of the mechanisms of NNK tumour induction.

NNK, like other procarcinogens, requires metabolic activation to exert its mutagenic, and thus presumably carcinogenic, effects (Hecht 1998). Metabolic activation of NNK occurs largely via cytochrome P450 (CYP)-catalysed hydroxylation of the carbon atoms adjacent to the nitroso moiety (i.e. α -hydroxylation)

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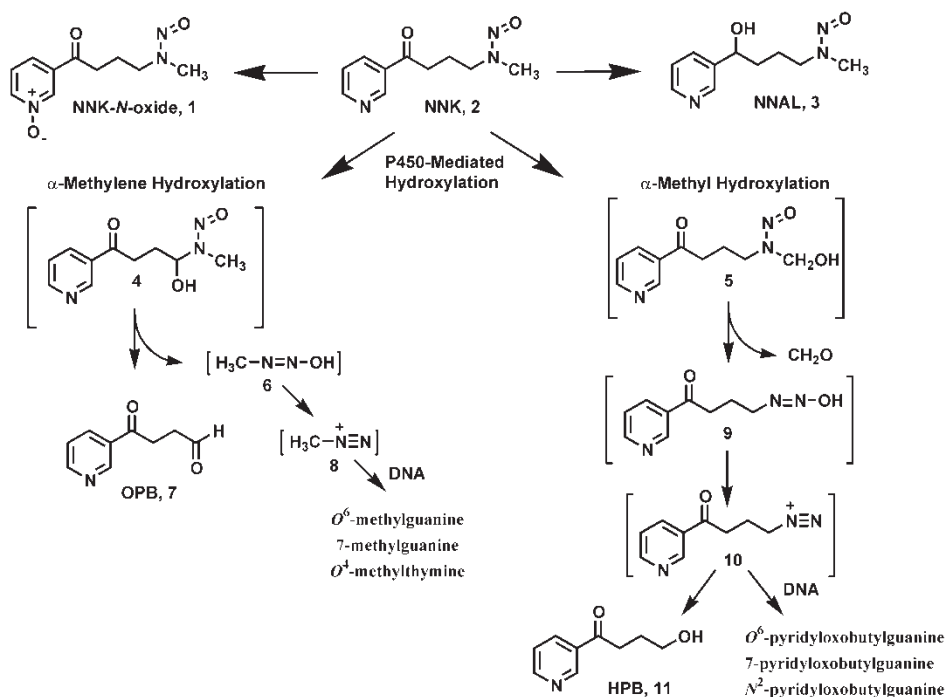


Figure 1. Metabolic transformations of NNK.

(Hecht 1998). As shown in figure 1, hydroxylation of the α -methylene and α -methyl carbons of NNK ultimately generates 4-oxo-4-(3-pyridyl)butanal (OPB) (7) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (11), respectively. The diazonium ions 8 and 10 formed during the breakdown of the first-formed α -hydroxynitrosamines 4 and 5, respectively, are the ultimate carcinogens derived from NNK and they react with DNA to form mutagenic DNA adducts (Hecht 1998, Pauly *et al.* 2002, Upadhyaya *et al.* 2003, Wang *et al.* 2003). In addition to α -hydroxylation, NNK undergoes pyridine N-oxidation to 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone (NNK-N-oxide) (1) and carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (3) (figure 1) (Hecht 1998).

A number of different CYP enzymes, including those in the 1A, 2A, 2B, 2D, 2E and 3A subfamilies, catalyse the α -hydroxylation of NNK (Guo *et al.* 1991, Patten *et al.* 1996, Smith *et al.* 1996, Hecht 1998, Felicia *et al.* 2000, Su *et al.* 2000, Zhang *et al.* 2002, Ding and Kaminsky 2003, J alas *et al.* 2003a, b). To date, the best catalysts of this reaction (i.e. those enzymes with the lowest K_m and highest V_{max}/K_m) are members of the CYP2A subfamily, notably mouse CYP2A5, rat CYP2A3 and human CYP2A13 (Hong *et al.* 1992, Felicia *et al.* 2000, Su *et al.* 2000, Zhang *et al.* 2002, J alas *et al.* 2003a, b, and an unpublished review).

Interestingly, despite relatively high primary sequence similarity among these three CYP2A enzymes ($\geq 87\%$), they demonstrate differing predilections for metabolism of NNK at the α -methylene and α -methyl carbon atoms. As shown in table 1, CYP2A3, 2A5 and 2A13 all exhibit comparable K_m values for NNK α -hydroxylation, but mouse CYP2A5 preferentially catalyses α -methyl

Table 1. Steady-state kinetic parameters for NNK metabolism by members of the CYP2A subfamily.

Enzyme	Metabolite and kinetic parameter						OPB/HPB ^b	Reference
	OPB ^a			HPB				
	$V_{\max}^c$	K_m (μM)	$V_{\max}/K_m^d$	$V_{\max}$	K_m (μM)	$V_{\max}/K_m$		
Rat CYP2A3	10.8±0.4	4.6±0.5	2.3±0.3	8.2±0.3	4.9±0.5	1.7±0.2	1.3	Jalas <i>et al.</i> (2003a) ^e
Mouse CYP2A4	7.3±0.4	97±19	0.075±0.015	1.8±0.1	67±17	0.027±0.007	4.1	Jalas <i>et al.</i> (2003b) ^f
	190±23	3900±995	0.049±0.014					Felicia <i>et al.</i> (2000) ^g
Mouse CYP2A5	2.0±0.1	4.3±0.8	0.47±0.09	6.5±0.2	4.5±0.4	1.4±0.1	0.31	Jalas <i>et al.</i> (2003b) ^h
				4.0±0.4	1.5±0.6	2.7±1.8		Felicia <i>et al.</i> (2000) ⁱ
Human CYP2A6	1.0	118	0.0085	0.42	141	0.0030	2.4	Patten <i>et al.</i> (1996) ^j
Rabbit CYP2A10/2A11	1.4	15	0.092	1.3	9	0.14	1.1	Hong <i>et al.</i> (1992) ^k
Human CYP2A13	13.8±0.8	3.6±0.7	3.9±0.8	4.6±0.2	3.2±0.5	1.4±0.2	3.0	Jalas <i>et al.</i> (2003a) ^l
	4.1±0.6	11±3.5	0.36±0.12	1.2±0.2	13±5	0.092±0.04	3.4	Su <i>et al.</i> (2000) ^m
	14.5±0.8	4.6±0.4	3.2±0.3	5.7±0.7	2.8±0.5	2.0±0.4	2.5	Zhang <i>et al.</i> (2002) ⁿ

^aDetected as an OPB-bisulfite adduct (Peterson *et al.* 1991).

^bRatio of $V_{\max}$ values for 4-oxo-4-(3-pyridyl)butanal (OPB) versus 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB).

^cUnits are pmol product min⁻¹ (pmol CYP)⁻¹.

^dUnits are pmol product min⁻¹ (pmol CYP)⁻¹ μM⁻¹.

^eNNK (0.25–50 μM) was incubated with *Spodoptera frugiperda* (Sf9)-expressed 2A3 (5 nM) for 10 min at 37°C (pmol NADPH-cytochrome CYP oxidoreductase [OR]:pmol CYP = 25:1).

^fNNK (0.50–3000 μM) was incubated with Sf9-expressed 2A4 (17 nM) for 10 min at 37°C (OR:CYP = 25:1).

^gNNK (1.0–5000 μM) was incubated with Sf9-expressed 2A4 (17 nM) for 10 min at 37°C (OR:CYP = 50:1).

^hNNK (0.25–50 μM) was incubated with Sf9-expressed 2A5 (3.8 nM) for 10 min at 37°C (OR:CYP = 25:1).

ⁱNNK (0.25–100 μM) was incubated with Sf9-expressed 2A5 (3.8 nM) for 10 min at 37°C (OR:CYP = 50:1).

^jNNK (5–2000 μM) was incubated with Sf9-expressed 2A6 (50 nM) for 30 min at 37°C (OR:CYP = 30 units nMol⁻¹ CYP; cytochrome b₅ (3 nMol nMol⁻¹ CYP) was also added).

^kNNK (2.9–154 μM) was incubated with purified, reconstituted 2A10/2A11 (25 nM) for 10 min at 37°C (OR:CYP = 3:1).

^lNNK (0.25–50 μM) was incubated with Sf9-expressed 2A13 (5 nM) for 10 min at 37°C (OR:CYP = 25:1).

^mNNK (2.0–160 μM) was incubated with Sf9-expressed 2A13 (25 nM) for 15 min at 37°C (cytochrome b₅:CYP = 4:1).

ⁿNNK (2.0–100 μM) was incubated with purified, reconstituted 2A13 (25 nM) for 10 min at 37°C (OR:CYP = 5:1).

hydroxylation (Felicia *et al.* 2000, Jalas *et al.* 2003b), rat CYP2A3 catalyses α -methylene and α -methyl hydroxylation to equivalent extents (Jalas *et al.* 2003a), and human CYP2A13 preferentially catalyses α -methylene hydroxylation (Su *et al.* 2000, Zhang *et al.* 2002, Jalas *et al.* 2003a). Mouse CYP2A4 and human CYP2A6, by contrast, exhibit higher K_m and lower V_{max}/K_m values than the other CYP2A enzymes listed in table 1; both CYPs preferentially metabolize NNK at the α -methylene carbon (table 1) (Patten *et al.* 1996, Felicia *et al.* 2000, Jalas *et al.* 2003b). These differences in K_m values and regioselectivity are particularly intriguing in the case of mouse CYP2A4 and 2A5, which are 98% identical in primary sequence, and human CYP2A6 and 2A13, which are 94% identical in primary sequence. Moreover, CYP2A4 and 2A5 exhibit stereoselectivity in the metabolism of NNK at the α -methylene position; they both preferentially abstract the pro-4*R* hydrogen relative to the pro-4*S* hydrogen (Jalas *et al.* 2003b). Presumably, subtle amino acid differences in the active site can account for this α -methylene stereoselectivity, as well as the K_m and regioselectivity differences apparent in table 1. The importance of putative active-site residues to the substrate specificity of CYP2A4 and CYP2A5 has been well established for coumarin, testosterone and other steroids using site-directed mutagenesis (Lindberg and Negishi 1989, Juvonen *et al.* 1991, 1993, 1997, Iwasaki *et al.* 1993a, 1994, 1995a, Negishi *et al.* 1996).

Unfortunately, only a few structural models of CYPs are currently available, and most of the available X-ray crystal structures are of soluble bacterial enzymes (Poulos *et al.* 1995, Podust *et al.* 2001, 2003). Relatively recently, the crystal structures of three mammalian, membrane bound enzymes — rat CYP2B4, a chimeric rabbit CYP2C5, and human CYP2C9 — have been solved (Scott *et al.* 2003, Wester *et al.* 2003a, b, Williams *et al.* 2000, 2003). Although a CYP2A crystal structure is not currently available, structural insight can be gained by constructing molecular models based on the structure of a related protein using sequence similarity. Homology modelling is used extensively both to explain and predict the metabolism of a wide variety of CYP substrates (Lewis 1999, 2002b, Gorokhov *et al.* 2003, Liu *et al.* 2003, Venhorst *et al.* 2003). The relatively high (compared with bacterial CYPs) primary sequence identity of about 51% between CYP2C5 and members of the CYP2A subfamily greatly facilitates the construction of molecular models based on the experimentally determined crystal structure (Williams *et al.* 2000). Moreover, the availability of a well-modelled solution structure of mouse CYP2A4 (Gorokhov *et al.* 2003) provides a convenient template upon which models of rat CYP2A3, mouse CYP2A5, and human CYP2A6 and human CYP2A13 may be built.

In an effort to elucidate critical active site residues governing the bioactivation of NNK, molecular models of a series of enzymes in the CYP2A subfamily were constructed based on a model of mouse CYP2A4 (which was developed using the crystal structure of rabbit CYP2C5 as a template) (Gorokhov *et al.* 2003). Homology modelling and molecular dynamics simulations were used to build the structural models and substrate-docking experiments were performed to rationalize experimental data regarding the stereo- and regioselectivity of NNK metabolism. The identity of the amino acid residues at critical positions, including those at positions 117, 209, 365, and 481, influences the orientation of NNK in the active site in a way consistent with the biochemical data (table 1). The molecular models provide important information about the molecular basis of

stereo- and regioselective NNK bioactivation, and are tools that may be used to screen for potential inhibitors of these CYPs.

Computational methods

All computations were carried out on a UNIX-based SGI workstation.

Sequence alignment

Amino acid sequences for rabbit CYP2C5/3LVdH (accession number P00179), rat CYP2A3 (P20812), mouse CYP2A4 (P15392) and 2A5 (P20852), and human CYP2A6 (P11509) and 2A13 (Q16696) were obtained from the Swiss-Prot database (<http://www.expasy.org>) and aligned using ClustalW 1.82 (Chenna *et al.* 2003). A full sequence alignment was performed with the default gap penalties.

Molecular models based on the modelled solution structure of mouse CYP2A4

The coordinates for the modelled solution structure of mouse CYP2A4 were kindly provided by Dr Lars Pedersen (NIEHS, Research Triangle Park, NC, USA). Molecular models of rat CYP2A3, mouse CYP2A5, and human CYP2A6 and 2A13 were constructed using the Homology module of InsightII and the modelled solution structure of mouse CYP2A4 as a template.

Once the homology models were built, structure refinement was performed using protocols similar to those previously reported for the CYP2A4 model (Gorokhov *et al.* 2003). Briefly, the models were first energy-minimized in vacuum for 20 000 steps using the SANDER module of AMBER version 7.0 (Perlman *et al.* 1995, Case *et al.* 2002), applying a combination of steepest descent and conjugate gradient methods. During minimization, the backbone was fixed and only the side chains were allowed to move. The parameters and charges for the treatment of the hexacoordinated haem with an oxygen atom at the sixth axial position were taken from Harris and Loew (1995). The haem model parameters had been previously developed to be adapted for the AMBER force field (Harris and Loew 1995).

After energy minimization, the models were neutralized by adding Cl⁻ ions and then placed in a periodic box of TIP3P water molecules (Jorgensen *et al.* 1983) extending out to 12 Å on all sides, making it about 104 × 93 × 97 Å in dimension. All simulations were performed using the SANDER module of AMBER version 7.0 and applying the ff99 force field (Wang *et al.* 2000) with periodic boundary conditions. During equilibration, the solvent and counterions were first minimized while a 50 kcal mol⁻¹ Å⁻² restraint was on the solute. With the restraints turned on, the solvent and ions were then allowed to relax with a short dynamics run. Over five rounds of 1000-step minimization, the solute restraints were gradually reduced by 5.0 kcal mol⁻¹ Å⁻² each run. The system was then heated from 100 to 300 K over 5 ps and the equilibration was terminated with a short 50-ps simulation. Production runs were initiated after this stage for 500 ps with an integration time step of 2.0 fs.

During the constant temperature and pressure molecular dynamics production runs, the temperature was maintained at 300 K using the Berendsen coupling algorithm (Berendsen *et al.* 1984) and pressure of 1 atm. The temperature and pressure coupling constants were 2.0 ps. SHAKE was used to constrain all hydrogen atoms (Ryckaert *et al.* 1977). The particle mesh Ewald method with a grid spacing of 1 Å was used to treat all long-range coulombic interactions

(Essmann *et al.* 1995). A non-bonded cut-off of 9.0 Å was applied. The final structures were then visualized using the InsightII molecular modelling package (Insight II User Guide 2002).

Docking studies

A model of NNK was constructed using the Builder module of InsightII. The small molecule was then energy minimized using the Discover module of InsightII by employing the consistent valence force field (cvff) force field and fixing all potentials. The NNK model was manually docked into the active sites of all five protein models and substrate–protein interactions were identified with the aid of InsightII. This program provides a means to determine, for example, hydrogen-bonding interactions between the protein and small molecule and provides total energies for these interactions.

Results

Primary sequence alignment and general CYP protein fold

The results of a primary sequence alignment between CYP2A3, 2A4, 2A5, 2A6, 2A13 and 2C5/3LVdH can be seen in figure 2. The substrate recognition sequences (SRSs) predicted by Gotoh (1992) based on alignment of CYP family 2 enzymes with bacterial CYPs of known structure are indicated as shaded regions in figure 2. The locations of α -helices in the crystal structure of the chimeric rabbit CYP2C5 are also indicated, as are non-conserved residues in the SRS regions (by asterisks). Among the 78 SRS residues, 20 are not conserved among the five CYP2A sequences displayed in figure 2 (asterisks). Mouse CYP2A4 and 2A5 are more similar within the six SRSs than are human CYP2A6 and 2A13. The mouse enzymes differ only at residues 116, 117, 209, 365 and 481, whereas the human enzymes differ at positions 110, 117, 208, 213, 239, 240, 300, 301, 365, 366, 369 and 372 (figure 2). The SRS residues of mouse CYP2A5 and rat CYP2A3 are identical at all positions except 117, where a Val is present in CYP2A5 and Ala in CYP2A3. The enzymes with low K_m values for NNK metabolism listed in table 1 share one common SRS residue that differs from the corresponding residue in the high K_m enzymes — a Met residue at position 365. Mouse CYP2A4 and human CYP2A6 have Leu and Val at this position, respectively (figure 2).

The α -helices in CYP enzymes are lettered sequentially from the N- to C-terminus, as depicted in the superposed model of mouse CYP2A4 and the crystal structure of rabbit CYP2C5/3LVdH shown in figure 3. All CYPs of known structure exhibit strikingly similar overall topology, despite relatively low primary sequence similarity among distantly related proteins (Poulos *et al.* 1995, Podust *et al.* 2003, Wester *et al.* 2003b, Williams *et al.* 2000, 2003). CYPs possess two domains, an α -domain consisting mostly of α -helices located on the C-terminal side of the enzyme (e.g. helices D, E, F, I and L; figure 3) and a β -domain consisting largely of β -sheets located on the N-terminal side of the molecule. The haem cofactor is sandwiched between the I- and L-helices (figure 3).

Model building based on the modelled solution structure of CYP2A4

The coordinates for a solvent-equilibrated CYP2A4 molecular model (constructed using CYP2C5/3LVdH as a template) were kindly provided by Dr Pedersen (Gorokhov *et al.* 2003). The modelled solution structure of CYP2A4

		< A helix	
Mouse CYP 2A4	MLTSGLLLVAAVAFSLVLMVSVKQRKLSGKLPFGPTPLPFVGNFLQLNTEQMYNSLMK	60	
Mouse CYP 2A5	MLTSGLLLVAAVAFSLVLMVSVKQRKLSGKLPFGPTPLPFVGNFLQLNTEQMYNSLMK	60	
Rat CYP 2A3	MLASGLLLVASVAFSLVLMVSVKQRKLSGKLPFGPTPLPFVGNFLQLNTEQMYNSLMK	60	
Human CYP 2A6	MLASGMLLVALLVCLTVMVLMVSVKQRKLSGKLPFGPTPLPFVGNFLQLNTEQMYNSLMK	60	
Human CYP 2A13	MLASGLLLVTLVLAFLTVMVLMVSVKQRKLSGKLPFGPTPLPFVGNFLQLNTEQMYNSLMK	60	
Rabbit CYP 2C5/3LVdH	MA-----KTSSGKGLPPGPTPFPIIGNILQIDAKDISKSLTK	56	
	> < B >		SRS 1
Mouse CYP 2A4	ISQRYGPFVFTIYLGSRRIIVLVCQGEAVKEALVDQAEFFS	120	GRGEQATFDWLFPKGYGIAFSS
Mouse CYP 2A5	ISQRYGPFVFTIYLGSRRIIVLVCQGEAVKEALVDQAEFFS	120	GRGEQATFDWLFPKGYGVVFPSS
Rat CYP 2A3	ISQRYGPFVFTIHLGPRRVVVLVCQGEAVKEALVDQAEFFS	120	GRGEQATFDWLFPKGYGVAFSS
Human CYP 2A6	ISERYGPFVFTIHLGPRRVVVLVCQGEAVKEALVDQAEFFS	120	GRGEQATFDWLFPKGYGVVFSN
Human CYP 2A13	ISERYGPFVFTIHLGPRRVVVLVCQGEAVKEALVDQAEFFS	120	GRGEQATFDWLFPKGYGVAFSS
Rabbit CYP 2C5/3LVdH	FSECYGPVFTVYLGMRKPTVVLHGVEAVKEALVDLGEFFA	116	CTGSGVPILEKYSKGLGIAFSS
	< C helix > < D helix >		< E helix
Mouse CYP 2A4	GERAKQLRRFSIATLRDFGVGKRGIEERIQQEAGFLIDSFRTKNGAFIDPTFFLSRTVSN	180	
Mouse CYP 2A5	GERAKQLRRFSIATLRDFGVGKRGIEERIQQEAGFLIDSFRTKNGAFIDPTFFLSRTVSN	180	
Rat CYP 2A3	GERAKQLRRFSIATLRDFGVGKRGIEERIQQEAGFLIDSFRTKNGAFIDPTFFLSRTVSN	180	
Human CYP 2A6	GERAKQLRRFSIATLRDFGVGKRGIEERIQQEAGFLIDAHRTGGANIDPTFFLSRTVSN	180	
Human CYP 2A13	GERAKQLRRFSIATLRDFGVGKRGIEERIQQEAGFLIDALRGTHGANIDPTFFLSRTVSN	180	
Rabbit CYP 2C5/3LVdH	AKTWKEMRRFSLMTLRNFGMGKRSIEDRIQEEARCLVEELRKTNASPCDPTFFILGCAPCN	176	
	> < F helix SRS 2 > < F' >		SRS 3
Mouse CYP 2A4	VISSIVFGDRFDYEDKEFLSLRMLMLGSLQFTATSMGQVYEMFSSVMKHLPGPQQQAFKE	240	
Mouse CYP 2A5	VISSIVFGDRFDYEDKEFLSLRMLMLGSPQFTATSMGQVYEMFSSVMKHLPGPQQQAFKE	240	
Rat CYP 2A3	VISSIVFGDRFDYEDKEFLSLRMLMLGSPQFTATSTGQVYEMFSSVMKHLPGPQQQAFKE	240	
Human CYP 2A6	VISSIVFGDRFDYEDKEFLSLRMLMLGIFQFTSTSTGQVYEMFSSVMKHLPGPQQQAFOL	240	
Human CYP 2A13	VISSIVFGDRFDYEDKEFLSLRMLMLGRFQFTSTSTGQVYEMFSSVMKHLPGPQQQAFKE	240	
Rabbit CYP 2C5/3LVdH	VICSVIFHNRFDYKDEEFLKLMESLHENVELLGLPWLQVYNNFPALLDYPPGIHKLTLKLN	236	
	G helix > < H >		SRS 4
Mouse CYP 2A4	LQGLEDFITKKVEHNQRTLDPNSPRDFIDSFILRMLEEKKNPNTFYMKNLVMTTLNLFF	300	
Mouse CYP 2A5	LQGLEDFITKKVEHNQRTLDPNSPRDFIDSFILRMLEEKKNPNTFYMKNLVMTTLNLFF	300	
Rat CYP 2A3	LQGLEDFITKKVEQNQRTLDPNSPRDFIDSFILRMLEEKKNPNTFYMKNLVMTTLNLFF	300	
Human CYP 2A6	LQGLEDFIAKKVEHNQRTLDPNSPRDFIDSFILRMQEEKKNPNTFYLKKNLVMTTLNLFI	300	
Human CYP 2A13	LQGLEDFIAKKVEHNQRTLDPNSPRDFIDSFILRMQEEKKNPNTFYLKKNLVMTTLNLFF	300	
Rabbit CYP 2C5/3LVdH	ADYIKNFIMEKVKEHEKLLDVNNPRDFIDCFLIKMEQEN--NLEFTLESVLAIVSDLFGL	293	
	I helix > < J helix > < J' > < K helix		
Mouse CYP 2A4	AGTETVSTTLRYGFLLLMKYPDIEAKVHEEIDRVIGRNRQPKYEDRMKMPYTEAVIHEIQ	360	
Mouse CYP 2A5	AGTETVSTTLRYGFLLLMKHPDIEAKVHEEIDRVIGRNRQPKYEDRMKMPYTEAVIHEIQ	360	
Rat CYP 2A3	AGTETVSTTLRYGFLLLMKHPDIEAKVHEEIDRVIGRNRQAKYEDRMKMPYTEAVIHEIQ	360	
Human CYP 2A6	GGTETVSTTLRYGFLLLMKHPEVEAKVHEEIDRVIGRNRQPKFEDRAKMPYTEAVIHEIQ	360	
Human CYP 2A13	AGTETVSTTLRYGFLLLMKHPEVEAKVHEEIDRVIGRNRQPKFEDRAKMPYTEAVIHEIQ	360	
Rabbit CYP 2C5/3LVdH	AGTETTSSTLRYSLLLLKHPEVAARVQEEIERVIGRHRSPCMQDRSRMPYTDVAIHEIQ	353	
	> SRS 5		
Mouse CYP 2A4	RFADLIPMGLARRVTKDKFRDFLLPKGTEVFPMLGSVLKDPKFFSNPKDFNPKHFLDDK	420	
Mouse CYP 2A5	RFADLIPMGLARRVTKDKFRDFLLPKGTEVFPMLGSVLKDPKFFSNPKDFNPKHFLDDK	420	
Rat CYP 2A3	RFADLIPMGLARRVTKDKFRDFLLPKGTEVFPMLGSVLKDPKFFSNPNDFNPKHFLDDK	420	
Human CYP 2A6	RFGDVIPLMGLARRVKKDKFRDFLLPKGTEVFPMLGSVLKDPKFFSNPQDFNPKHFLNEK	420	
Human CYP 2A13	RFGDVIPLMGLARRVKKDKFRDFLLPKGTEVFPMLGSVLKDPKFFSNPQDCSPQHFLDEK	420	
Rabbit CYP 2C5/3LVdH	RFIDLPLTNLPHAVTRDVRFRNYFIPKGTDIITSLTSVLHDEKAFNPKNKVFDPGHFLDES	413	
	< L helix >		SRS 6
Mouse CYP 2A4	GQFKKSDAFVFPFSIGKRYCFGEGLARMELFLFLTNIMQNFHFKSTQAPQDID	480	VSPRLVGF
Mouse CYP 2A5	GQFKKSDAFVFPFSIGKRYCFGEGLARMELFLFLTNIMQNFHFKSTQAPQDID	480	VSPRLVGF
Rat CYP 2A3	GQFKKSDAFVFPFSIGKRYCFGEGLARMELFLFLTNIMQNFCKSPQAPQDID	480	VSPRLVGF
Human CYP 2A6	GQFKKSDAFVFPFSIGKRNCGEGLARMELFLFFTTVMQNFRLKSSQSPKIDID	480	VSPKLVGF
Human CYP 2A13	GQFKKSDAFVFPFSIGKRYCFGEGLARMELFLFFTTIMQNFRLKSSQSPKIDID	480	VSPKLVGF
Rabbit CYP 2C5/3LVdH	GNFKKSDYFMFPFSAGKRCMVGEGGLARMELFLFLTSILQNFQLQSLVEPKDLDTAVVNGF	473	
Mouse CYP 2A4	VIIPPTYTMSFLSR	494	
Mouse CYP 2A5	ATIPPTYTMSFLSR	494	
Rat CYP 2A3	ATIPPNYTMSFLSR	494	
Human CYP 2A6	ATIPRNYTMSFLPR	494	
Human CYP 2A13	ATIPRNYTMSFLPR	494	
Rabbit CYP 2C5/3LVdH	VSVPPSYQLCFIPI	487	

Figure 2. Sequence alignment of mouse CYP2A4 and CYP2A5, rat CYP2A3, human CYP2A6 and CYP2A13, and the chimeric rabbit CYP2C5/3LVdH. The location of helices in CYP2C5/3LVdH is designated with < > symbols. Substrate recognition sites (SRSs) predicted by Gotoh (1992) are shown as shaded regions and labelled according to the colour scheme used in figures 3–9. *SRS residues not conserved among the 2A enzymes.

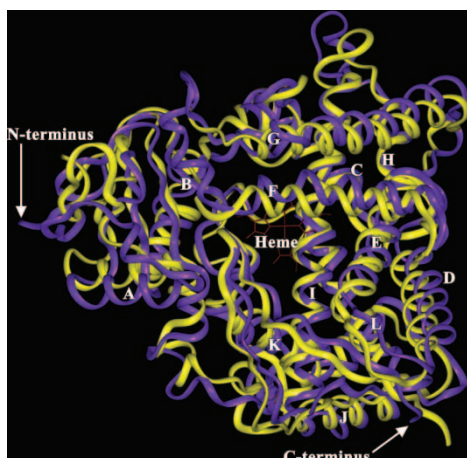


Figure 3. Superposition of the solvent-equilibrated CYP2A4 model (Gorokhov *et al.* 2003) (purple) and the X-ray structure of CYP2C5 (yellow). The N- and C-termini of the CYP2A4 model are indicated. Note that the 2A4 model has undergone significant movements relative to the CYP2C5 template after 3 ns of molecular dynamics in solution, especially helices A, D, F and G.

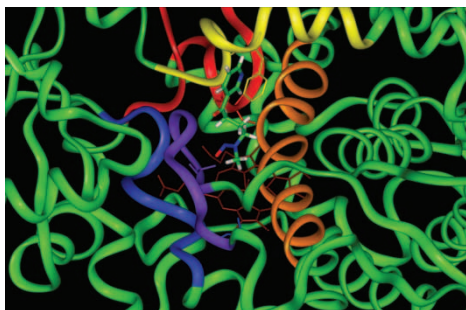


Figure 5. Ribbon diagram of NNK docked in the 'α-methyl' pocket of mouse CYP2A5. SRS regions are coloured as in figure 4. In this orientation, NNK is perpendicular to the haem plane due to the interaction between the pyridine and phenyl rings of Phe209 (yellow, the only side-chain displayed); this alignment of NNK in the active site effects α-methyl hydroxylation. The α-methyl protons are within 3 Å of the iron-oxo oxygen atom, whereas the α-methylene protons are over 5 Å away. SRSs are coloured according to figure 1.

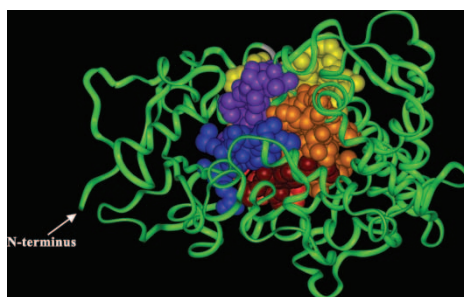


Figure 4. Side view of CYP2A5 with space-filling representation of SRSs forming the active site. Each SRS region is a unique colour. SRS 2 (yellow) forms the top of the active site; SRS 4 (orange) forms the 'left' side, SRS 5 (blue) the lower 'right', and SRS 6 (purple) the upper 'right' side. Barely discernible is SRS 1 (red) forming the far wall of the active site. The haem prosthetic group forms the floor of the active site (maroon).

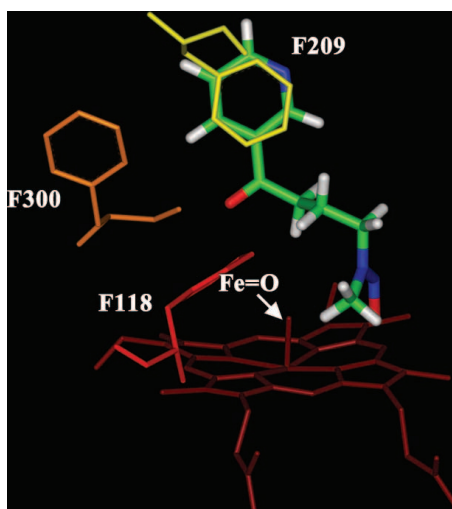


Figure 6. Close-up view of NNK docked in the active site of human CYP2A13 illustrating the α-methyl hydroxylation-binding mode. In this orientation, the pyridine ring π-stacks directly with Phe209 instead of within the 'π-pocket' formed by Phe118, Phe209 and Phe300 (shown in figure 7).

was constructed according to essentially the same sequence alignment presented in figure 2. After F–G and H–I loop repair to correct flaws in the published crystal structure, this model was subjected to extensive (3 ns) molecular dynamics simulations in water, leading to significant movements of backbone and side chain atoms relative to the CYP2C5 template (figure 3) (Gorokhov *et al.* 2003).

The appropriate residues in the CYP2A4 template were then mutated to the corresponding amino acids in CYP2A3, 2A5, 2A6 or 2A13 according to the sequence alignment presented in figure 2. Following residue replacement (conducted using the Homology module of InsightII) and energy minimizations to relieve unfavourable local side-chain interactions, all four models were subjected to 500 ps molecular dynamics in solution. Upon completion of these computational experiments, four new models were obtained: mouse CYP2A5, rat CYP2A3, and human CYP2A6 and 2A13. The root-mean-square deviations between the CYP2A4 model and CYP2A5, CYP2A3, CYP2A6, and CYP2A13 models were 3.756, 3.447, 3.666 and 3.428 Å, respectively.

Considering that the CYP2A4 model underwent extensive testing to ensure its stability and reliability as a CYP model (Gorokhov *et al.* 2003), the newly generated models were assumed to possess similar qualities. Before docking experiments, however, the validity of all molecular models was assessed by confirming that the predicted SRSs (Gotoh 1992) lined the active site; this may be seen for CYP2A5 in figure 4. SRS 1, consisting of amino acids in the B–C loop, forms the ‘right’-side wall of the active site (figure 4). The haem plane forms the floor of the active site, and SRS 2, which consists of the F-helix running parallel to the haem plane, forms the top of the active site. A majority of the I-helix makes a close approach to the haem and lines one wall of the active site (SRS 4; figure 4). SRSs 5 and 6 form the bottom and top of the ‘left’ wall of the active site, respectively. These latter two SRS regions are formed from loops connecting β -sheets in the β -sheet N-terminal domain. Clearly, the SRS regions form the core of the protein and line the active site, while the remainder of the protein, depicted as a green ribbon diagram, forms the scaffold within which the SRS residues lie (figure 4).

NNK docked in the active sites of mouse CYP2A4 and CYP2A5, rat CYP2A3, and human CYP2A6 and CYP2A13

A model of NNK was constructed using the Builder module of InsightII (Insight II User Guide 2002). The model was then subjected to energy minimization using the cvff force field as implemented in the Discover module of InsightII. Once constructed, NNK was manually docked in the active sites of all five protein models. NNK was oriented to provide close (approximately 3–4 Å) approach of known sites of metabolism to the iron-oxo oxygen atom of the putative active oxidant (Ortiz de Montellano 1995). From these starting conformations, the substrate was manipulated to avoid steric conflicts and identify favourable hydrophobic, hydrogen bonding, and π -stacking interactions between the substrate and active site residues. The InsightII suite of programs provides a measure of some of these interactions and identifies likely hydrogen-bonding interactions.

Because CYP2A enzymes form more than one product from a single substrate (table 1), multiple substrate binding modes likely exist within the active site. Docking studies of NNK in the active site of CYP2A models revealed two general binding modes that align the substrate for α -methyl or α -methylene hydroxylation.

As illustrated in figure 5, using CYP2A5 as an example, an active site cavity exists directly over the haem plane that accommodates NNK. A triangular approach gate for the substrate formed by helices I, K and F could be easily visualized. In this binding mode, NNK is oriented perpendicular to the haem plane and is involved in a π -stacking interaction between the pyridine and phenyl rings of Phe209. Phe209 resides in SRS 2 (F-helix, yellow; figure 5) and hangs down almost directly over the haem plane. Other residues of CYP2A5, the side chains of which are not shown in figure 5 for clarity, that make substrate contacts in this binding mode include Gln210, Thr212, Ala213 and Thr214 of SRS 2 (yellow); Phe299, Phe300, Ala301, Glu304, Thr305 and Thr308 of SRS 4 (I-helix, orange); Met365, Ile366 and Leu370 of SRS 5 (blue); and Val478, Gly479, Phe480 and Ala481 of SRS 6 (purple). The side chains of these residues, as well as some of their backbone amide moieties, provide hydrophobic and hydrogen-bonding interactions with NNK.

Many of the important enzyme–substrate interactions discussed above for mouse CYP2A5 are also present in the other CYP2A enzymes. As shown clearly in figure 6 with CYP2A13, when NNK is within the CYP2A13 active site such that the π -stacking interaction exists between the pyridine ring and Phe209, the α -methyl hydrogen atoms are positioned much closer to the iron-oxo species (about 3 Å) than the α -methylene hydrogen atoms (over 5 Å). Thus, this may be termed an ' α -methyl' binding mode for NNK because it preferentially facilitates α -methyl hydroxylation (i.e. HPB formation) (figure 1 and table 1).

A second probable NNK binding mode — the ' α -methylene' binding mode — is illustrated within the active site of human CYP2A13 (figure 7). The phenyl moieties of Phe118, Phe209 and Phe300 form a ' π -pocket' that may facilitate NNK binding via π -stacking interactions with the pyridine ring. Other residues that contact the substrate in this binding mode include those at positions 117, 212, 296, 299, 365, 366 and 370 (not shown in figure 7 for clarity). NNK is oriented parallel to the haem plane such that the pyridine ring lies within the π -pocket and the α -methylene protons lie over the iron-oxo species. Thus, this orientation may be considered an ' α -methylene' binding mode because the α -methylene protons lie much closer to the active oxidant (about 3 Å) than do the α -methyl protons (about 6 Å). Hydroxylation of the α -methylene carbon ultimately generates OPB (figure 1 and table 1). Note that Phe209 contributes to both the α -methylene and α -methyl binding modes.

The relative degrees to which the α -methylene and α -methyl binding modes contribute to NNK metabolism for each individual CYP2A enzyme may have a bearing on the biochemical results presented in table 1. Therefore, comparisons of the active sites of these enzymes, especially within species, are necessary to elucidate the physical basis for regioselective NNK metabolism.

CYP2A6 versus CYP2A13

A comparison of 10 non-conserved SRS residues between CYP2A6 and CYP2A13 can be seen in figure 8. One notable difference between the model structures for these two enzymes is within the F-helix (SRS 2), which is slightly unwound in CYP2A13 relative to CYP2A6 (not shown). This unwinding may be caused by a Phe residue at position 300 in the I-helix of CYP2A13 (SRS 4). CYP2A6 has an Ile at this position. Phe300 of CYP2A13 juts up into the F-helix and displaces Arg208 considerably more than Ile300 displaces Ile208 in CYP2A6 (figure 8). In addition, Ile300 of CYP2A6 protrudes into the π -pocket and exhibits

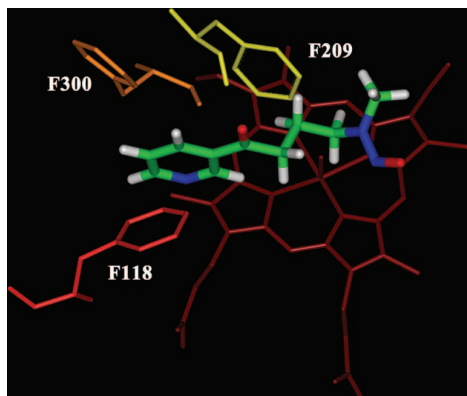


Figure 7. Close-up view of the 'π-pocket' of CYP2A13 illustrating interactions of the phenyl rings of Phe118, Phe209 and Phe300 with the pyridine ring of NNK. These interactions position NNK in the active site such that α -methylene hydroxylation is effected.

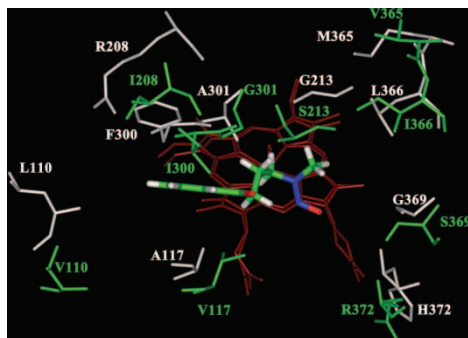


Figure 8. Close-up view of NNK docked in the active site of superposed human CYP2A6 (green) and 2A13 (white) demonstrating 10 non-conserved SRS residues. NNK is docked with the pyridine ring in the 'π-pocket' and is positioned to effect α -methylene hydroxylation. CYP2A6 possesses an Ile residue at position 300 (Phe in 2A13) and thus has a less well developed π-pocket than does CYP2A13. The presence of Phe300 in 2A13 displaces Arg208 higher above the haem plane than Ile208 in 2A6; this creates a larger binding pocket in 2A13 versus 2A6.

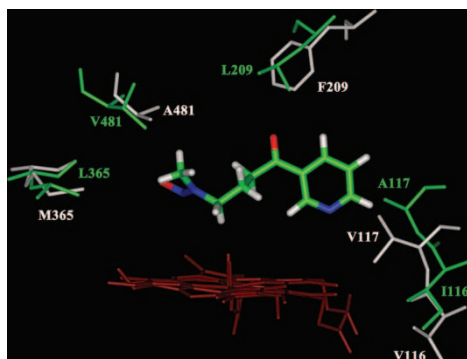


Figure 9. Superposition of mouse CYP2A4 (green) and CYP2A5 (white) active sites demonstrating the five non-conserved SRS residues between these two enzymes. NNK is docked with the pyridine ring in the 'π-pocket' and is positioned for α -methylene hydroxylation. Note that Val117 in CYP2A5 protrudes into the π-pocket more so than does Ala117 of CYP2A4.

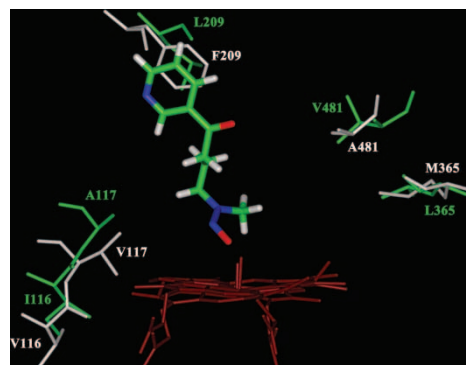


Figure 10. Superposition of mouse CYP2A4 (green) and CYP2A5 (white) active sites demonstrating the five non-conserved SRS residues between these two enzymes. NNK is docked in the 'α-methyl' pocket facilitated by π-stacking between the pyridine and phenyl rings of Phe209 (2A5, white). Note that in CYP2A4, the presence of Leu at this position instead of Phe abolishes this favourable interaction. In addition, from this angle, one can better appreciate the greater projection of Val117 into the active site relative to Ala117.

steric clashes with the pyridine ring of NNK, as opposed to the favourable π -stacking interaction provided by Phe300 of CYP2A13. Finally, CYP2A6 has a Val residue at position 117 that juts into the π -pocket more so than the equivalent Ala residue at the same position in CYP2A13 (figure 8). The net effect of these amino acid differences between human CYP2A6 and CYP2A13 is a more capacious π -pocket in the latter enzyme that is better able to bind NNK in the α -methylene binding mode; thus more efficiently effecting α -methylene hydroxylation.

When comparing the residues lining the α -methyl pockets of CYP2A6 and CYP2A13, it can be seen that the critical Phe residue at position 209 is present in both enzymes (figure 2), and that this residue occupies the same relative space in both model structures (not shown). However, dramatic differences are apparent when comparing the residues in SRS 5 (the residues in SRS 6 are identical between the two enzymes, see figure 2). Four residues in SRS 5 differ between these two enzymes, at positions 365 (Val in CYP2A6, Met in CYP2A13), 366 (Ile in CYP2A6, Leu in CYP2A13), 369 (Ser in CYP2A6, Gly in CYP2A13), and 372 (Arg in CYP2A6, His in CYP2A13) (figures 2 and 8). The relative coordinates of these non-conserved residues do not differ as dramatically between the two enzymes as they do in the π -pocket, but there are significant differences in the identity of certain residues. Most significant are the Val365Met, Ser369Gly and Arg372His changes between CYP2A6 and CYP2A13, respectively. Thus, the presence of more accommodating α -methylene and α -methyl pockets of CYP2A13 compared with CYP2A6 may account for the dramatic differences in K_m values between these two enzymes (table 1).

CYP2A3 versus CYP2A5

Interestingly, mouse CYP2A5 and rat CYP2A3 possess identical residues at all SRS positions except one — residue 117 (figure 2). The rat enzyme, like mouse CYP2A4 and human CYP2A13, has Ala at this position (figure 2), and would thus be expected to have a constructive α -methylene binding mode. This notion is supported by the observation that the other residues making up the CYP2A3 π -pocket have similar coordinates to those of CYP2A13 (not shown), which is clearly an efficient catalyst of α -methylene hydroxylation (table 1). On the other hand, the α -methyl pocket of CYP2A3 looks quite similar to that of CYP2A5 (not shown), and thus it also possesses favourable substrate interactions leading to HPB formation. Perhaps the presence of two relatively efficient binding modes explains why CYP2A3 does not exhibit appreciable regioselectivity for either the α -methylene or α -methyl carbons of NNK, but is still an efficient catalyst of both reactions (table 1).

CYP2A4 versus CYP2A5

Mouse CYP2A4 and 2A5 are the most similar among the CYP2A enzymes investigated in this study; they only differ by 11 amino acids out of 494, but five of these non-conserved amino acids fall within the SRS regions (figure 2). Thus, these enzymes differ in five of 78 SRS residues, but in only six of the remaining 416 residues. The superposed active sites of CYP2A4 and CYP2A5 illustrating these five amino acid differences may be seen in figure 9. NNK is docked into the composite active site such that the pyridine ring lies within the π -pocket and the substrate is poised to undergo α -methylene hydroxylation. As was the case

with the human 2A enzymes, mouse CYP2A4 and 2A5 differ in the residue present at position 117 (figure 2). CYP2A5 has a Val at this position that sticks into the π -pocket, just as was seen with human CYP2A6. CYP2A4, like CYP2A13, possesses the more accommodating Ala residue at this position. The presence of the more obtrusive Val residue at position 117 may retard the α -methylene hydroxylation pathway by making this binding mode unfavourable in CYP2A5 and CYP2A6. CYP2A4 has a rather ambivalent π -pocket, as it possesses the 'favourable' Ala residue at position 117 (instead of Val), but does not have a Phe at position 209. Thus, CYP2A4 does not have as 'well-developed' a π -pocket as do CYP2A5, 2A6, and 2A13. The lack of a Phe at position 209 would also be expected to make NNK binding in the α -methyl pocket less efficient in CYP2A4, as illustrated in figure 10. The presence of a Phe at position 209 is important to facilitate alignment of NNK in the active site such that α -methyl hydroxylation is effected. The lack of this residue in CYP2A4 may explain why CYP2A5 is a much better catalyst of HPB formation than is CYP2A4 (figure 10 and table 1). It may also partially explain the higher K_m values for CYP2A4 relative to CYP2A5 for both HPB and OPB formation (table 1).

One final point of interest regarding CYP2A-catalysed NNK metabolism concerns the relative K_m values listed in table 1. The only common residue among all the low K_m enzymes (CYP2A3, CYP2A5 and CYP2A13) that differs from the residue at the corresponding position in the high K_m enzymes (CYP2A4 and CYP2A6) occurs at position 365, which lies within SRS 5; CYP2A3, 2A5 and 2A13 all possess a Met residue at this position, whereas CYP2A4 (Leu) and CYP2A6 (Val) do not.

Discussion

Molecular models of mouse CYP2A5, rat CYP2A3, and human CYP2A6 and 2A13 were constructed using the solvent-equilibrated model of mouse CYP2A4 (Gorokhov *et al.* 2003) as a template. NNK was then docked into the active sites of these protein models in an attempt to rationalize biochemical data regarding the efficiency, stereoselectivity, and regioselectivity of metabolism of this substrate (table 1). Note that the docking studies described here were performed by manually manipulating the substrate in the active site. However, binding of substrate in the CYP active site is likely to induce significant conformational changes in the protein (Modi *et al.* 1996, Gorokhov *et al.* 2003, Wester *et al.* 2003b, Williams *et al.* 2003). In future work, docking programs such as Autodock 3.05 (Morris *et al.* 1998) will be employed in conjunction with site-directed mutagenesis experiments to further substantiate the binding modes investigated in this work.

As is summarized in table 2, several key CYP2A residues that make important contacts with NNK have been identified. The identity of residues at these positions may aid in explaining the biochemical data presented in table 1. As stated above, the presence of a Val residue at position 117 leads to unfavourable steric interactions with the pyridine ring of NNK. Because CYP2A3 and CYP2A5 have identical residues at all SRS positions except 117, it is possible that the presence of Val117 in CYP2A5 lowers the affinity of the π -pocket for NNK sufficiently such that NNK preferentially binds in the α -methyl hydroxylation pocket.

The importance of the residue at position 117 of CYP2A4 and CYP2A5 has been well documented by the Negishi group. They have found that substitution of Val for Ala in two polymorphic mouse CYP2A5 enzymes leads to a much higher coumarin 7-hydroxylase activity in the Val-containing protein (Aida *et al.* 1994). 7-Hydroxylation of coumarin likely occurs when the substrate is docked in the 'α-methyl' pocket identified for NNK (Lewis 2002a). Thus, the presence of Val at position 117 precludes access of coumarin to the alternative 'α-methylene' binding mode and thereby indirectly promotes efficient conversion of coumarin to 7-hydroxycoumarin.

Consistent with a role for Val117 in defining the 'α-methyl' pocket is our recent data that CYP2A3, CYP2A4 and CYP2A13 all catalyse coumarin 3,4-epoxidation, either exclusively or in addition to 7-hydroxylation (Von Weymarn and Murphy 2001, 2003). CYP2A3, CYP2A4 and CYP2A13 all have Ala at position 117, which may allow reorientation of coumarin for 3,4-epoxidation. However, modelling for the interaction of coumarin in this orientation has not been carried out.

Negishi and co-workers have also demonstrated that the Ala117Val mutation in CYP2A4 transforms the enzyme from a Δ^4 , 3-ketone steroid hydroxylase to an efficient Δ^4 , 3-hydroxy steroid hydroxylase (Iwasaki *et al.* 1994). Regarding NNK metabolism, Hong and co-workers recently reported the Ala117Val mutation in human CYP2A13 led to decreased α-methylene and increased α-methyl hydroxylation of NNK (He *et al.* 2003). Clearly, very subtle amino acid changes at this position can have a profound effect on the regioselectivity of NNK metabolism.

The identity of the residue at position 209 also profoundly affects substrate specificity of CYP2A enzymes. Phe209 occupies a critical position directly above the haem plane and contributes to both the α-methylene and α-methyl binding modes for NNK (figures 5–7). CYP2A4, which possesses a Leu at this position, is a much poorer catalyst of NNK metabolism than are CYP2A3, 2A5 or 2A13 (table 1). However, human CYP2A6 is, like CYP2A4, a poor catalyst of NNK metabolism, but it does possess a Phe residue at position 209 (table 2). Regardless, the importance of this residue has been elegantly demonstrated by Negishi and co-workers. They showed that the single mutation Phe209Leu was sufficient to convert the substrate specificity of CYP2A5 from coumarin 7-hydroxylation to testosterone 15α-hydroxylation (a prototypical CYP2A4 reaction) (Lindberg and Negishi 1989). Single amino acid mutations are also sufficient to alter the regioselectivity of other CYP enzymes (Schalk and Croteau 2000).

When comparing all five CYP2A enzymes investigated here, only Met365 (of the 78 SRS residues) is unique to the enzymes that exhibit low K_m values for NNK α-hydroxylation (CYP2A3, CYP2A5 and CYP2A13; table 1), whereas the high K_m enzymes possess a Leu (CYP2A4) or Val (CYP2A6) at this position. Although not modelled in this work, rabbit CYP2A10/11 also exhibit a low K_m for NNK metabolism (table 1) and possess a Met residue at position 365. The presence of Met at position 365 in only the low K_m enzymes may be a coincidental finding, but it would be interesting to determine if introduction of a Met residue at position 365 of CYP2A4 and CYP2A6 resulted in a lowering of K_m values for NNK metabolism. The effect of Met365 is not clear, but it is possible that the easily polarizable sulfur atom stabilizes NNK via dipole–dipole interactions with the partial positive charge on the nitroso nitrogen atom. Negishi and co-workers have found that replacement of Met365 with Lys significantly decreased the

Table 2. Summary of some amino acid residues in CYP2A enzymes that make important substrate contacts with NNK.

Residue number	Enzyme and residue					Remarks
	2A3	2A4	2A5	2A6	2A13	
117	A	A	V	V	A	Val117 protrudes into the π -pocket making α -methylene hydroxylation less likely
209	F	L	F	F	F	Phe209 is a key residue contributing to both the π -pocket leading to α -methylene hydroxylation and the α -methyl binding mode
300	F	F	F	I	F	Phe300 contributes to the π -pocket. Ile300 in 2A6 clashes with NNK bound in the α -methylene hydroxylation mode
365	M	L	M	V	M	Sulfur atom of Met365 may be involved in a dipole–dipole interaction with the partially positively charged nitroso nitrogen atom. CYP2A4 and 2A6 lack a Met at this position and exhibit much higher K_m values for NNK α -hydroxylation
481	A	V	A	A	A	Ala481 provides a hydrophobic interaction with NNK in the α -methyl binding mode. The presence of Val at this position in 2A4 may compress this binding pocket

CYP2A5-mediated metabolism of the food carcinogen aflatoxin B₁ (Pelkonen *et al.* 1997).

Site-directed mutagenesis studies involving mutation of the residue at position 481 of CYP2A4 and CYP2A5 have demonstrated that substitution at this position with larger residues (e.g. Ile for Val) leads to increased regiospecificities for the C15 position of androstendione (Iwasaki *et al.* 1995a). When NNK is docked in the α -methyl binding pocket of CYP2A4, Val481 projects into the binding cavity somewhat more than does Ala481 of CYP2A5. This substitution may therefore contribute to the decreased ability of CYP2A4 to catalyse the methyl hydroxylation of NNK (table 2).

Molecular modelling has been used extensively to investigate the mechanisms of CYP reactivity as well as to elucidate structure–function relationships (Bathelt *et al.* 2002, De Groot *et al.* 2002, Kumar *et al.* 2003, Liu *et al.* 2003, Park and Harris 2003, Venhorst *et al.* 2003). The models presented here compare favourably to those published previously for CYP2A enzymes (Lewis *et al.* 1999, Lewis 2002a, Xu *et al.* 2002). Specifically, the identities of key active site residues, such as those at positions 117, 209 and 365, are in good agreement with previous modelling work as well as site-directed mutagenesis data (Lindberg and Negishi 1989, Juvonen *et al.* 1991, Negishi *et al.* 1992, 1996, Iwasaki *et al.* 1993a, b, 1994, 1995a, b, Pelkonen *et al.* 1997, Lewis *et al.* 1999, Lewis 2002a, Xu *et al.* 2002). Recently, the crystal structure of the first human CYP enzyme (2C9) was solved, and two new structures of CYP2C5 were reported to higher resolution than the original structure (Wester *et al.* 2003a, b, Williams *et al.* 2003). As improvements are made in refining and defining the atomic coordinates for these enzymes, the predictive power of molecular modelling techniques will continue to increase.

More work needs to be done to determine the effects of amino acid substitutions on the stereo- and regioselectivity of NNK metabolism by CYP2A enzymes. It is interesting to note when NNK binds in the ' α -methylene' mode, the pro-4*R* α -methylene hydrogen is closer to the iron-oxo oxidant than the pro-4*S* hydrogen (figures 7 and 8), consistent with previous isotope-effect studies (Jalas *et al.* 2003b).

It would be interesting to determine if CYP2A enzymes from other species (e.g. CYP2A3 and CYP2A13) also exhibit pro-4*R* stereoselectivity. In addition, it would be interesting to determine the effects of active-site mutations on the stereoselectivity of α -methylene hydroxylation. It is hoped that biochemical data (table 1), the models generated in this work, and future improved versions of these models will assist in the development of highly specific and efficacious inhibitors of NNK bioactivation.

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***In vitro* assessment of intestinal permeability and hepatic metabolism of 4'-bromoflavone, a promising cancer chemopreventive agent**

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1. The intestinal permeability and hepatic metabolism of the investigational cancer chemoprevention agent 4'-bromoflavone were investigated *in vitro* using human intestinal Caco-2 cell monolayers, human liver microsomes and human hepatocytes. Liquid chromatography-mass spectrometry and tandem mass spectrometry were used for quantitative analysis in support of the Caco-2 cell studies and for the characterization of metabolites of 4'-bromoflavone.

2. The Caco-2 cell model indicated that 4'-bromoflavone would be absorbed by the intestine at a moderate rate by means of direction-independent, passive diffusion. There was no indication of active transport or efflux.

3. Three monohydroxylated metabolites and one monohydroxylated, hydrated metabolite of 4'-bromoflavone were detected at relatively low levels in the human liver microsomal and hepatocyte incubations. The structures of these metabolites were confirmed by comparison with synthetic standards. Hydroxylation occurred on the A-ring of 4'-bromoflavone but not on the B-ring, probably due to deactivation of the B-ring by bromine. No phase II metabolites were detected following incubation of 4'-bromoflavone in these *in vitro* systems.

4. In conclusion, these studies predict that 4'-bromoflavone should show moderate oral bioavailability, and that it would probably be excreted as unchanged compound and monohydroxylated metabolites. The results might be helpful in the design of clinical trials and in the interpretation of pharmacokinetic studies of 4'-bromoflavone.

Introduction

Cancer chemoprevention is defined as the use of specific natural or synthetic chemical agents to reverse or suppress carcinogenesis and prevent the development of invasive cancer (Hong and Sporn 1997, Kucuk 2002). One mechanism for cancer chemoprevention involves the induction of detoxification enzymes, such as quinone reductase and glutathione *S*-transferase, which are associated with protection against chemical stress and the initiation of carcinogenesis (Talalay *et al.* 1995). Several cancer chemopreventive agents have been identified based on this mechanism including sulforaphane (Zhang *et al.* 1992), brassinin (Mehta *et al.* 1995), sulforamate (Gerhauser *et al.* 1997) and 4'-bromoflavone (Song *et al.* 1999).

4'-Bromoflavone has been shown to induce quinone reductase activity at the transcriptional level in Hepa 1c1c7 murine hepatoma cells and to induce two major detoxification isoforms of glutathione *S*-transferases (α - and μ -class) in H4IIE rat

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hepatoma cells without observed toxic effects (Song *et al.* 1999). In studies with Sprague–Dawley rats, 4'-bromoflavone induced quinone reductase activity and increased glutathione levels in all the tissues examined including liver, mammary gland, colon, stomach and lung (Song *et al.* 1999). In addition, 4'-bromoflavone inhibited the covalent binding of metabolically activated benzo[α]pyrene to cellular DNA in HepG2 and MCF-7 cells (Song *et al.* 1999). Finally, 4'-bromoflavone was found to inhibit or delay 7,12-dimethylbenz[*a*]anthracene-induced tumour formation in a rat mammary tumorigenesis model (Song *et al.* 1999, Mehta and Pezzuto 2002).

As a promising cancer chemopreventive agent, 4'-bromoflavone is under consideration for clinical trials. However, there is no information available about its bioavailability and metabolism. The evaluation of the intestinal absorption and metabolism of an investigational new drug is essential during preclinical drug development (Lin and Lu 1997, Li 2001), and this is the subject of the present investigation.

In vitro models such as the human intestinal epithelial Caco-2 cell monolayer assay for drug permeability and human liver microsomes and hepatocytes for drug metabolism have become standard during early drug discovery and development (Hidalgo *et al.* 1989, Ekins *et al.* 2000, Artursson *et al.* 2001, Li *et al.* 2001). In addition, the speed, sensitivity and selectivity of high-performance liquid chromatography-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS-MS) have made these analytical tools essential for preclinical studies of metabolism and bioavailability (Poon *et al.* 1999, Niessen 2003). In the present study, the permeability and metabolism of 4'-bromoflavone were investigated using Caco-2 cell monolayers and human hepatic microsomes and hepatocytes in combination with LC-MS and LC-MS-MS. The results should be useful for the prediction of *in vivo* intestinal permeability and hepatic metabolism in preparation for clinical trials.

Materials and methods

Chemicals and materials

4'-Bromoflavone and its hydroxylated analogues were synthesized as described below. Pooled human liver microsomes and cryopreserved human hepatocytes were obtained from In Vitro Technologies (Baltimore, MD, USA). The cytochrome P450 content in the human liver microsomes was 0.17 nmol P450 mg⁻¹ protein. HPLC-grade solvents were purchased from Fisher Scientific (Fair Lawn, New York, USA), and all other chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

The Caco-2 human colorectal carcinoma cell line, Eagle's minimum essential medium and foetal bovine serum were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Trypsin-EDTA (0.25%), Hank's balanced salt solution (HBSS), penicillin and streptomycin were purchased from Life Technology (Grand Island, New York, USA). Transwell cell culture plates with polycarbonate inserts (24.4 mm in diameter, 4.71 cm² surface area and 3.0 μ m pore size) were purchased from Corning Costar (Cambridge, MA, USA). Transepithelial electrical resistance (TEER) of each Caco-2 cell monolayer was measured using a Millipore (Bedford, MA, USA) Millicell-ERS instrument.

Caco-2 cell permeability studies

Caco-2 cells were cultured at 37°C in Eagle's minimum essential medium with 10% foetal bovine serum, 100 units ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin in an atmosphere of 5% carbon dioxide and 90% relative humidity. Confluent monolayers were subcultured every 7 days by treatment with 0.25% trypsin containing EDTA. For permeability studies, Caco-2 cells (passage number 23) were seeded on polycarbonate membranes at a density of 2×10^5 cells/insert and cultured until late confluence (20–21 days). The medium was changed every other day after seeding. The integrity of

Caco-2 cell monolayers was monitored by measuring the TEERs, and only monolayers $> 300 \Omega \text{ cm}^2$ were utilized.

Caco-2 cell monolayer permeability incubations were carried out at 37°C with continuous agitation on a plate shaker. Immediately before each experiment, the monolayers were rinsed with HBSS buffer containing 25 mM HEPES (pH 7.4) followed by pre-incubation for 30 min. 4'-Bromoflavone was added either to the apical side (for apical to the basolateral permeability measurements) or to the basolateral side (for basolateral to the apical measurements) of the inserts. The receiving chambers contained only HBSS buffer. Propranolol (10 μM) and mannitol (100 μM) were added to the apical sides of each monolayer as markers of high and low (paracellular only) permeability, respectively. The total volumes of buffer in the apical and basolateral chambers were 1.5 and 2.6 ml, respectively. After incubation for 5, 10, 20, 40 or 60 min, the medium in the receiving compartments was collected and replaced by fresh HBSS buffer. The concentrations of 4'-bromoflavone, mannitol and propranolol in the samples of medium that were removed were then determined using LC-MS as described below.

The apparent permeability coefficients (P_{app} , cm s^{-1}) were calculated using the following equation:

$$P_{\text{app}} = V_{\text{R}} \times \text{d}C/\text{d}t \times 1/AC_0,$$

where V_{R} is the volume of the solution in the receiving compartments, $\text{d}C/\text{d}t$ is the transport rate ($\mu\text{M s}^{-1}$), A the surface area of the membrane (4.71 cm^2) and C_0 is the initial concentration of compounds in the donor chambers (μM). P_{app} values were measured under 'sink conditions', i.e. less than 10% transported, so that the diffusion of the solute back from the receiving to the donor side was minimized. All experiments were carried out in triplicate. After 1 h incubation, the TEERs were measured again to verify the integrity of the monolayers, which in all cases remained $> 300 \Omega \text{ cm}^2$. The permeability coefficients of mannitol from the apical to the basolateral sides were also measured and remained low, about $3 \times 10^{-7} \text{ cm s}^{-1}$, and the P_{app} values of propranolol were about $5 \times 10^{-5} \text{ cm s}^{-1}$. These data indicated that the Caco-2 cell monolayers utilized in this study possessed suitable monolayer integrity.

To investigate the possible metabolism of 4'-bromoflavone by Caco-2 cell monolayers, 4'-bromoflavone was added to the apical chambers to produce a final concentration of 20 μM . After incubation for 4 h, both apical and basolateral samples were collected for LC-MS analysis of 4'-bromoflavone and its metabolites. In addition, Caco-2 cell monolayers were washed twice with HBSS buffer and then incubated in 1 ml of methanol for 30 min. The methanol extracts were collected for LC-MS analysis of the 4'-bromoflavone metabolites.

Incubation of 4'-bromoflavone with human liver microsomes and hepatocytes

4'-Bromoflavone (10 μM) was incubated with pooled human liver microsomes (1 mg ml^{-1}) in a total volume of 400 μl containing 50 mM phosphate buffer (pH 7.4). NADPH (1 mM) was added after a 5 min pre-incubation at 37°C . The reaction was terminated after 1 h by adding 1.2 ml ice-cold ethanol/acetonitrile (1:1, v/v). After centrifugation, the supernatant was removed and evaporated to dryness *in vacuo*. The residue was reconstituted in 150 μl methanol/water (1:1, v/v) for analysis using LC-MS. Controls included incubations without the addition of NADPH or without microsomes.

In a separate experiment, cryopreserved hepatocytes were thawed according to the supplier's instructions, and approximately 10^6 cells in a 1 ml suspension were incubated with 4'-bromoflavone (10 μM) at 37°C for 4 h. The reaction was terminated by adding 3 ml ice-cold methanol followed by centrifugation. The supernatant was evaporated to dryness *in vacuo*, and the residue was reconstituted in 150 μl methanol/water (1:1, v/v) for LC-MS analysis. Control samples were incubated with heat-killed hepatocytes.

Enzymatic kinetic studies

For the investigation of drug metabolism kinetics using enzymes in human liver microsomes, each incubation (total volume 400 μl) contained 50 mM phosphate buffer (pH 7.4), 0.25 mg ml^{-1} human liver microsomal protein, 1 mM NADPH, and 0.1–100 μM 4'-bromoflavone. After incubating for 10 min, the reactions were terminated, and the samples were processed as described above. Incubations were carried out in duplicate. Kinetic parameters were calculated using non-linear regression analysis with GraphPad 3.0 software (San Diego, CA, USA).

Synthesis of 4'-bromoflavone and analogues for metabolite identification

4'-Bromoflavone was synthesized as reported (Song *et al.* 1999). To confirm the structures of the metabolites, analogues of 4'-bromoflavone were synthesized in three steps beginning with readily available dihydroxyacetophenones (**1–3**) and 4-bromobenzaldehyde (figure 1). Briefly, dihydroxyacetophenone **1**, **2** or **3** was mono-protected as a THP ether (Adams and Main 1991) and condensed with 4-bromobenzaldehyde under alkaline conditions. The resulting chalcone **4**, **5** or **6** was then cyclized and deprotected in one step using a catalytic amount of iodine in refluxing DMSO to give the desired flavone **7**, **8** or **9**. The synthetic scheme is summarized in figure 1, and details of the synthetic procedures are described below.

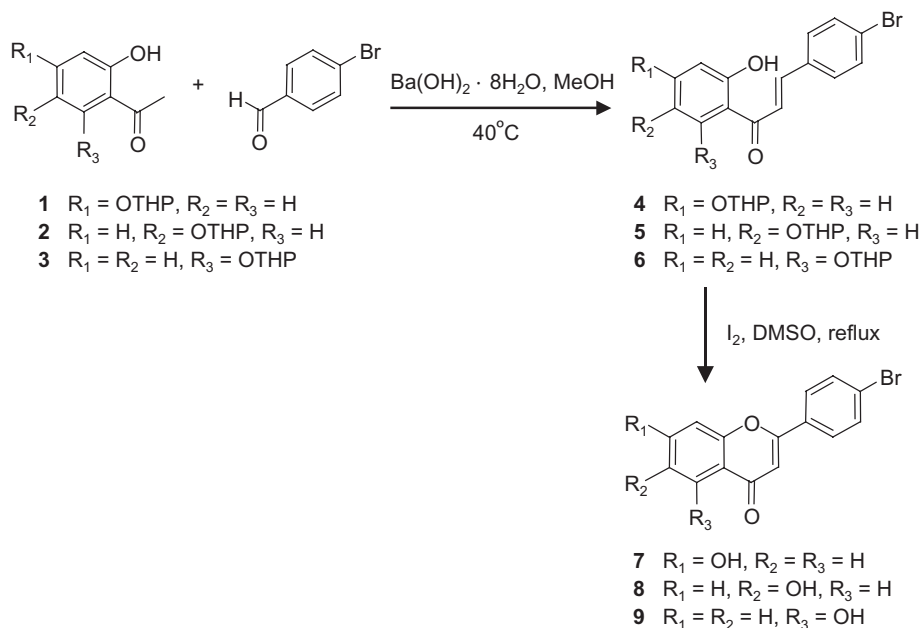


Figure 1. Synthetic scheme for the preparation of monohydroxylated derivatives of 4'-bromoflavone.

4-Bromobenzaldehyde (1 eq.) and a mono-protected dihydroxyacetophenone (1 eq.) were dissolved in methanol (8 ml mmol⁻¹). Barium hydroxide heptahydrate (1 eq.) was added, and the reaction mixture was stirred at 40°C for 24 h and then cooled to room temperature. The methanol was removed under reduced pressure, water was added and the solution was acidified to pH 4 with 1 M aqueous HCl. The mixture was extracted with dichloromethane, and the combined organic extracts were washed with brine, dried over Na₂SO₄ and evaporated under reduced pressure to give a crude oil. The resulting reaction mixture was purified by chromatography over silica gel using ethyl acetate/hexane (1:9, v/v) as the eluent to obtain the chalcones **4–6** as described below.

3-(4-Bromophenyl)-1-[2-hydroxy-4-(tetrahydropyran-2-yloxy)-phenyl]-propenone **4**: yield 71%, yellow needles m.p. = 155–157°C, $R_f = 0.56$ (ethyl acetate/hexane; 1:4, v/v); FTIR (NaCl, film): $\nu_{\text{max}} = 1632$ (C=O), 1580 (C=C, aromatic), 1221 (C-O-C) cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 1.65 (m, 3H), 1.86 (m, 1H), 1.97 (m, 2H), 3.6 (m, 1H), 3.83 (m, 1H), 5.50 (t, $J = 2.8$ Hz, 1H, O-CH-O), 6.59 (dd, $J = 8.8, 2.2$ Hz, 1H, 5'-H), 6.62 (d, $J = 2.3$ Hz, 1H, 3'-H), 7.48 (d, $J = 8.4$ Hz, 2H, 2,4-H), 7.54 (d, $J = 8.8$ Hz, 2H, 3,5-H), 7.56 (d, $J = 8.8$ Hz, 1H, 6'-H), 7.77 (d, $J = 12.4$ Hz, 1H, α -H), 7.81 (d, $J = 12.1$ Hz, 1H, β -H); ¹³C-NMR (400 MHz, CDCl₃): 18.49, 24.97, 29.96, 62.18, 96.13, 104.22, 108.67, 114.62, 120.88, 124.92, 129.86 (2C), 131.20, 132.22 (2C), 133.68, 142.93, 163.80, 166.31, 191.59; HRMS [M - H]⁻ calculated for C₂₀H₁₉O₄Br 401.0388, measured m/z 401.0399.

3-(4-Bromophenyl)-1-[2-hydroxy-5-(tetrahydropyran-2-yloxy)-phenyl]-propenone **5**: yield 65%, orange needles m.p. = 115–117°C, $R_f = 0.59$ (ethyl acetate/hexane; 1:4, v/v); FTIR (NaCl, film): $\nu_{\text{max}} = 1643$ (C=O), 1580 (C=C, aromatic), 1256 (C-O-C) cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 1.64 (m, 3H), 1.85 (m, 2H), 1.96 (m, 1H), 3.61 (d, $J = 11.2$ Hz, 1H), 3.92 (t, $J = 9.0$ Hz, 1H), 5.32 (s, 1H, O-CH-O), 6.92 (d, $J = 9.0$ Hz, 1H, 3'-H), 7.26 (dd, $J = 6.2, 2.7$ Hz, 1H, 4'-H), 7.47 (d, $J = 8.5$ Hz, 2H, 2,4-H), 7.51 (d, $J = 8.8$ Hz, 2H, 3,5-H), 7.53 (d, $J = 2.7$ Hz, 1H, 6'-H), 7.55 (d, $J = 15.5$ Hz, α -H), 7.77 (d, $J = 15.5$ Hz, β -H); ¹³C-NMR (400 MHz, CDCl₃): 18.78, 25.18, 30.44, 62.12, 97.63, 116.54, 119.22, 119.58, 120.73, 125.24, 130.02 (2C), 130.96, 132.28 (2C), 132.44, 144.02, 149.12, 158.67, 191.04; HRMS [M - H]⁻ calculated for C₂₀H₁₉O₄Br 401.0388, measured m/z 401.0381.

3-(4-Bromophenyl)-1-[2-hydroxy-6-(tetrahydropyran-2-yloxy)-phenyl]-propenone **6**: yield 58%, orange needles m.p. = 114–116°C, $R_f = 0.54$ (ethyl acetate/hexane; 1:4, v/v); FTIR (NaCl, film): $\nu_{\text{max}} = 1629$ (C=O), 1590 (C=C, aromatic), 1226 (C-O-C) cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 1.59 (m, 2H), 1.70 (m, 1H), 1.88 (m, 3H), 3.66 (m, 1H), 3.86 (m, 1H), 5.28 (t, $J = 2.5$ Hz, 1H), 6.57 (d, $J = 8.1$ Hz, 1H, 3'-H), 6.69 (d, $J = 8.1$ Hz, 1H, 5'-H), 7.32 (t, $J = 8.3$ Hz, 1H, 4'-H), 7.44 (d, $J = 8.5$ Hz, 2H, 2',6'-H), 7.51 (d, $J = 8.5$ Hz, 2H, 3',5'-H), 7.68 (d, $J = 15.6$ Hz, 1H, α -H), 7.86 (d, $J = 15.6$ Hz, 1H, β -H); ¹³C-NMR (400 MHz, CDCl₃): 18.72, 24.90, 31.56, 62.37, 97.31, 105.31, 111.38, 112.63, 124.54, 128.39, 129.67 (2C), 132.15 (2C), 134.12, 136.08, 141.02, 158.31, 164.20, 194.31; HRMS [M - H]⁻ calculated for C₂₀H₁₉O₄Br 401.0388, measured m/z 401.0386.

Flavones **7–9** were synthesized from the chalcone intermediates **4–6** using the following approach. Each chalcone was dissolved in DMSO (1 mmol^{-1}), and a chip of I_2 was added. The resulting reaction mixture was refluxed for 8 h and then cooled to room temperature. A saturated aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$ was added, and the mixture was allowed to stand at room temperature for 2 h. The reaction mixture was then extracted with dichloromethane, and the combined organic extracts were washed with water and brine, dried over Na_2SO_4 , and the solvent removed under reduced pressure to give a crude solid. The resulting solid was purified by using preparative thin layer chromatography over silica gel (ethyl acetate/hexane, 1:4 or 2:3, v/v) to obtain the following flavones **7–9**.

2-(4-Bromophenyl)-7-hydroxy-chromen-4-one [7-hydroxy-4'-bromoflavone] **7**: yield 67%, white powder m.p. = $281\text{--}283^\circ\text{C}$, $R_f = 0.11$ (ethyl acetate/hexane; 3:7, v/v); FTIR (NaCl, film): $\nu_{\text{max}} = 1646$ (C=O), 1548 (C=C, aromatic), 1226 (C-O-C) cm^{-1} ; $^1\text{H-NMR}$ (400 MHz, MeOH) δ (ppm): 6.81 (s, 1H, 3-H), 6.94 (dd, $J = 8.8, 2.2$ Hz, 1H, 6-H), 6.98 (d, $J = 2.2$ Hz, 1H, 8-H), 7.72 (d, $J = 8.6$ Hz, 2H, 2',4'-H), 7.92 (d, $J = 8.6$ Hz, 2H, 3',5'-H), 7.98 (d, $J = 8.8$ Hz, 1H, 5-H); $^{13}\text{C-NMR}$ (400 MHz, MeOH): 102.16, 106.15, 115.28, 115.87, 125.84, 126.46, 127.70(2C), 130.61, 132.06(2C), 158.36, 162.77, 163.98, 178.64; HRMS $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{15}\text{H}_9\text{O}_3\text{Br}$ 338.9633, measured m/z 338.9646.

2-(4-Bromophenyl)-6-hydroxy-chromen-4-one [6-hydroxy-4'-bromoflavone] **8**: yield 71%, white powder m.p. = $256\text{--}258^\circ\text{C}$, $R_f = 0.13$ (ethyl acetate/hexane; 3:7, v/v); FTIR (NaCl, film): $\nu_{\text{max}} = 1637$ (C=O), 1592 (C=C, aromatic), 1228 (C-O-C) cm^{-1} ; $^1\text{H-NMR}$ (400 MHz, MeOH) δ (ppm): 6.86 (s, 1H, 3-H), 7.29 (dd, $J = 8.9, 3.0$ Hz, 1H, 7-H), 7.41 (d, $J = 3.0$ Hz, 1H, 5-H), 7.60 (d, $J = 9.0$ Hz, 1H, 8-H), 7.72 (d, $J = 8.6$ Hz, 2H, 2',4'-H), 7.93 (d, $J = 8.6$ Hz, 2H, 3',5'-H); $^{13}\text{C-NMR}$ (400 MHz, MeOH): 105.54, 107.32, 119.36, 123.49, 124.03, 125.93, 127.76, 127.76(2C), 130.64, 132.06(2C), 150.26, 155.40, 162.92, 179.08; HRMS $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{15}\text{H}_9\text{O}_3\text{Br}$ 338.9633, measured m/z 338.9633.

2-(4-Bromophenyl)-5-hydroxy-chromen-4-one [5-hydroxy-4'-bromoflavone] **9**: yield 52%, white powder m.p. = $172\text{--}174^\circ\text{C}$, $R_f = 0.63$ (ethyl acetate/hexane; 3:7, v/v); FTIR (NaCl, film): $\nu_{\text{max}} = 1655$ (C=O), 1619 (C=C, aromatic), 1264 (C-O-C) cm^{-1} ; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 6.69 (s, 1H, 3-H), 6.80 (d, $J = 8.4$ Hz, 1H, 6-H), 6.97 (d, $J = 8.2$ Hz, 1H, 8-H), 7.54 (t, $J = 8.4$ Hz, 1H, 7-H), 7.65 (d, $J = 8.7$ Hz, 2H, 2',4'-H), 7.76 (d, $J = 8.7$ Hz, 2H, 3',5'-H); $^{13}\text{C-NMR}$ (400 MHz, CDCl_3): 106.14, 107.04, 110.80, 111.64, 126.87, 127.79 (2C), 130.06, 132.44 (2C), 135.57, 156.28, 160.76, 163.39, 183.41; HRMS $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{15}\text{H}_9\text{O}_3\text{Br}$ 338.9633, measured 338.9633.

LC-MS and LC-MS-MS

Quantitative analysis of 4'-bromoflavone, propranolol and mannitol for the Caco-2 cell monolayer permeability study was carried out using an Agilent (Palo Alto, CA, USA) MSD single quadrupole mass spectrometer equipped with a model 1100 HPLC system. A Waters (Milford, MA, USA) Xterra C_{18} column (2.1×100 mm, $3.5\text{ }\mu\text{m}$) was used for HPLC separations with an isocratic mobile phase consisting of methanol/0.5% aqueous acetic acid (65:35, v/v) at a flow rate of 0.2 ml min^{-1} . Flavone ($0.1\text{ }\mu\text{M}$) was used as an internal standard to mix with the aliquots of the transport medium (1:1) for these LC-MS measurements. Samples ($10\text{ }\mu\text{l}$ each) were injected directly into the LC-MS without further preparation. For quantitative analysis, selected ion monitoring was carried out of the protonated molecules of 4'-bromoflavone at m/z 301 and of the internal standard flavone at m/z 223. The LC-MS peak areas of 4'-bromoflavone (eluting at 11.3 min) and flavone (retention time 6.0 min) were determined and used in the calculation of 4'-bromoflavone concentration. For the analysis of propranolol, selected ion monitoring of the protonated molecule of m/z 260 was carried out, and for mannitol, the deprotonated molecule of m/z 181 was monitored. (For a review of the use of LC-MS in Caco-2 studies, see Li *et al.* 2003.)

Metabolites of 4'-bromoflavone were analysed using positive-ion electrospray mass spectrometry and tandem mass spectrometry (MS-MS) with collision-induced dissociation (CID) on a Micromass (Manchester, UK) QTOF2 hybrid tandem mass spectrometer interfaced to a Waters 2690 HPLC system. HPLC separations were carried out using a Waters Xterra column (as described above) with a 40 min linear gradient from 5:95 to 95:5 (v/v) methanol/0.5% aqueous acetic acid. Positive-ion mass spectra were acquired over the range m/z 100–700, and tandem mass spectra were obtained using CID with nitrogen as the collision gas at 30 eV. The resolving power of the first quadrupole (HM and LM values) was set at 4.0 during LC-MS-MS to accept both of the bromine isotope peaks (M and $\text{M} + 2$) during ion selection. For elemental composition analysis, exact mass measurements of the synthetic 4'-bromoflavone derivatives and one 4'-bromoflavone metabolite were also obtained using the QTOF2 mass spectrometer.

Results

Caco-2 cell assay of intestinal permeability

4'-Bromoflavone contained in aliquots of the transport medium from the Caco-2 incubations was quantified using LC-MS as described above. The

standard curve for this assay was linear from 1.6 nM to 5 μ M and is described by the equation $y = 3.521x - 0.0001$, with a correlation coefficient $r^2 = 0.9999$. The limit of quantitation was 16 fmol (1.6 nM in a 10 μ l injection) based on a signal-to-noise ratio of 10:1. The intraday coefficient of variation for the measurement of 4'-bromoflavone ranged from 2.3 to 4.4% ($n = 3$), and the interday coefficient of variation for analysis of the same samples on three consecutive days ranged from 2.6 to 4.4% ($n = 9$) (table 1).

Caco-2 cell monolayers were used to evaluate the rates of 4'-bromoflavone passage in the apical-to-basolateral and in the basolateral-to-apical directions. The P_{app} values of 4'-bromoflavone in both directions and at two concentrations were calculated and are summarized in table 2. The *in vitro* Caco-2 permeability of 4'-bromoflavone ($3\text{--}5 \times 10^{-6} \text{ cm s}^{-1}$) was intermediate between the high permeability standard propranolol (about $5 \times 10^{-5} \text{ cm s}^{-1}$) and the low permeability standard mannitol (about $0.3 \times 10^{-6} \text{ cm s}^{-1}$).

Since Caco-2 cells express the drug transporters P-glycoprotein and multidrug resistance proteins that can affect drug absorption in a directional manner (Hunter *et al.* 1993, Walle *et al.* 1999), the P_{app} values for 4'-bromoflavone were investigated in both the apical-to-basolateral and basolateral-to-apical directions. However, no significant difference was observed between P_{app} values in the two directions (table 2). In addition, the P_{app} values were similar when the initial concentrations of 4'-bromoflavone were 2 and 20 μ M, which suggests also that 4'-bromoflavone is not a substrate for saturable drug transporters such as P-glycoprotein or the multidrug resistance proteins. In summary, these Caco-2 cell monolayer assays showed that 4'-bromoflavone crossed the monolayers at a moderate rate through a direction-independent, passive diffusion mechanism.

Metabolism of 4'-bromoflavone

The *in vitro* metabolism of 4'-bromoflavone was investigated using human liver microsomes, human hepatocytes, and Caco-2 cells. LC-MS analysis of the media

Table 1. Intra- and interday accuracy and precision of the LC-MS determination of 4'-bromoflavone in the transport medium.

4'-Bromoflavone concentration (μ M)	Intraday assay		Interday assay	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.02	95.4	4.4	99.4	4.4
0.4	98.2	4.0	98.1	4.4
2	96.3	2.3	94.2	2.6

Table 2. Apparent permeability coefficients of 4'-bromoflavone and the high and low permeability standards propranolol and mannitol using the Caco-2 cell monolayer model.

Analyte	Concentration (μ M)	P_{app}^a (AP $\rightarrow$ BL ^b)	P_{app} (BL $\rightarrow$ AP ^c)
4'-Bromoflavone	2	4.60 ± 0.57^d	4.27 ± 0.95
	20	3.04 ± 0.46	3.03 ± 0.03
Propranolol	10	54.1 ± 2.60	—
Mannitol	100	0.34 ± 0.02	—

^a P_{app} , apparent permeability coefficient ($\text{cm s}^{-1} \times 10^{-6}$).

^bAP $\rightarrow$ BL, solute movement in the apical-to-basolateral direction.

^cBL $\rightarrow$ AP, solute movement in the basolateral-to-apical direction.

^dData are mean $\pm$ SD, $n = 3$.

from the apical and basolateral sides of the Caco-2 cell monolayers and the methanolic extract of the Caco-2 cells following incubation with 4'-bromoflavone showed no indication of metabolites (data not shown). Next, 4'-bromoflavone was incubated with human liver microsomes and, separately, with human hepatocytes, and the reaction mixtures were analysed using LC-MS and LC-MS-MS. Compared with the control incubations, four additional peaks were detected in both the microsomal and hepatocyte incubations. The computer-reconstructed mass chromatograms for the LC-MS analyses of the microsomal incubation are shown in figure 2. The hepatocyte incubations produced similar results (data not shown). Each of these chromatograms represents a composite of signals corresponding to the protonated molecules of 4'-bromoflavone, three monooxygenated ($M+16$) metabolites, and one monooxygenated plus hydrated ($M+16+18$) metabolite. Since arene oxides should be too unstable to be isolated using HPLC, the monooxygenated metabolites were probably hydroxylated 4'-bromoflavones.

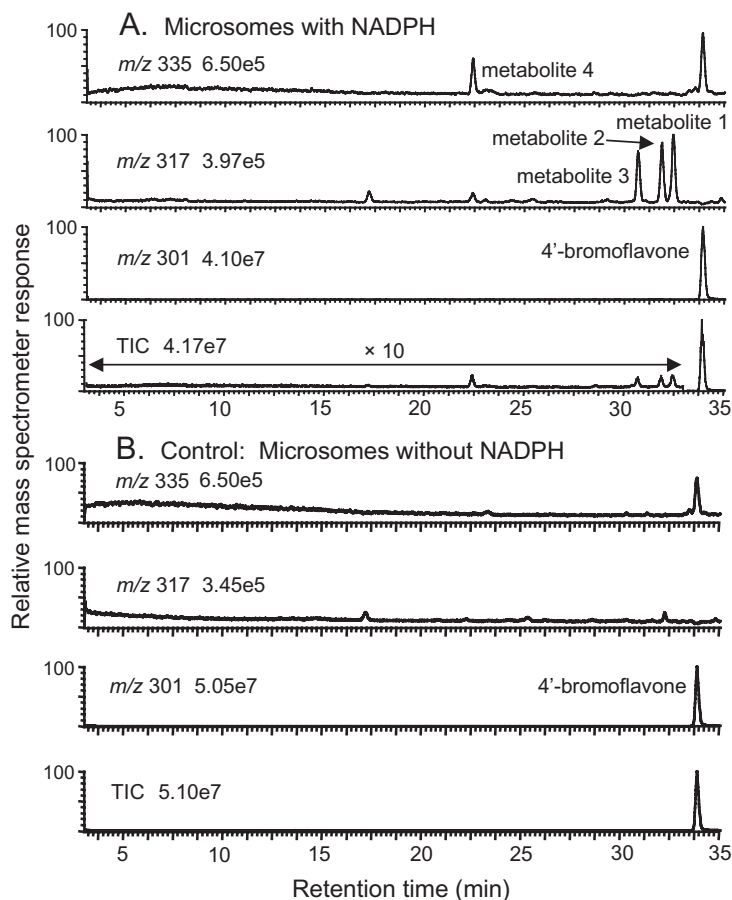


Figure 2. Computer-reconstructed positive-ion electrospray LC-MS chromatograms of 4'-bromoflavone and its metabolites following (A) incubation of 4'-bromoflavone with human liver microsomes and the cofactor NADPH and (B) control incubation of 4'-bromoflavone with human liver microsomes but without NADPH. Four metabolites 1–4 were detected. The selected ion chromatograms of m/z 301, 317 and 335 correspond to the protonated molecules of 4'-bromoflavone and its monohydroxylated and monohydroxylated/hydrated metabolites, respectively.

The four metabolites as well as intact 4'-bromoflavone were analysed using LC-MS-MS with CID to obtain structurally significant fragment ions. Detected at a retention time of 33.9 min, the protonated molecule of 4'-bromoflavone of m/z 301/303 fragmented during CID to form several structurally significant fragment ions that were useful for the characterization of its oxygenated metabolites (figures 3 and 4A). Product-ions in the tandem mass spectra are labelled according to the nomenclature of Ma *et al.* (1997). The major fragmentation pathway for the substrate 4'-bromoflavone was retro-Diels–Alder reaction (RDA) to produce $^{1,3}\text{A}^+$ and $^{1,3}\text{B}^+$ ions at m/z 121 and 181/183, respectively (note that the pair of ions of m/z 181/183 represents a single structure with a one-bromine isotope pattern). Another structurally significant fragment ion was detected at m/z 207/209, which was formed by 1,4-cleavage of the C-ring as indicated in figure 3. In addition, losses of bromine or HBr from the protonated molecule were detected at m/z 222 or 221, respectively, and ions corresponding to elimination of CO from the ions of m/z 222 and 121 were observed at m/z 194 and 93, respectively.

Eluting at 32.5 min (figure 2), metabolite 1 produced protonated molecules of m/z 317 and 319, which suggested that metabolite 1 contained a single bromine atom and was a monohydroxylated derivative of 4'-bromoflavone. The positive-ion MS-MS product-ion mass spectrum of metabolite 1 (figure 4B) showed abundant $^{1,3}\text{A}^+$ ions of m/z 137 and $^{1,3}\text{B}^+$ ions of m/z 181/183, which were analogous to the retro-Diels–Alder fragment ions of 4'-bromoflavone at m/z 121 and 181/183. Since the ion of m/z 181/183 was identical to that of 4'-bromoflavone but the diagnostic product-ion of m/z 121 increased by 16 u to m/z 137, the B-ring must be unchanged and oxygenation must have occurred on the A-ring of 4'-bromoflavone. The other product-ions such as m/z 207/209, which was formed by 1,4-cleavage of the C-ring of the protonated molecule, and m/z 109 formed by loss of CO from the ion of m/z 137, were also consistent with hydroxylation on the A-ring. Therefore, the structure of metabolite 1 was assigned as an A-ring monohydroxylated 4'-bromoflavone. The exact position of the hydroxyl group on the A-ring was determined by comparison with synthetic standards as described below.

To determine the site of hydroxylation of each monohydroxylated isomer, 5-hydroxy-4'-bromoflavone, 6-hydroxy-4'-bromoflavone, and 7-hydroxy-4'-bromoflavone were synthesized for comparison with the metabolites using LC-MS-MS. The structures of the synthetic standards were verified using exact mass

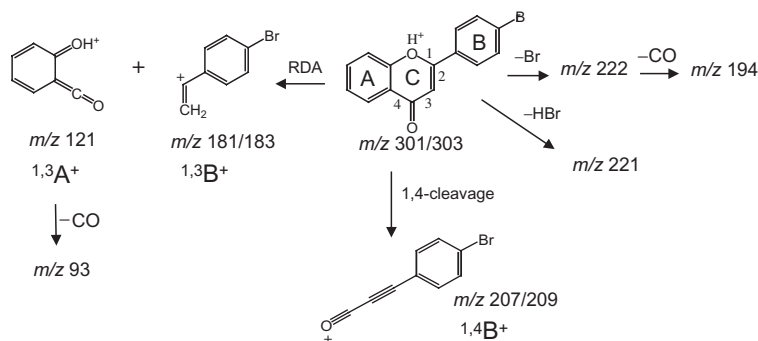


Figure 3. Proposed fragmentation pathways of the protonated molecule of 4'-bromoflavone at m/z 301 during positive-ion electrospray MS-MS with CID.

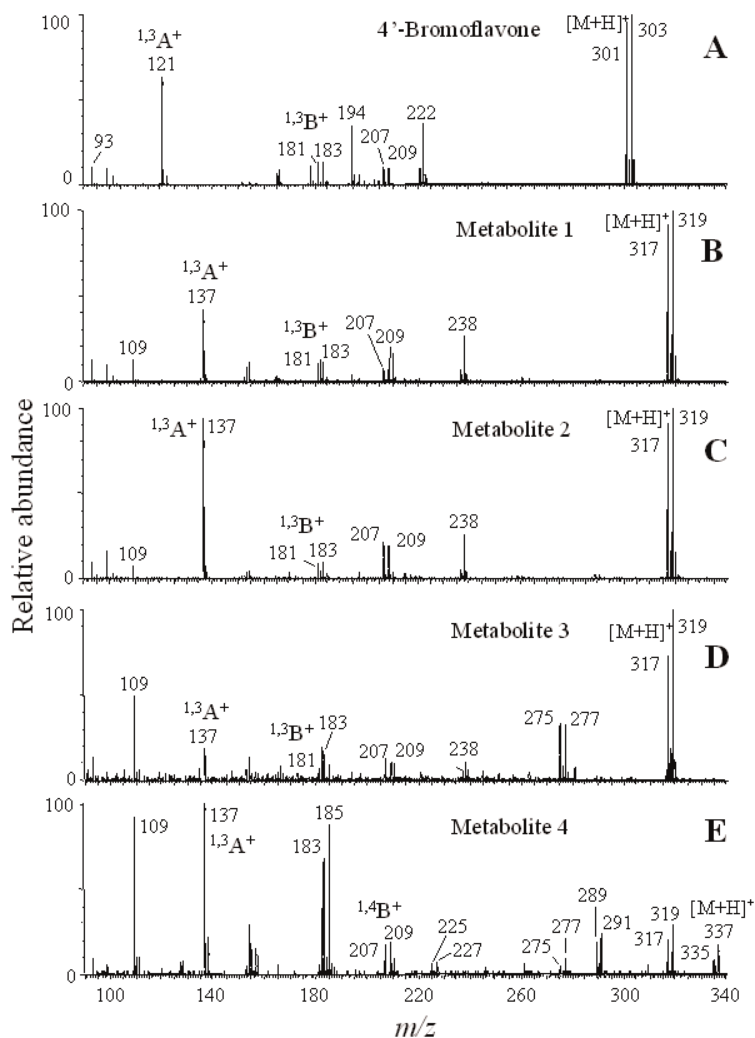


Figure 4. Product-ion tandem mass spectra with CID of (A) 4'-bromoflavone and four oxygenated metabolites eluting at (B) 32.5 (metabolite 1; 6-hydroxy-4'-bromoflavone); (C) 31.9 (metabolite 2; 7-hydroxy-4'-bromoflavone); (D) 30.7 (metabolite 3; 8-hydroxy-4'-bromoflavone); and (E) 22.4 min (metabolite 4; see the proposed structures in figure 5) in the mass chromatogram shown in figure 2.

measurements (for elemental composition), ^1H -NMR, and ^{13}C -NMR (see the Materials and methods). By comparing the LC-MS-MS retention times and product-ion mass spectra (including co-injection of synthetic compounds with the metabolite mixtures), metabolite 1 was identified as 6-hydroxy-4'-bromoflavone (figure 5).

The other two monohydroxylated 4'-bromoflavones, metabolites 2 and 3, eluted at LC-MS-MS retention times of 31.9 and 30.7 min, respectively (figure 2). The product-ion mass spectra of metabolites 2 and 3 were similar to that of metabolite 1 (figure 4), suggesting that they, too, were hydroxylated on the A-ring of 4'-bromoflavone. By comparison with the synthetic standards using LC-MS-MS, metabolite 2 was identified as 7-hydroxy-4'-bromoflavone (figure 5).

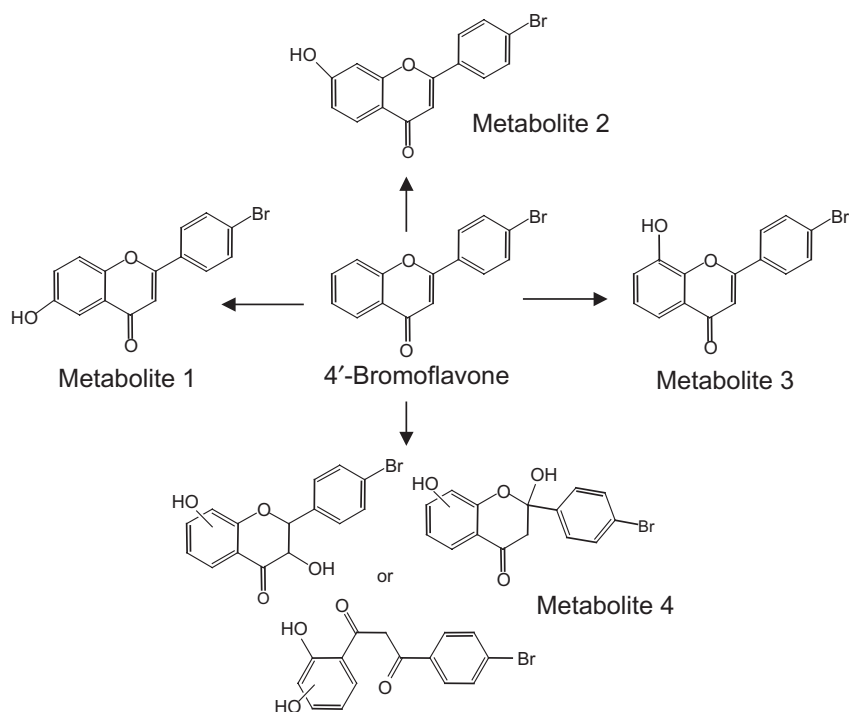


Figure 5. Metabolic pathways of 4'-bromoflavone. Although the structures of the monohydroxylated metabolites 1–3 were determined as described in the text, metabolite 4 was characterized using mass spectrometer but not unambiguously identified.

Table 3. Kinetic parameters for the formation of the most abundant metabolites (1 and 2) of 4'-bromoflavone by human liver microsomes.

Metabolite	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{pmol}^{-1}$ P450)	$V_{\max}/K_m$
6-Hydroxy-4'-bromoflavone (1)	0.67 ± 0.09	0.0059 ± 0.0001	0.0088
7-Hydroxy-4'-bromoflavone (2)	2.20 ± 0.74	0.0024 ± 0.0001	0.0011

Data are the standard error of the best-fit values determined using non-linear regression.

Although metabolites 1 and 2 were identified by direct comparison with synthetic standards, the 5-hydroxy-4'-bromoflavone synthetic standard (retention time 36.5 min) did not correspond to metabolite 3 (retention time 30.7). LC-MS-MS analysis indicated that metabolite 3 was a monooxygenated derivative of 4'-bromoflavone, the hydroxyl group was on the A-ring, and this compound was neither 5-hydroxy-, 6-hydroxy- or 7-hydroxy-4'-bromoflavone. Note that metabolite 3 produced the unique product-ions of m/z 275/277, which retained the single bromine isotope pattern and probably were formed by loss of $\text{CH}_2 = \text{C} = \text{O}$ from positions 3 and 4 of the C-ring of the protonated molecule of m/z 317/319 (figure 4D). The structure of the ion of m/z 275/277 was probably stabilized by the formation of a new six-membered ring involving the hydroxyl group on the A-ring, which therefore must be in the 8-position. Based on all of these data, the structure of metabolite 3 was determined to be 8-hydroxy-4'-bromoflavone, which was the only hydroxylated A-ring derivative of 4'-bromoflavone that was not synthesized (figure 5).

The lack of formation of 5-hydroxy-4'-bromoflavone is probably due to a deactivation effect by the ketone on the C ring. Although 5-hydroxy-4'-bromoflavone was not identified as a metabolite, it should be noted that this synthetic compound eluted at 36.5 min during reversed phase HPLC, which was later than 4'-bromoflavone and all of its metabolites. This prolonged retention time was probably the result of the formation of an intramolecular hydrogen bond between the hydroxyl group at the 5-position on the A-ring and the ketone oxygen on the C-ring.

Next, the kinetics of the hydroxylation of 4'-bromoflavone by human liver microsomes was investigated. The formation of 6-hydroxy-4'-bromoflavone (metabolite 1) and 7-hydroxy-4'-bromoflavone (metabolite 2) which are the most abundant metabolites of 4'-bromoflavone followed Michaelis–Menten kinetics, and the K_m and V_{max} for these reactions are shown in table 3. The kinetics of the formation of 6-hydroxy-4'-bromoflavone and 7-hydroxy-4'-bromoflavone by human liver microsomes indicate that the hydroxylation rate of 4'-bromoflavone is relatively slow.

Eluting at 22.4 min, metabolite 4 was the most polar of the 4'-bromoflavone derivatives. Exact mass measurement of the protonated molecule of m/z 335/337 indicated that the elemental composition of this ion was $C_{15}H_{12}O_4Br$. Therefore, metabolite 4 appeared to be both monohydroxylated and hydrated. The positive-ion product-ion tandem mass spectrum of metabolite 4 is shown in figure 4E. The fragment ions of m/z 137 ($^{1,3}A^+$) and 109 indicated that oxygenation occurred on the A-ring. Like metabolite 3, metabolite 4 also produced product-ions of m/z 275 and 277, suggesting that monohydroxylation might have occurred at the 8-position. Detection of the $^{1,4}B^+$ ion of m/z 207/209 indicated that the B-ring was unchanged. Two additional types of B-ring fragment ions were detected in metabolite 4 that were not observed in 4'-bromoflavone. The most abundant of these ions, m/z 183/185, probably corresponded to a *p*-bromophenyl acylium ion, and the other ion, m/z 225/227, was probably a *p*-bromophenyl ion corresponding to $[C_6H_4BrCOCH_2CO]^+$. All of these data indicate that hydration occurred on the C-ring. The only site for hydration on the C-ring would be the double bond between carbons 2 and 3, but whether the hydroxyl group was on carbon-2 or carbon-3 could not be determined based on these data alone. Three possible structures for metabolite 4 that are consistent with these data are shown in figure 5.

Discussion

As a potential cancer chemoprevention agent, 4'-bromoflavone must be orally bioavailable, and the purpose of this investigation was to assess various aspects of bioavailability using *in vitro* assays before clinical investigation. In our studies using the Caco-2 cell monolayer model, 4'-bromoflavone was shown to have moderate permeability through the monolayers. A Caco-2 transport rate of $\geq 2 \times 10^{-6} \text{ cm s}^{-1}$ is considered equivalent to complete absorption of a pharmaceutical agent from the human intestine (Gres *et al.* 1998). Since the P_{app} values of 4'-bromoflavone across the Caco-2 monolayers just exceeded this threshold ($3\text{--}5 \times 10^{-6} \text{ cm s}^{-1}$; table 2), 4'-bromoflavone should be well absorbed in humans. Furthermore, the mechanism of absorption was simple direction-independent passive diffusion. These properties of 4'-bromoflavone indicate that it will have sufficient oral absorption for use as a chemoprevention agent.

The next aspect of bioavailability that was investigated in this study was the metabolism of 4'-bromoflavone. Although Caco-2 cells express UDP-glucuronosyltransferases and sulfotransferases that can form phase II conjugates of various natural products (Li *et al.* 2003), these cells usually express only low levels of cytochrome P450 enzymes. Therefore, no hydroxylated metabolites of 4'-bromoflavone were observed in the Caco-2 incubation mixture. Since 4'-bromoflavone lacks hydroxyl groups or other functional groups that are suitable for conjugation, no glucuronide or sulfate conjugates were observed following incubation with Caco-2 cells.

Two *in vitro* models of hepatic metabolism were used, which were the incubation of 4'-bromoflavone with human liver microsomes and incubation with human hepatocytes. Both models of hepatic metabolism produced only phase I oxidation products, and the same phase I oxygenated metabolites were formed in each system. Three monohydroxylated metabolites of 4'-bromoflavone were identified and an additional metabolite was partially characterized that was probably monohydroxylated and hydrated 4'-bromoflavone. However, the bioactivities of these metabolites and their involvement in cancer chemoprevention remain to be established.

Hepatocytes are known to conjugate many natural products such as the flavonoid galangin (Otake *et al.* 2002) and resveratrol (Yu *et al.* 2002) to form glucuronides and sulfates, which probably enhances the rate of excretion of these compounds. However, since 4'-bromoflavone lacks functional groups suitable for phase II conjugation, no such conjugates were observed following incubation of 4'-bromoflavone with hepatocytes. Furthermore, the rates of formation of hydroxylated metabolites of 4'-bromoflavone were relatively slow, which might also explain why no conjugates of these phase I metabolites were observed in the hepatocyte incubations. It should be noted that the remaining human hepatocytes from these experiments were incubated with the chemoprevention agents brassinin and oxamate and formed abundant glucuronides and sulfates (data not shown). These additional incubations confirmed that the particular batch of hepatocytes used in this 4'-bromoflavone study were capable of phase II conjugation reactions. Based on these observations, it is predicted that the rate of excretion *in vivo* of 4'-bromoflavone might be slow compared with other experimental chemoprevention agents such as resveratrol.

In conclusion, we have used *in vitro* models to predict the intestinal permeability and *in vivo* metabolism of the experimental cancer chemoprevention agent 4'-bromoflavone. To the best of our knowledge, the metabolism and Caco-2 cell permeability of 4'-bromoflavone have not been reported previously. These results should be helpful in predicting and then interpreting the *in vivo* activity and pharmacokinetics of this promising cancer chemopreventive agent.

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Homology modelling of CYP3A4 from the CYP2C5 crystallographic template: analysis of typical CYP3A4 substrate interactions

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1. The results of homology modelling of cytochrome P4503A4 (CYP3A4), which is a human enzyme of major importance for the Phase 1 metabolism of drug substrates, from the CYP2C5 crystal structure is reported.

2. The overall homology between the two protein sequences was generally good (46%) with 24% of amino acid residues being identical and a 22% similarity between matched pairs in the CYP3A4 and CYP2C5 aligned sequences, thus indicating that CYP2C5 represents a viable template for modelling CYP3A4 by homology.

3. The CYP3A4 model appears to show consistency with the reported findings from the extensive site-directed mutagenesis studies already published.

4. Typical CYP3A4 substrates, such as midazolam, testosterone, nifedipine and verapamil, are shown to fit the putative active site of the enzyme structure in a manner consistent with their known positions of metabolism.

Introduction

Cytochrome P450 enzymes mediate in the majority of Phase 1 metabolism for pharmaceutical agents in man (Lewis 1996, 2001, Evans and Relling 1999). P450s from families CYP1, CYP2 and CYP3 have been associated with the metabolism of drugs and other xenobiotics (Ingelman-Sundberg 2001), most of which are catalysed by the CYP3A4 enzyme (Rendic and DiCarlo 1997, Thummel and Wilkinson 1998, Wrighton *et al.* 2000, Lewis and Dickins 2002, Rendic 2002). It has been reported that the levels of CYP3A4 in human liver exhibit considerable variation (Guengerich 1995) and allelic variants of the enzyme are also known, some of which display altered catalytic activities (Sata *et al.* 2000). The regulation of CYP3A4 has been reviewed (Guengerich 1999) and it is generally accepted that both the pregnane-X receptor (PXR) and the glucocorticoid receptor are associated with CYP3A4 induction (Honkakoski and Negishi 2000, Pascucci *et al.* 2000). Furthermore, CYP3A4 is known to exhibit diverse substrate selectivity (Guengerich 1995, 1999, Li *et al.* 1995) and it appears that there might be more than one substrate binding site within the haem environment (Kenworthy *et al.* 1999, 2001, Hosea *et al.* 2000) and, indeed, possibly a total of three. Moreover, the existence of allosteric effects for substrate binding to CYP3A4 has also been reported (Shou *et al.* 1994, 1999, Ueng *et al.* 1997, Atkins *et al.* 2001, Hlavica and

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Lewis 2001), which suggests that certain compounds, such as α -naphthoflavone, might represent activators (or effectors) for the binding of other CYP3A4 substrates. QSAR studies on typical substrates of CYP3A4 have been reported (Ekins *et al.* 1999, 2001, Lewis *et al.* 2001, 2002, De Groot and Ekins 2002, Singh *et al.* 2003) and molecular modelling investigations based on sequence homology with CYP102 have also been carried out (Lewis *et al.* 1996, Szklarz and Halpert 1997, 1998, De Rienzo *et al.* 2000, Kuhn *et al.* 2001, Renwick *et al.* 2001). Additionally, extensive site-directed mutagenesis experiments have been reported for CYP3A4 (Harlow and Halpert 1997, 1998, He *et al.* 1997, Domanski *et al.* 1998, Fraser *et al.* 1999, Stevens *et al.* 1999, 2000, 2001, Khan and Halpert 2000, Roussel *et al.* 2000, Domanski and Halpert 2001, Eiselt *et al.* 2001, Khan *et al.* 2001, 2002a, b, Xue *et al.* 2001), which point to the importance of certain key residues for the binding of various substrates of this enzyme, and epitope mapping studies have also been performed (Leeder *et al.* 1996). In addition, enzymatic studies highlighting the differences between CYP3A4, CYP3A5 and CYP3A7 have been published (Williams *et al.* 2002). The publication of the first mammalian P450 crystal structure, namely that of CYP2C5 (Williams *et al.* 2000), has afforded an opportunity for using this P450 as a template for modelling human P450s involved in drug metabolism (Lewis 2002) and the primary sequence identity between the two enzymes is 24% with a further 22% residues exhibiting similarity between their side chains, such that the overall homology is 46% between the target and template enzymes.

Materials and methods

Figure 1 shows a multiple sequence alignment between various CYP3A subfamily proteins and that of CYP2C5, the crystallographic template (Williams *et al.* 2000). The alignment was generated using the GCG software suite (Genetics Computer Group, Madison, WI, USA) with some manual adjustment to preserve the secondary structural elements of the CYP2C5 enzyme (pdb code: 1dt6). There was an overall good concordance between putative substrate binding site regions (Gotoh 1992) and known evidence from site-directed mutagenesis within CYP3A4 (reviewed in Domanski and Halpert 2001). Using the Sybyl Biopolymer module (Tripos Associates, St Louis, MO, USA), a model of CYP3A4 was constructed from CYP2C5 based on the alignment shown in figure 1. This was a relatively straightforward procedure and involved the deletion of only six residues out of 452. Also, a small number of residue insertions were required by the alignment, seven in total, and these were produced via loop searching of the protein databank using Sybyl Biopolymer.

Following construction of the raw model, a number of unfavourable steric contacts between residues were relieved manually before energy minimization (Tripos force field) via molecular mechanics that included parameterization for the haem iron. This resulted in a low energy geometry after 200 iterative cycles of minimization, which showed a satisfactory protein conformation. Substrates were docked interactively using the Sybyl molecular modelling software; this also used a dynamic hydrogen bond feature that facilitates the search for favourable substrate orientations within the putative active site region. Movement of the substrate relative to the enzyme gives rise to the making and breaking of potential hydrogen bonds, thus enabling one to optimize interactively the substrate's position in relation to the haem moiety with appropriate hydrogen bonding to amino acid residues present in the binding site. This process was also assisted by knowledge of the substrate's site of metabolism and by reported findings from site-directed mutagenesis (SDM), which has indicated the likely residue contacts within CYP3A4–substrate interactions (reviewed in Domanski and Halpert 2001).

The fitting of substrates within the putative active site using the position of metabolism and location of SDM residues provides an internal method of validation based on available experimental evidence, and this is the approach adopted in the present study, in addition to assessment of the energy minimized structure in terms of acceptable protein geometry. Figure 2 indicates the sites of metabolism and hydrogen bonding for a number of typical CYP3A4 substrates, showing the location of a common hydrogen bond donor (*) close to the major metabolic site (†) in each case. Table 1 shows information of the actual routes of metabolism, together with the K_D (or K_m) values for CYP3A4-mediated catalysis and distances between the haem iron and metabolic site for those substrates studied in this work.

All molecular modelling was conducted on a Silicon Graphics Indigo² 10000 high-impact graphics workstation operating under UNIX. The Sybyl molecular modelling software was used for all molecular modelling studies and its minimization procedures have been investigated in comparison with X-ray crystal structures of proteins (Christensen and Jorgensen 1997).

Results and discussion

Figure 1 shows the multiple sequence alignment between CYP2C5 and proteins of the CYP3A subfamily, showing the likely location of substrate recognition sites (SRS regions). The residues probed by site-directed mutagenesis in these regions have been emboldened for clarity. Figure 2 provides the structures of selected CYP3A4 substrates, including those studied herein. The CYP3A4 structure minimized smoothly over 200 iterative cycles of molecular mechanics, and the final model with an energy of $-1309.75 \text{ kcal mol}^{-1}$ showed no evidence of disallowed protein conformation according to the Sybyl Biopolymer test criteria. The putative active site region appears to be somewhat more enclosed than that obtained from homology modelling of CYP3A4 (Lewis *et al.* 1996) from the CYP102 bacterial template, however. Nevertheless, it is apparent that typical CYP3A4 substrates are likely to bind within the putative active site region due to the overall size and shape of the haem pocket, together with the presence of complementary residues for interacting with aromatic rings and hydrogen bond donor/acceptor atoms on substrates. Table 2 indicates the particular residues that have been identified as likely contacts with the CYP3A4 substrates studied, together with their actual alignment positions.

In fact, the previously reported results of site-directed mutagenesis experiments in this area (reviewed in Domanski and Halpert 2001) clearly indicate which amino acid residues are likely to interact with various CYP3A4 substrates, thus facilitating the modelling of such interactions. For example, serine-119 tends to represent a common hydrogen bond donor site for many CYP3A4 substrates, irrespective of their route of metabolism. There appears to be at least one other hydrogen bond-forming residue in the CYP3A4 active site, and two phenylalanine residues have also been identified as likely candidates for aromatic π - π stacking with certain types of CYP3A4 substrate (Domanski and Halpert 2001). In fact, a substantial number of typical CYP3A4 substrates can be shown to fit the putative active site and bind to one or more of these residues (table 2). These complementary contacts apparently can orientate substrates for metabolism by positioning the known site of oxidation close to the haem iron. Although we focused on the major sites of substrate metabolism, it is known that some compounds such as midazolam and progesterone are metabolized at two sites via CYP3A4. It is interesting to note, therefore, that it is in fact possible to orientate such examples in two ways that are consistent with their known routes of metabolism, and these substrate positions involve interactions (e.g. hydrogen bonding) to residues shown by SDM to be important. In fact, for the CYP3A4 substrates investigated in the work, there is an excellent agreement with the previously published findings from site-specific mutagenesis experiments, and table 3 represents a summary of these studies reported in the literature. The mode of binding of typical CYP3A4 substrates will now be discussed in relation to figures 3-9, whereas figure 10 presents a template of typical CYP3A4 substrates.

ALIGNMENT BETWEEN CYP102, CYP2C5 AND CYP3A FAMILY MEMBERS

	Membrane anchor region										
	70	80	85	90	100	110	120	130	140	150	160
cyp102											
cyp2c5	MD..P.VV.	.LVLGLCCLL	LLSIW..	KQS	GRG.KL	PPG	PTFPPIIGNI	LQDDAKDISK	SLTRFSECYQ	PVFTVILGMK	PTVVLHGVEA
cyp3a1	MDLLSALTLE	TWLLAVLV	LLYFGTRTH	GLFKKGIPG	KPLPLFFGTG	LNYYKGLG	.K	FDVECHKKYG	KIWGLFDGQM	PLFAITDTEM	IKNVL..VKE
cyp3a2	MDLLSALTLE	TWLLAVLV	LLYRLGTRH	GIFKKGIPG	PKPLPFLGTG	LNYYKGLG	.R	FDMECHKKYG	KIWGLFDGQM	PVFAITDTEM	IKNVL..VKE
cyp3a3	.ALIPDLAME	TWLLAVSLV	LLYLYGTHSH	GLFKKLGIPIG	PTPLPFLGNI	LSYHKG	.CM	FDMECHKKYG	KWGFYDGGQ	PVLAITDPM	IKTVL..VKE
cyp3a4	.ALIPDLAME	TWLLAVSLV	LLYLYGTHSH	GLFKKLGIPIG	PTPLPFLGNI	LSYHKG	.CM	FDMECHKKYG	KWGFYDGGQ	PVLAITDPM	IKTVL..VKE
cyp3a5	MDLIPNLAVE	TWLLAVSLV	LLYLYGTRTH	GLFKKLGIPIG	PTPLPFLGNI	LSYHKG	.WK	FDTECYKKYK	KWGFYDGGQ	PVLAITDPM	IKTVL..VKE
cyp3a6	MDLI..FSLE	TWLLAAASLV	LLYLYGTRTH	GLFKKLGIPIG	PTPLPFLGNI	LSYHKG	.WK	FDTECYKKYK	KWGFYDGGQ	PVLAITDPM	IKTVL..VKE
cyp3a7	MDLIPNLAVE	TWLLAAASLV	LLYLYGTRTH	GLFKKLGIPIG	PTPLPFLGNI	LSYHKG	.WT	FDMECYKKYK	KWGFYDGGQ	PVLAITDPM	IKTVL..VKE
cyp102	DKN..LSQA	LKVRDFAGDGLFTSWTHEK	NWKAHNLPSFSQ.QAMKG.	YHAMVDIAV	QLVQKWERLNAD	H.IEVPE	DMTRLTLDTI	GLCGFNYRFN			
cyp2c5	GEFAGTG	SVPILEKVSGLGIAFSNAK	TWKEMRRFSMLTRNFGMKRS	IEDRIQEEAR	CLVEELRKTN...	ASPCDPTF	ILGCAPCNVI	CSVIFHNRF			
cyp3a1	CFS..VETN	RDFFGPGVINGKAVSVAKDE	EWKRYRALLSPTFTS.GRLKE.	MFPIIEQYGD	ILVKYLKQEA.	ETGKPYTMKK	VFGAYSMVDI	TSTSFVGNVD			
cyp3a2	CFS..VETN	RDFFGPGVINGKAVSVAKDE	EWKRYRALLSPTFTS.GRLKE.	MFPIIEQYGD	ILVKYLKQEA.	ETGKPYTMKK	VFGAYSMVDI	TSTSFVGNVD			
cyp3a3	CYS..VETN	RPFGPGVGMKSAISIAEDE	EWKRLRSLLSPTFTS.GRLKE.	MVPIIAQYGD	VLVRNLRRER.	ETGKPYTMKK	VFGAYSMVDI	TSTSFVGNVD			
cyp3a4	CYS..VETN	RPFGPGVGMKSAISIAEDE	EWKRLRSLLSPTFTS.GRLKE.	MVPIIAQYGD	VLVRNLRRER.	ETGKPYTMKK	VFGAYSMVDI	TSTSFVGNVD			
cyp3a5	CYS..VETN	RRSLGPGVGMKSAISIAEDE	EWKRIRSLLSPTFTS.GRLKE.	MFPIIAQYGD	VLVRNLRRER.	EKGKPYTMKK	IFGAYSMVDI	TGTSFGVNI			
cyp3a6	CYS..VETN	RRSLGPGVGMKSAISIAEDE	EWKRIRSLLSPTFTS.GRLKE.	MFPIIAQYGD	VLVRNLRRER.	EKGKPYTMKK	IFGAYSMVDI	TGTSFGVNI			
cyp3a7	CYS..VETN	RRSGPGVGMKNAISIAEDE	EWKRIRSLLSPTFTS.GRLKE.	MVPIIAQYGD	VLVRNLRRER.	ETGKPYTMKK	VFGAYSMVDI	TSTSFVGSID			
cyp102	SFYRQQ.PHP	FITSMVRALD	EAMNKLQRA.	.NPDD.PAYD	.ENKRFQ	QEDIKVMND	LVDKIADRK	ASGE.QSDDL	ITHMLNGK..	.DPETGEPI	
cyp2c5	YKDEFLKLM	ESINENVRII	SS.PWLOVYN	NFPALLDYFP	GIHKTL	KNADYIKNFI	MEKVKEHEKL	LDVNNPR.DF	IDCFLLKME.	QE...NNLEF	
cyp3a1	SLNNPKDPFV	EKTKKLIRFD	FEDPLFLSV	LFPELTPIYE	MLNICMF	PKDSIEFFKK	FVYRMKETRL	DSVQKHRVDF	LQLMNNAHD	SKDKESHAI	
cyp3a2	SLNNPKDPFV	EKTKKLIRFD	FEDPLFLSV	LFPELTPIYE	MLNICMF	PKDSIAFFQK	FVHRIKETRL	DSKHKHRVDF	LQMLNNAHN	SKDEVSHKAI	
cyp3a3	SLNNQDPLV	ENTKKLIRFD	FEDPFFLSIT	VFPFLPILE	VLNICVF	PREVTNFLRK	AVKRMKESRL	EDTQKHRVDF	LQLMIDSHKN	SKTESHKA	
cyp3a4	SLNNQDPLV	ENTKKLIRFD	FEDPFFLSIT	VFPFLPILE	VLNICVF	PREVTNFLRK	SVKRMKESRL	EDTQKHRVDF	LQLMIDSQ.N	SKTESHKA	
cyp3a5	SLNNQDPLV	ESTKKFLKFG	FEDPFLSII	LFPELTPIYE	ALNVSIF	PKDTINFLSK	SVNRMKESRL	NDKQKHRVDF	LQLMIDSQ.N	SKTESHKA	
cyp3a6	SLRNPQDPFV	KNVRLIKES	FEDPFLSIT	LFPELTPIFE	ALHISMF	PKDVMDFLKT	SVEKIKDDR	KDKQKRRVDF	LQLMINSQ.N	SKIDSHKAI	
cyp3a7	SLNNQDPLV	ENTKKLIRN	PLDPFVLISIK	VFPFLTPILE	ALNITVF	PKKVISFLTK	SVKQIKEGR	KETQKHRVDF	LQLMIDSQ.N	SKDSETHKAI	

	250	260	<i>SRS4</i>	270	280	290	300	310	320	<i>SRS5</i>	330	340
cyp102	DDENIRYQII	TFLIAGHETT	SGLLSFALYF	LVKNPHVLQK	AAEEAARVLV	DPV.PSYKQV	KQLKYVGMVL	NEALRLWETA	PAFSLYAKED	TVLGGEYPLE		
cyp2c5	TLESLSIAVS	DLFGAGTETT	STTLRYSLLL	LLKHPEVAAR	VQEEIERVIG	RHRSPCMQDR	SRMPYTDAMI	HEIQRFIDLLPTNLPHAVTRD	VRFRNYF.IP			
cyp3a1	SDMEITAQSI	IFIFAGYEPT	SSTLSFVLHS	LATHPDTQKK	LQEEIDRALP	NKAPPTYDTV	MEMEYLDMLV	NETLRLYPIG	NRLERVCKKD	VEINGVF.MP		
cyp3a2	SDVEIIAQSV	IFIFAGYEPT	SSTLSFVLHF	LATHPDIQKK	LQEEIDGALP	SKAPPTYDIV	MEMEYLDMLV	NETLRLYPIG	NRLERVCKKD	IELDGLF.IP		
cyp3a3	SDLELVAQSI	IFIFAGYEPT	SSVLSFIMYE	LATHPDVQKK	LQEEIDAVLP	NKAPPTYDTV	LQMEYLDMLV	NETLRLFPPIA	MRLERVCKKD	VEINGMF.IP		
cyp3a4	SDLELVAQSI	IFIFAGYEPT	SSVLSFIMYE	LATHPDVQKK	LQEEIDAVLP	NKAPPTYDTV	LQMEYLDMLV	NETLRLFPPIA	MRLERVCKKD	VEINGMF.IP		
cyp3a5	SDLELVAQSI	IFIFAGYEPT	SSVLSFTLYE	LATHPDVQKK	LQKEIDAVLP	NKAPPTYDAV	VQMEYLDMLV	NETLRLFPPIA	IRLERTCKKD	VEINGVF.IP		
cyp3a6	DDIEVVQSI	IILFAGYEPT	SSTLSFIMHL	LATHPDVQKK	LQEEIDTLLP	NKELATYDTL	VKMEYLDMLV	NETLRLYPIA	GRLERVCKKD	VDINGTF.IP		
cyp3a7	SDLELVAQSI	IFIFAGYEPT	SSVLSFIIYE	LATHPDVQKK	VQKEIDTVLP	NKAPPTYDTV	LQLEYLDMLV	NETLRLFPPIA	MRLERVCKKD	VEINGMF.IP		
	350	360	370	380	390	400	410	420	430	<i>SRS6</i>	440	
cyp102	KGDELMVLIP	QLHRDKTIWG	DDVEEFRPER	F..ENPSAIP	QHAFKPFNG	QRACIGQQFA	LHEATLVLM	MLKHFDDEDHTN	YELDIKET	L..TLKPE.G		
cyp2c5	KGTDIITSLT	SVLHDEKAFP	.NPKVFDPGH	FLDESGNFKK	SDYFMFSSAG	KRMCVGEGLA	RMELFLFLTS	ILQNFKLQSLVEPKDLDTAV	VNGFVS.VFP			
cyp3a1	KGSVVMIPSY	ALHRDPQHWP	.EPEEFRPER	FSKENKGSID	PYVYLPFGNG	PRNCIGMRFA	LMNMKLALTK	VLQNFQFQCKE	TQIPLKLS	RQGLLQPTKP		
cyp3a2	KGSVVTIPTY	ALHHDPPQHW	.KPEEFHPER	FSKENKGSIH	PYVYLPFGNG	PRNCIDMRFA	LMNMKLALTK	VLQNFQFQCKE	TQIPLKLS	RQAILPEPKP		
cyp3a3	KGWVVMIPSY	ALHRDPKYWT	.EPEKFLPER	FSKKNKDNID	PYIYTPFGSG	PRNCIGMRFA	LMNMKLALIR	VLQNFQFQCKE	TQIPLKLS	LGGLLQPEKP		
cyp3a4	KGWVVMIPSY	ALHRDPKYWT	.EPEKFLPER	FSKKNKDNID	PYIYTPFGSG	PRNCIGMRFA	LMNMKLALIR	VLQNFQFQCKE	TQIPLKLS	LGGLLQPEKP		
cyp3a5	KGSMVVIPTY	ALHHDPPQHW	.EPEEFRPER	FSKK.KDSID	PYIYTPFGTG	PRNCIGMRFA	LMNMKLALIR	VLQNFQFQCKE	TQIPLKLD	TQGLLQPEKP		
cyp3a6	KGTIVM.PTY	ALHRDPQHW	.EPDEFRRPER	FSKKNKDNIN	PYIYHPFGAG	PRNCLGMRFA	LMNKLALVR	LMQNFQFKLCKE	TQVPLKLG	KQGLLQPEKP		
cyp3a7	KGVVVMIPSY	VLHHDPPQWT	.EPEKFLPER	FSKKNKDNID	PYIYTPFGSG	PRNCIGMRFA	LVNMKLALVR	VLQNFQFQCKE	TQIPLKLR	FGGLLITEKP		
	450											
cyp102	FVVKAKSKKIPLGGI											
cyp2c5	SYQLCFIPI											
cyp3a1	IILKVPRDEIITGS											
cyp3a2	IVLKVLPDRAVINGA											
cyp3a3	VVLKVESRDGTVSGA											
cyp3a4	VVLKVESRDGTVSGA											
cyp3a5	IVLKVDSRDGTLSGE											
cyp3a6	IVLKVSESIGIRGA											
cyp3a7	IVLKAESRDETIVSGA											

Figure 1. Multiple sequence alignment of several CYP3A subfamily enzymes matched with the CYP2C5 sequence. The putative substrate recognition sites (SRS regions) are shown as shaded boxes.

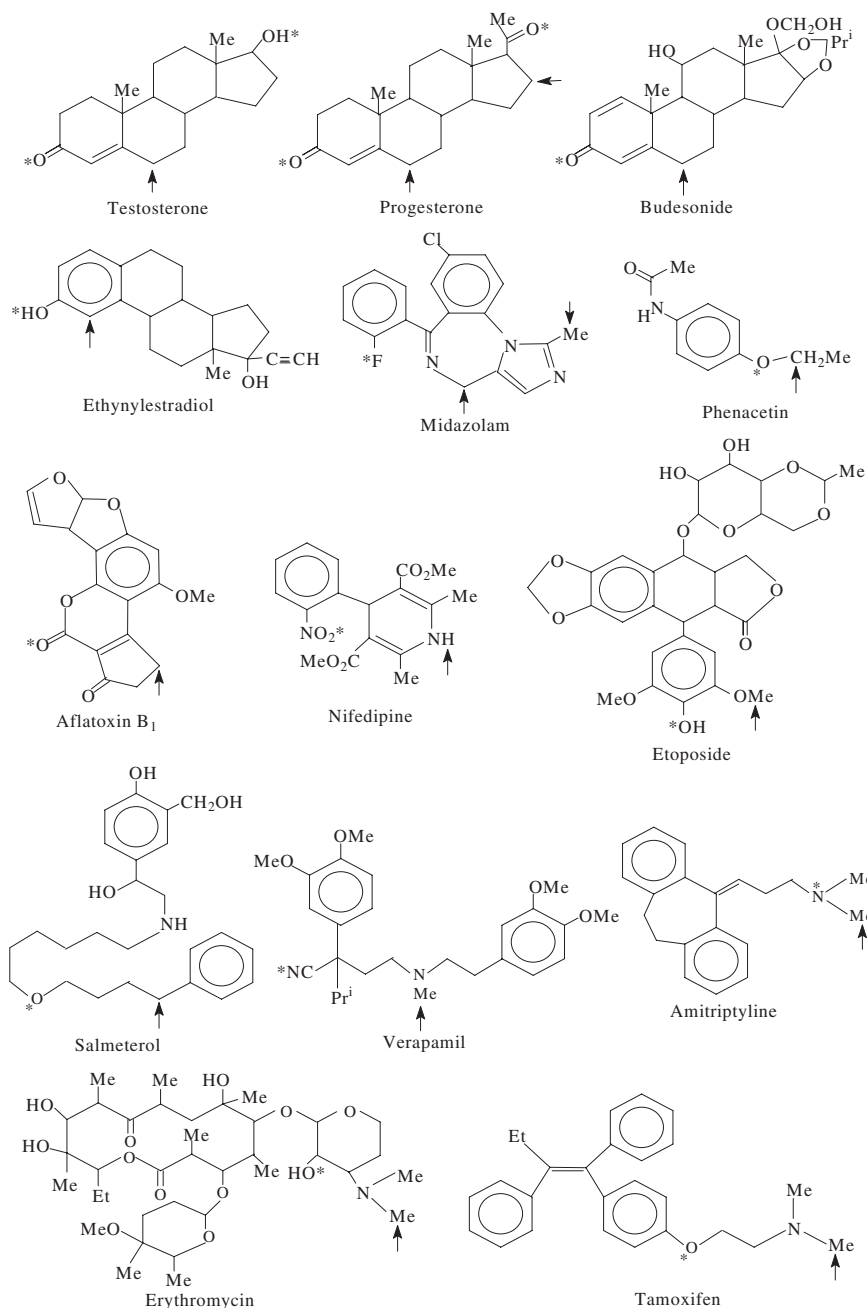


Figure 2. Structures of typical CYP3A4 substrates showing sites of CYP3A4-mediated metabolism (↑) and hydrogen bond donors (*) associated with modelled interactions to serine-119 in CYP3A4.

Midazolam

Figures 3 and 4 show the likely orientations of midazolam within the putative active site of CYP3A4. Although two sites of oxidation are known, it would appear that different amino acid residues are involved in binding to the substrate such that

Table 1. CYP3A4 substrate metabolism and distances between sites of metabolism and haem iron.

Compound	Routes of metabolism	K_D (K_m)	Reference	Distance (Å)
1. Midazolam	1'- and 4-hydroxylation	8.8 (8.7)	Gorski <i>et al.</i> (1994)	2.803 (1') and 4.600 (4)
2. Testosterone	6 β -hydroxylation	21.7 (41)	Hosea <i>et al.</i> (2000)	4.553
3. Nifedipine	N-oxidation	19 (15)	Pelkonen <i>et al.</i> (1998)	4.025
4. Aflatoxin B ₁	3 α -hydroxylation	(43)	Ueng <i>et al.</i> (1997)	4.537
5. Phenactin	O-deethylation	9.1	Lewis <i>et al.</i> (2001, 2002)	4.675
6. Progesterone	6 β - and 16 α -hydroxylation	(80)	Yamazaki and Shimada (1997), He <i>et al.</i> (1997)	3.945 (6 β) and 4.245 (16 α)
7. Ethynylestradiol	2-hydroxylation	6.5	Lewis <i>et al.</i> (2001, 2002)	3.264
8. Etoposide	O-demethylation	23.2	Lewis <i>et al.</i> (2001, 2002)	3.016
9. Budesonide	6 β -hydroxylation	6.4	Lewis <i>et al.</i> (2001, 2002)	3.833
10. Verapamil	N-demethylation	3.5	Lewis <i>et al.</i> (2001, 2002)	3.339
11. Salmeterol	Debenzylation	165	Lewis <i>et al.</i> (2001, 2002)	2.686
12. Erythromycin	N-demethylation	1.8	Lewis <i>et al.</i> (2001, 2002)	1.916
13. Amitriptyline	N-demethylation	348	Lewis <i>et al.</i> (2001, 2002)	4.310
14. Ketoconazole	Inhibition	0.1 (K_i)	Pelkonen <i>et al.</i> (1998)	2.037 (azole nitrogen)

General references to CYP3A4 substrate metabolism information include: Rendic and DiCarlo (1997), Rendic (2002) and Ekins *et al.* (1999).

Table 2. Amino acid residues contacting CYP3A4 substrates in the model.

Position	CYP3A4	SRS
F102	108	1
S113	119	1
L205	211	2
F293	304	4
A294	305	4
T298	309	4
S301	312	4
L363	373	5
L473	482	6
L474	483	6
E476	486	6

F, phenylalanine; S, serine; L, leucine; A, alanine; T, threonine; E, glutamic acid.

*Alignment position relative to the CYP2C5 template.

Residues that have been the subject of SDM experiments are emboldened.

SRS, substrate recognition site (Gotoh 1992).

it might be positioned in two ways (Khan *et al.* 2002). Consequently, there is an optimal orientation directed towards 1'-hydroxylation (figure 3) and corresponding one for 4-hydroxylation (figure 4). In this way, the results of molecular modelling are in agreement with known substrate metabolism and reported site-directed mutagenesis information (Khan *et al.* 2002).

Testosterone

Figure 5 shows a possible mode of binding for the CYP3A4 substrate, testosterone, in the putative active site. The steroid appears to form hydrogen

Table 3. Site-directed mutagenesis of CYP3A4.

Change	CYP2C5*	SRS	Comments	Reference
F108L	I102	1	substrate contact residue (π - π stacking)	Domanski <i>et al.</i> (2001)
S119A	A113	1	substrate contact residue (hydrogen-bonding)	Domanski <i>et al.</i> (2001) Khan <i>et al.</i> (2002)
I120W	F114	1	within site binding region	Khan <i>et al.</i> (2002)
N206S	S200	2	within site binding region	Xue <i>et al.</i> (2001)
L210A	N204	2	close to binding site region	Khan <i>et al.</i> (2002)
L211A	V205	2	substrate contact residue (hydrophobic)	Domanski <i>et al.</i> (2001)
F213A	I207	2	close to binding site region	Harlow and Halpert (1997)
D214A	L208	2	substrate contact residue (hydrogen-bonding)	Harlow and Halpert (1997)
I301A	D290	4	within binding site region	Xue <i>et al.</i> (2001)
F304A	G293	4	substrate contact residue (π - π stacking)	Xue <i>et al.</i> (2001)
A305V	A294	4	substrate contact residue (hydrophobic)	Halpert (1998)
Y307A	T296	4	within binding site region	Khan <i>et al.</i> (2002)
T309A	T298	4	conserved distal threonine residue	Domanski <i>et al.</i> (2001)
I369V	L358	5	substrate contact residue (hydrophobic)	Halpert (1998)
A370V	L359	5	within binding site region	Halpert (1998)
L373F	L363	5	substrate contact residue (hydrophobic)	Halpert (1998)
S478D	V469	6	surface residue close to binding site	Khan <i>et al.</i> (2002)
L479T	V470	6	surface residue close to binding site	Khan and Halpert (2000)

*Residue at the equivalent position in CYP2C5 according to the alignment in figure 1.
SRS, substrate recognition site (Gotoh 1992).

bonds with complementary amino acid residues in the haem environment, including serine-119, which is also involved in midazolam binding (Khan *et al.* 2002). This residue and another hydrogen bond-forming side chain in the F-helix cooperatively assist in orientating the steroidal substrate for 6 β -hydroxylation by positioning the 6 β -hydrogen directly over the haem iron. In fact, progesterone can be orientated within the putative active site of CYP3A4 for both 6 β and 16 α -hydroxylations in accordance with experimental observations (He *et al.* 1997, Yamazaki and Shimada 1997).

Nifedipine

An orientation for the selective substrate, nifedipine, in the CYP3A4 active site is shown in figure 6. In this case, the nitro group of the substrate forms a hydrogen bond with serine-119 and this, together with other complementary interactions with active site residues such as phenylalanine-108, positions the molecule for N-oxidation as the hydrogen attached to the ring nitrogen lies directly above the haem iron. In addition to serine-119, the aromatic residue, phenylalanine-108 mentioned previously, has also been the subject of site-directed mutagenesis experiments on CYP3A4 (Khan *et al.* 2002).

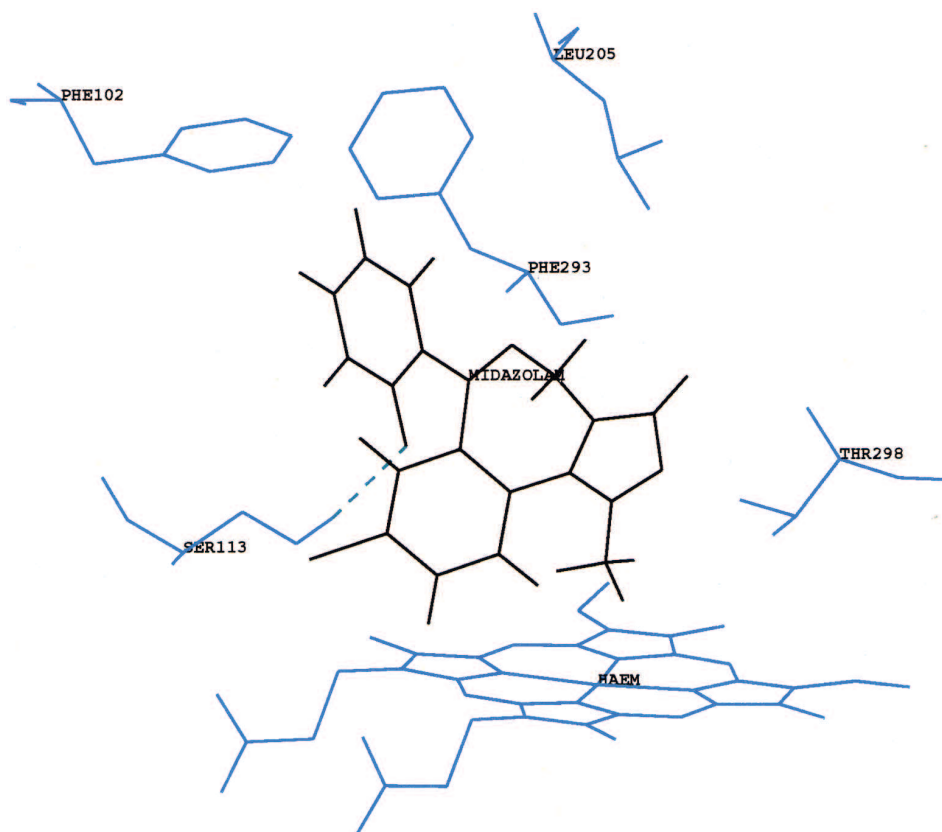


Figure 3. Midazolam is shown orientated within the putative active site of CYP3A4 for 1'-hydroxylation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.

Phenacetin

Figure 7 shows a possible mode of binding for phenacetin in the putative active site of CYP3A4. There is a hydrogen bond between serine-119 and the ether oxygen of the substrate, and this interaction assists in orientating the molecule such that the ethyl group, which is the site of metabolism via *O*-deethylation, lies directly above the haem moiety. Consequently, the modelled interaction is in agreement with the results of substrate metabolic studies and site-directed mutagenesis investigations, which indicate the importance of serine-119 for binding and orientation of substrates (Khan *et al.* 2002).

Aflatoxin B₁

In figure 8, a possible orientation of aflatoxin B₁ within the putative active site of CYP3A4 is shown. In this case, a combination of hydrogen bonding to serine-119 and π - π stacking with aromatic residues positions the substrate for 3 α -hydroxylation. The 3 α -hydrogen is placed 4.5 Å from the haem iron, and is the closest atom in the substrate relative to the active centre. Both of the residues involved in this interaction have been investigated via site-directed mutagenesis,

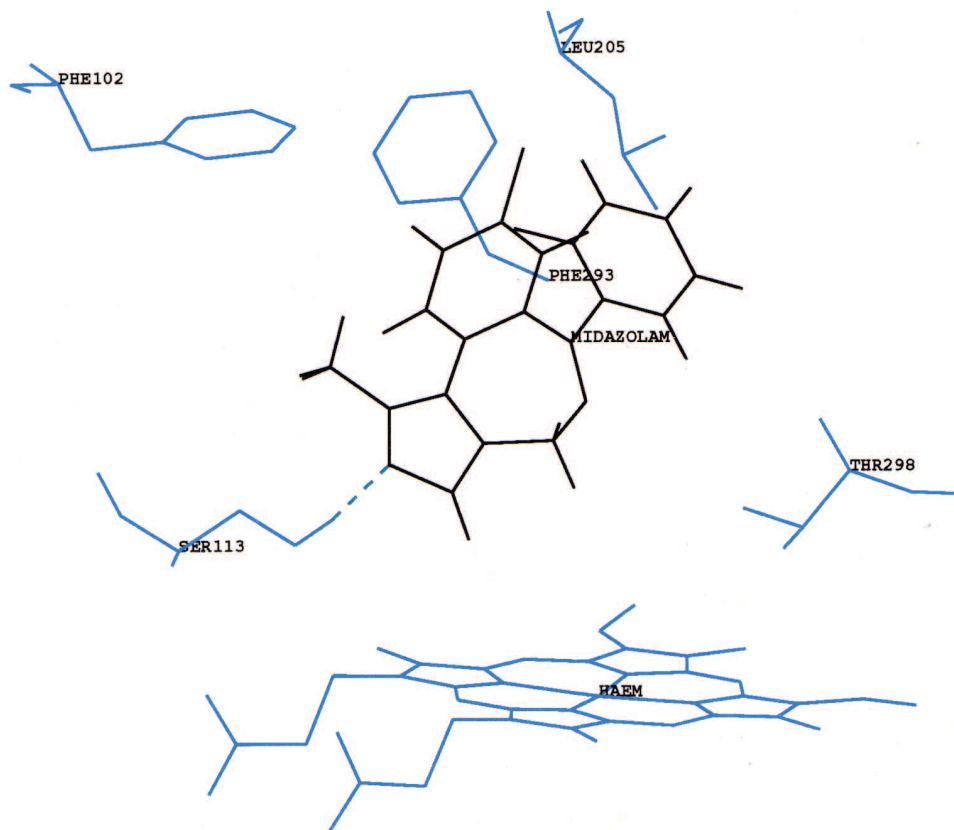


Figure 4. Midazolam is shown orientated within the putative active site of CYP3A4 for 4-hydroxylation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.

albeit on other substrates (reviewed in Domanski and Halpert 2001). However, the modelling appears to show consistency with experimental observations within the CYP3A4 area.

Verapamil

Figure 9 shows a likely orientation for verapamil within the CYP3A4 model. It is the cyano nitrogen on the substrate which forms a hydrogen bond with serine-119, and there are also several hydrophobic interactions with complementary amino acids, some of which have been probed via site-specific mutation (reviewed in Domanski and Halpert 2001). The N-methyl group of verapamil is positioned directly over the haem iron in this orientation, and the substrate also overlaps closely with a molecular template of typical CYP3A4 substrates shown in figure 10a.

Substrate template

This template of superimposed CYP3A4 substrates (figure 10a) within the putative active site contains 12 molecules, including midazolam, testosterone, nifedipine, phenacetin, aflatoxin B₁, verapamil, ethynylestradiol, progesterone,

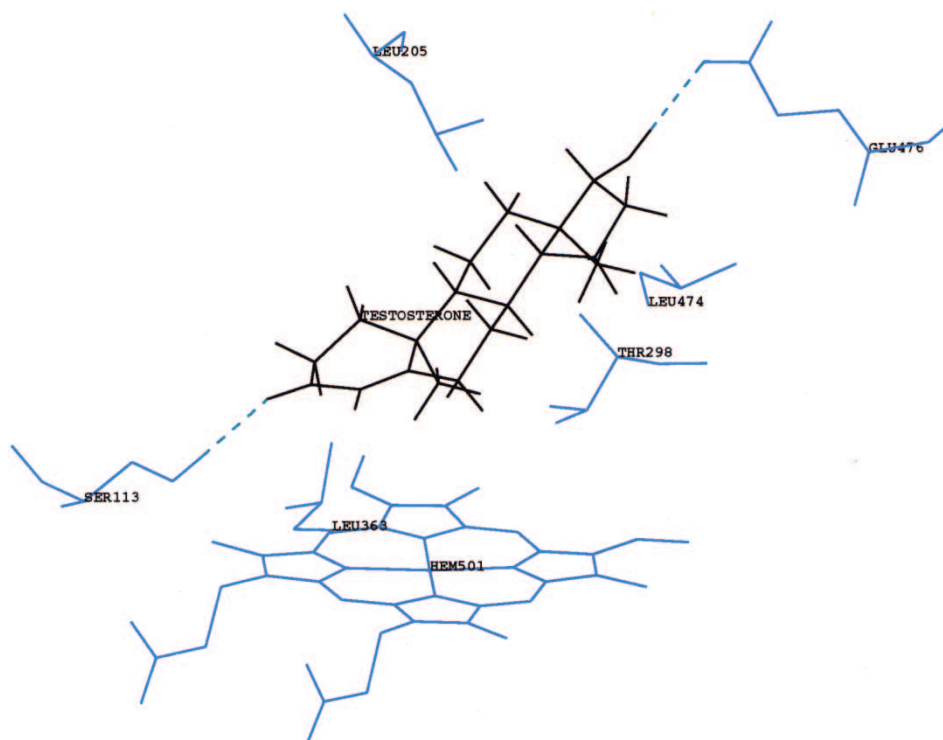


Figure 5. Testosterone is shown orientated within the putative active site of CYP3A4 for 6 β -hydroxylation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.

etoposide, budesonide, salmeterol and amitriptyline. Interestingly, although there is structural diversity in these compounds, they all contain a grouping which can form a hydrogen bond with the active site serine residue, serine-119. Mapping the molecular electrostatic potential energy onto the Connolly solvent-accessible surface (Connolly 1983) of this template reveals two compact regions of positive and negative electrostatic potential (figure 10b) corresponding to the sites of metabolism and hydrogen bonding, respectively. The distance between the centres of these areas at about 7 Å conforms closely to the 6.66 Å for the distance between serine-119 and the haem iron atom. Figure 2 shows the structures of the CYP3A4 substrates studied in this work and also indicates the hydrogen bond acceptor atoms which can bind with serine-119, together with their preferred sites of metabolism. Despite considerable structural diversity in these compounds, they all fit remarkably well into the putative active site region and overlay closely on the basis of their hydrogen bonded interactions with serine-119 and with their respective sites of metabolism mediated by CYP3A4. The overall similarity in molecular size is reflected in their diameters (ranging from 8.2 to 11.6 Å) as shown in table 4. Furthermore, most compounds exhibit a relatively high dipole moment and this is likely to result from the presence of hydrogen bond donor/acceptor atoms in the substrate molecules. Consequently, it is possible to explain the substrate selectivity of CYP3A4 on the basis of

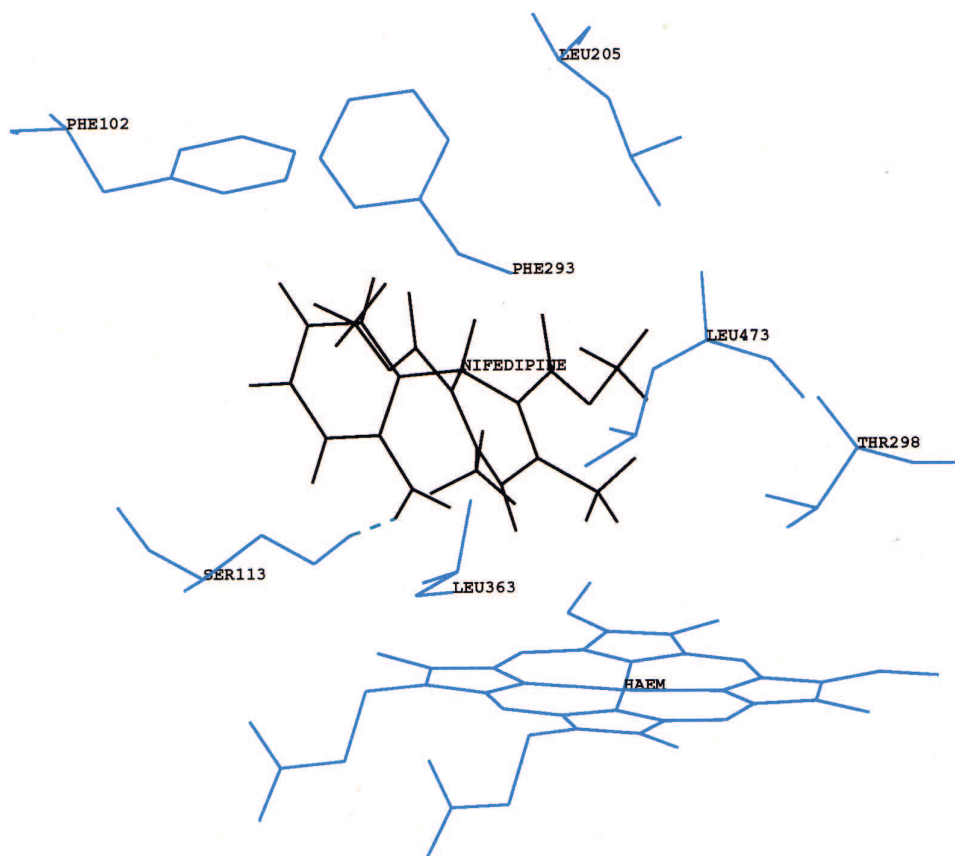


Figure 6. Nifedipine is shown orientated within the putative active site of CYP3A4 for N-oxidation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.

compound structure, particularly the presence of a hydrogen bond donor/acceptor atom at a relatively short distance from the known site of metabolism, together with a relatively high molecular diameter.

Based on the known interactions between substrates and the active site residues in the CYP3A4 model, it is possible to derive estimates for substrate binding affinity as shown in table 4, which includes consideration of compound lipophilicity in the form of $\log P$ values (Lewis *et al.* 1998, Lewis 2000, 2002, 2003). In addition to this measure, for the full procedure it is required that one makes estimates of the energy contributions arising from hydrogen bonding, π - π stacking and desolvation of the active site which accompany substrate binding, together with considering of the loss in energy due to restricted bond rotations in the bound substrate molecule (table 3). The desolvation term is easily calculated from the $\log P$ value using the energy partitioning relationship ($\Delta G_{\text{part}} = -RT \ln P$, where R is the gas constant and T is the absolute temperature). Combination of these terms leads to an estimated free energy of binding (ΔG_{bind}) for substrate and enzyme (table 4). This can then be compared with experimental values obtained from either K_D or K_m determinations, and table 1

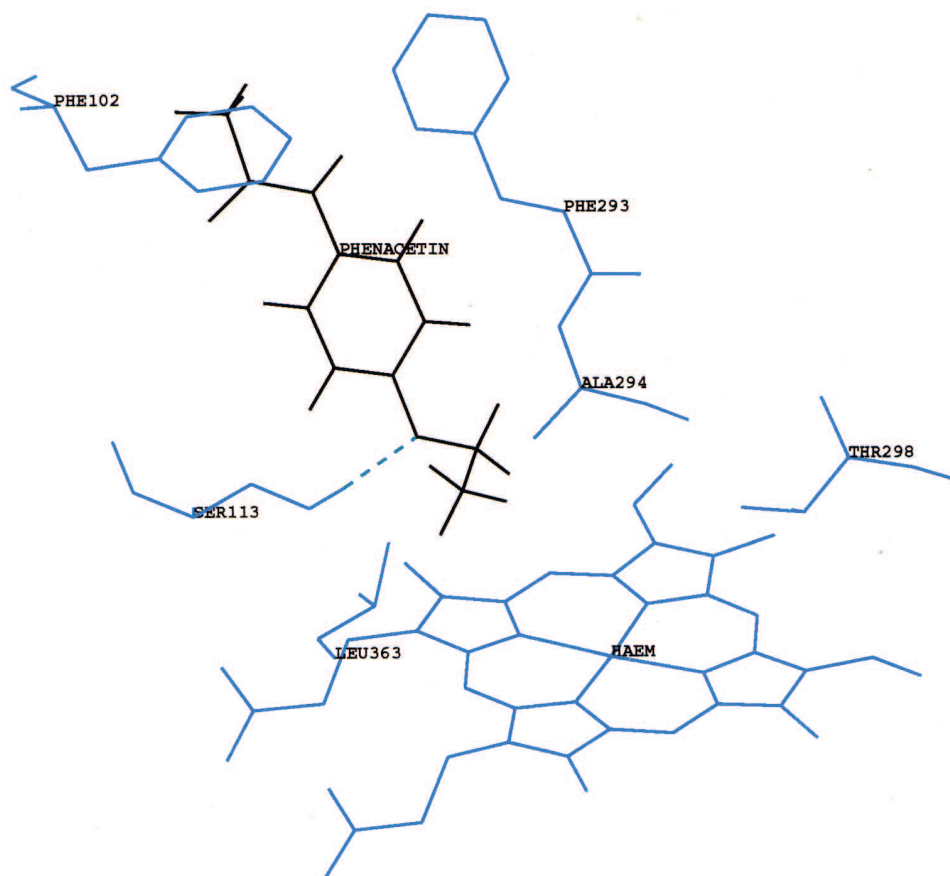


Figure 7. Phenacetin is shown orientated within the putative active site of CYP3A4 for *O*-deethylation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.

provides this information whereas table 4 lists the relevant contributions to the overall binding energy for several CYP3A4 substrates. The correlation between calculated and experimental values of ΔG_{bind} is 0.978 and the slope of this relationship is very close to unity (1.007) thus indicating that the approach appears to be a valid one. Furthermore, quantitative structure-activity relationships (QSARs) can be formulated for substrates of CYP3A4 based on their physicochemical and structural characteristics (Lewis *et al.* 2001, 2002) and table 5 shows data for a number of typical CYP3A4 substrates. Using a combination of frontier orbital energies and active site interactions in the form of number of hydrogen bond and π - π stacking contacts, there is a good correlation ($R=0.96$) with binding energy obtained from K_{D} values (Lewis *et al.* 2001, 2002). This suggests that the substrate binding energy is probably dependent upon charge-transfer interactions between substrate and enzyme, which are frontier orbital-dependent, together with specific contacts with amino acid residues in the enzyme's active site. Consequently, there is a good agreement between the results of QSAR and homology modelling.

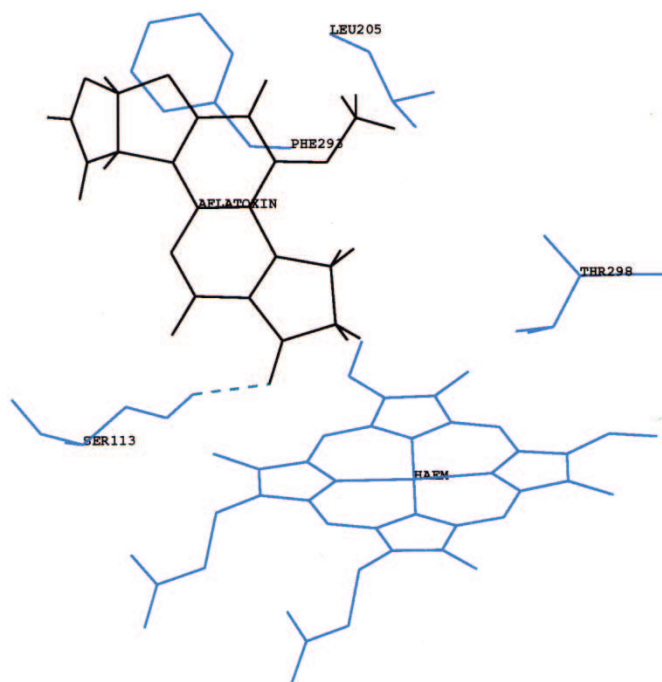


Figure 8. Aflatoxin B₁ is shown orientated within the putative active site of CYP3A4 for 3 α -hydroxylation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.

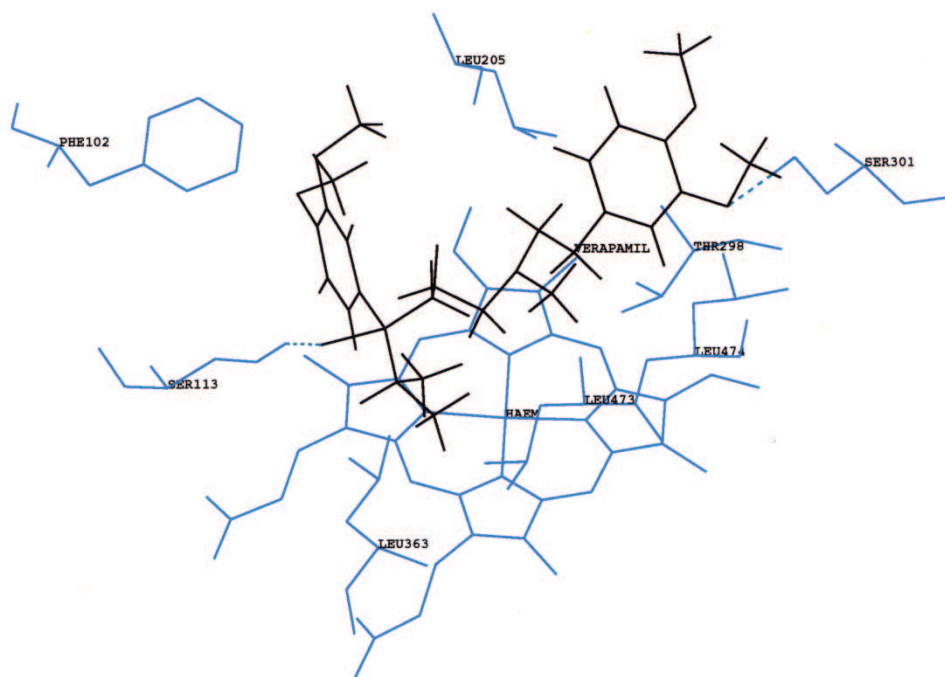
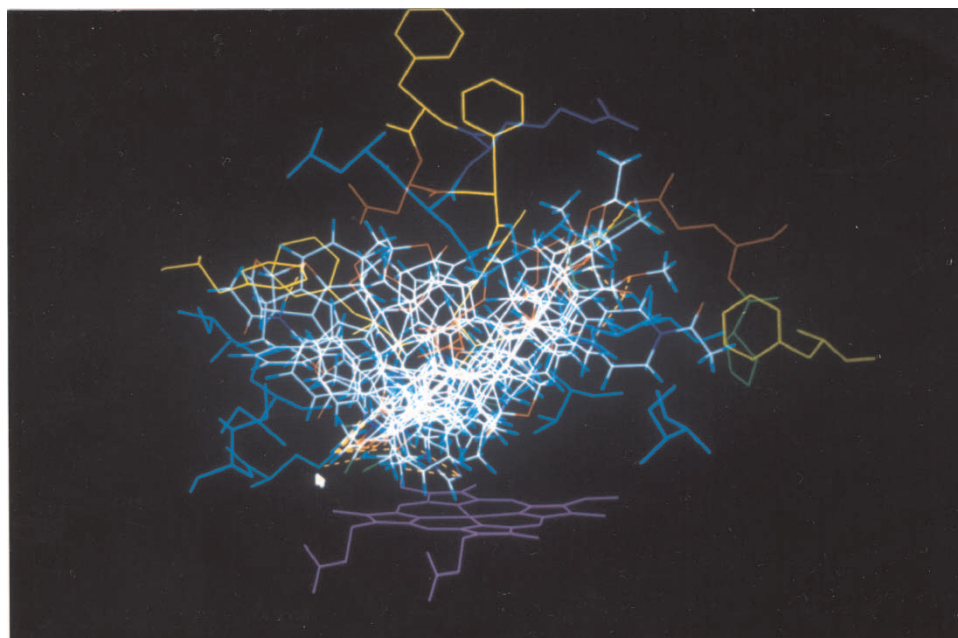
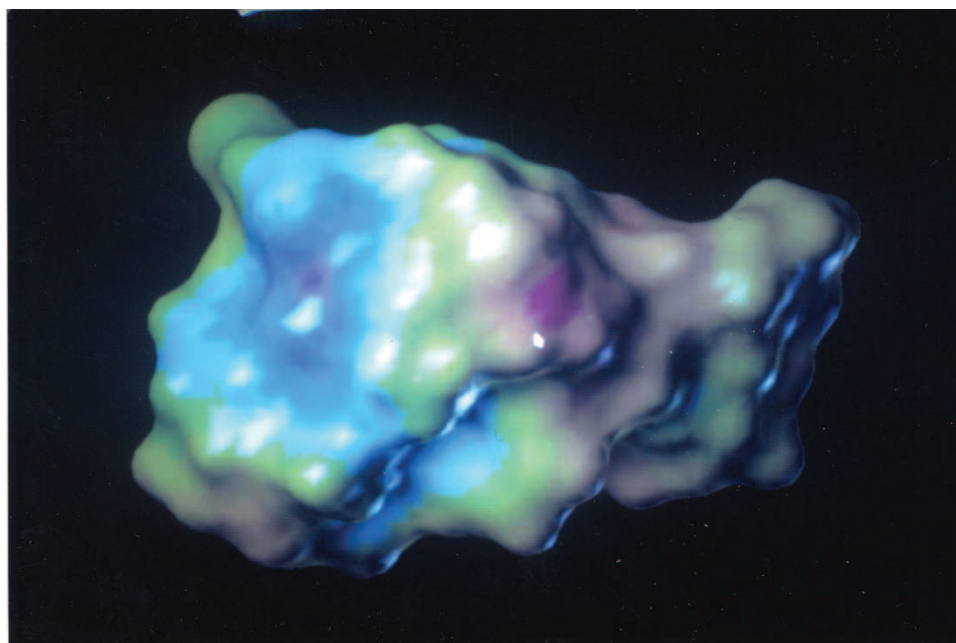


Figure 9. Verapamil is shown orientated within the putative active site of CYP3A4 for N-demethylation. Hydrogen bonds are shown as dashed lines and the residues are numbered according to the CYP2C5 alignment.



(a)



(b)

Figure 10. (a) Template of 12 superimposed structures of midazolam, testosterone, nifedipine, phenacetin, aflatoxin B₁, verapamil, ethynylestradiol, progesterone, etoposide, budesonide, salmeterol and amitriptyline within the CYP3A4 active site. (b) Molecular template of 12 superimposed CYP3A4 substrates shown in figure 9 depicted as a Connolly solvent-accessible surface, colour-coded according to molecular electrostatic potential energy showing regions of positive (red) and negative (blue) potential energy.

Table 4. ΔG_{bind} estimation of CYP3A4 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. Amitriptyline	5.04	-7.1491	0	0	2.4	-4.7491	-4.7474
2. Budesonide	3.28	-4.6526	-4.0	0	1.2	-7.4526	-7.1296
3. Etoposide	1.00	-1.4185	-4.0	-0.9	0	-6.3185	-6.3618
4. Midazolam	1.53	-2.1703	-4.0	-0.9	0	-7.0703	-6.9398
5. Phenacetin	1.57	-2.2270	-4.0	-1.8	1.2	-6.8270	-6.9198
6. Verapamil	3.79	-5.3760	-4.0	-0.9	3.0	-7.2760	-7.4894
7. Ethynylestradiol	2.11	-2.9930	-4.0	-0.9	0.6	-7.2930	-7.1204
8. Testosterone	3.32	-4.7093	-2.0	0	0.6	-6.1093	-6.4017
9. Erythromycin	2.48	-3.5178	-4.0	0	0	-7.5178	-7.8858
10. Salmeterol	3.71	-5.2651	-4.0	0	4.2	-5.0651	-5.1923

$\log P$, logarithm of the octanol–water partition coefficient; $\Delta G_{\text{part}} = -RT \ln P$; ΔG_{hb} , hydrogen bond contribution to binding (-2 kcal mol^{-1} per hydrogen bond); $\Delta G_{\pi-\pi}$, π - π stacking contribution to binding ($-0.9 \text{ kcal mol}^{-1}$ per π - π stack); ΔG_{rot} , loss in bond rotational energy on binding ($+0.6 \text{ kcal mol}^{-1}$ per rotatable bond); $\Delta G_{\text{calc}} = \Delta G_{\text{part}} + \Delta G_{\text{hb}} + \Delta G_{\pi-\pi} + \Delta G_{\text{rot}}$; $\Delta G_{\text{expt}} = RT \ln K$, where K is either the K_{D} or K_{m} ; the correlation between experimental and calculated values of ΔG_{bind} is 0.978 and the slope is 1.007, indicating very good agreement between the estimated and observed binding affinities.

Table 5. Electronic and structural data for CYP3A4-selective compounds.

Compound	E_{HOMO}	E_{LUMO}	ΔE	μ	a/d^2	l/w	SA	Volume	Diameter
1. Midazolam	-9.30653	-0.74119	8.56534	3.195	1.2358	1.0994	290.1134	293.9126	8.2491
2. Testosterone	-10.04336	0.07800	10.12136	3.630	2.1783	1.8041	283.0224	322.4390	8.5078
3. Nifedipine	-8.86965	-0.29027	8.57938	2.239	1.3507	1.1790	311.0292	325.2833	8.5327
4. Aflatoxin B ₁	-9.49000	-1.08967	8.40033	7.161	2.6529	1.0888	274.8124	266.9135	7.9884
5. Phenacetin	-8.65370	0.38364	9.03734	4.156	6.0634	1.7125	208.7931	183.9113	7.0556
6. Progesterone	-10.09526	0.02478	10.12004	1.964	1.8099	1.5218	305.5888	353.3789	8.7716
7. Ethynyl E2	-8.88648	0.41904	9.30552	1.492	1.8805	1.8083	293.5231	318.4330	8.4724
8. Etoposide	-9.05834	-0.28808	8.77026	5.957	3.7105	1.3672	497.3360	550.5217	10.1685
9. Budesonide	-10.08315	-0.31626	9.76689	3.662	2.4708	1.1242	386.9251	451.8571	9.5207
10. Verapamil	-8.82574	-0.07252	8.75322	5.016	1.6023	1.5656	499.9064	523.2324	9.9977
11. Salmeterol	-8.98856	0.36866	9.35722	4.780	2.3645	2.0568	457.0169	465.7608	9.6173
12. Erythromycin	-9.19925	0.85607	10.05532	6.132	2.8677	1.1261	594.2150	810.4607	11.5676
13. Amitriptyline	-8.98917	0.18175	9.17092	0.987	1.4228	1.2232	306.9413	312.0148	8.4151
14. Tamoxifen	-8.50925	0.00187	8.51112	1.770	2.2797	1.1203	396.8759	415.7200	9.2598
15. Ketoconazole	-7.82112	-0.82165	6.99947	5.813	1.6419	1.5292	473.8901	511.2672	9.9209

E2, 17 β -oestradiol; volume = volume of the Connolly solvent-accessible surface ($\text{\AA}^3$); μ , dipole moment (Debye) diameter = molecular diameter estimated from volume ($\text{\AA}$); a/d^2 , molecular area-to-depth-squared ratio; E_{HOMO} , energy of the highest occupied molecular orbital (eV); l/w , length-to-width ratio; E_{LUMO} , energy of the lowest unoccupied molecular orbital (eV); SA, surface area of the Connolly solvent-accessible surface ($\text{\AA}^2$); $\Delta E = E_{\text{LUMO}} - E_{\text{HOMO}}$ (eV).

Conclusions

The homology model of CYP3A4, derived from sequence homology with the rabbit enzyme CYP2C5, appears to show a good overall degree of consistency with the currently available information from site-specific mutagenesis experiments and substrate metabolism. It is found that all substrates investigated within this new model are able to form hydrogen-bonded contacts with serine-119, which has been the subject to SDM investigations, and appears to lie close to the haem where its distance to the central iron atom (6.7 Å) is consistent with its possible role as an 'anchor' for typical CYP3A4 substrates. All of the substrates studied in this work show the presence of a hydrogen bond acceptor within the molecule which is located about five heavy atoms from the site of metabolism with an average distance of approximately 7 Å according to the EIP-coded surface of the substrate template. This model also enables the estimation of substrate binding affinity, based on active site contacts and compound lipophilicity, which agrees favourably with the experimental values for ΔG_{bind} obtained from K_{D} determinations. Consequently, it should be possible to use this new model of CYP3A4 for the screening of new chemical entities of relevance to drug development. Although not yet in the public domain, a recently disclosed crystal structure of CYP3A4 (Astex Technology, Cambridge, UK) should make an interesting structural comparison with this model as well as providing an improved starting point for investigating CYP3A4-mediated substrate metabolism in structural terms.

Acknowledgements

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Prediction of clinical pharmacokinetic parameters of linezolid using animal data by allometric scaling: applicability for the development of novel oxazolidinones

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1. Allometric scaling has previously been used as an effective tool for the prediction of human pharmacokinetic data. The pharmacokinetic data for linezolid, a novel oxazolidinone to treat Gram-positive pathogens, in mice, rats and dogs were subjected to simple allometric scaling. Generated allometric equations for parameters such as clearance (CL), volume of distribution (V_{ss}) and elimination rate constant (K_{10}) were used to predict human pharmacokinetic parameters including elimination half-lives. In addition, the human plasma concentration–time curve was simulated using a one-compartmental model.

2. Application of simple allometry ($Y = aW^b$) for animal parameters such as CL, V_{ss} , and K_{10} showed excellent allometric fit ($r \geq 0.98$). The allometric equations for CL, V_{ss} , and K_{10} were $-0.5465W^{0.6595}$, $-0.1369W^{0.9246}$, and $-0.4117W^{-0.3139}$, respectively. The confidence in predictability of CL and V_{ss} parameters was particularly high since the allometric exponents of CL and V_{ss} almost approached the suggested values of 0.75 and 1.00, respectively.

3. Animal pharmacokinetic parameters generated in the present authors' laboratories for linezolid were in close agreement with reported literature values. The predicted human values for CL (4.68 l h^{-1}), V_{ss} (37.07 litres), and K_{10} (0.10 h^{-1}) were within the range observed for linezolid in the literature (CL = $4\text{--}10.5 \text{ l h}^{-1}$; V_{ss} = $21\text{--}53$ litres; K_{10} = $0.09\text{--}0.3 \text{ h}^{-1}$). The human half-life ($t_{1/2}$) predicted using allometry (6.9 h) was similar to reported values in humans of 5 h. In summary, the retrospective analysis for linezolid suggests that allometric scaling can be used as a prospective tool for predicting human pharmacokinetic parameters of novel oxazolidinones.

Introduction

The emergence of clinical drug resistance among bacteria has been on the rise, necessitating the need for novel agents to treat multidrug-resistant infections, especially those related to Gram-positive pathogens. In this context, the oxazolidinone class as a whole, which selectively inhibits bacterial protein synthesis, has been a focus of relentless research in both pharmaceutical industries and academic institutions. Special attention to this novel class has been reserved in recent international meetings dealing with antimicrobial agents. Perhaps this is attributable to the fact that after several decades of painstaking research, linezolid has been approved for the treatment of serious infections due to sensitive and resistant Gram-positive pathogens (Ford *et al.* 2001, Perry and Jarvis 2001, Ament *et al.* 2002). Linezolid's spectrum of activity covers several important pathogens, notably methicillin-resistant staphylococci, penicillin-resistant pneumococci,

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macrolide-resistant streptococci and vancomycin-resistant enterococci (Moellering 2003). Some of the most frequently reported adverse events in the clinic following linezolid administration are diarrhoea, headache, nausea and vomiting (Perry and Jarvis 2001).

The pharmacokinetic and pharmacodynamic profiles of linezolid have been studied extensively (Bouza and Munoz 2001, Slatter *et al.* 2001, Sisson *et al.* 2002, MacGowan 2003, Stalker and Jungbluth 2003, Stalker *et al.* 2003). It has been reported to be rapidly and completely absorbed (F approaching 100%) after oral dosing to human subjects. Typically, the peak concentration is reached within 2 h after dosing. Owing to plasma levels tending to be close to its minimum inhibitory concentrations (MICs) (approximately $2\text{--}4\text{ }\mu\text{g ml}^{-1}$) by 12 h after dosing, the use of linezolid in therapy is via b.i.d. dosing. Literature data suggest that repeated daily doses up to 625 mg b.i.d. are well tolerated in human subjects. The clinical pharmacology attributes of linezolid suggest it is a useful compound from a pharmacokinetic point of view with respect to age, renal impairment or hepatic dysfunction having no influence on its disposition (Hendershot *et al.* 2001, Sisson *et al.* 2002, Brier *et al.* 2003, Stalker and Jungbluth 2003). Although somewhat higher levels are observed in women as compared to men, the difference is not sufficient to warrant an adjustment in dosage (Sisson *et al.* 2002). Owing to its complete bioavailability by the oral route of administration, linezolid offers the unique advantage of interchange between intravenous (i.v.) and oral dosing. In addition, it is neither a substrate nor an inhibitor of major human cytochrome P450 enzymes (CYP3A4, 2D6, 2C9, 2C19, 1A2), making it unlikely to have a drug interaction capability with other co-administered agents (Wienkers *et al.* 1999). In terms of pharmacodynamic effects, it has been observed that both time above MIC ($t > \text{MIC}$) and the quotient of total exposure and MIC (area under the curve (AUC)/MIC) correlate well with bacteriological and clinical outcomes of linezolid (MacGowan 2003). In addition, both AUC and the duration of treatment of linezolid could also be potentially related to the risk of thrombocytopenia occurring in the patient population (MacGowan 2003).

Since linezolid kinetics have been thoroughly investigated in animals and humans, of interest were the animal data of linezolid reported in the literature and those generated in the present authors' laboratories to predict pharmacokinetic parameters in humans. Following which, the next part of the study was to compare the predicted versus observed human pharmacokinetic data for linezolid. As the present authors have an ambitious novel oxazolidinones programme in place, the above exercise(s) would aid in lead optimization and the clinical development of novel oxazolidinones.

Materials and methods

Chemicals and reagents

Linezolid was synthesized by the Medicinal Chemistry Group, Discovery Research, Dr. Reddy's Laboratories Ltd (Hyderabad, India) and was characterized using chromatographic and spectral techniques by the Analytical Research Group of the same company. Purity was >99% for linezolid. Dichloromethane, ethyl acetate, acetonitrile and methanol were of high-performance liquid chromatography (HPLC) grade and obtained from Merck (Mumbai, India), Rankem (New Delhi, India), Qualigens (Mumbai, India) and Rankem (India), respectively. Potassium dihydrogen orthophosphate was procured from Spectrochem Pvt. Ltd (Mumbai, India); orthophosphoric acid was purchased from Qualigens. All the primary and working stocks for analytical purpose were prepared using dimethyl sulfoxide purchased from Sigma-Aldrich Co. (St. Louis, USA). All aqueous solutions including

the buffer for the HPLC mobile phase was prepared with Milli Q (Millipore, Massachusetts, USA)-grade water.

Intravenous pharmacokinetic studies

Male Wistar rats (220–230 g, $n=6$) and male Swiss Albino mice (25–30 g, $n=12$) were procured from the Discovery Research animal house facility. Animals were quarantined and acclimatized to laboratory conditions for 24 h. Feed (Amruth Laboratory Rat and Mice Feed, Hyderabad, India) and water were provided *ad libitum*. The institutional ethics committee of Discovery Research, Dr. Reddy's Laboratories, approved all protocols in the experimental design.

Linezolid was administered i.v. as a solution in water to non-fasted male Wistar rats and male Swiss Albino mice at 10 mg kg^{-1} dose (tail vein bolus). Post-dose blood samples (0.25 ml) were collected (in case of mice by sparse sampling) from retro-orbital plexus at designated time points post-dosing (mice, 0.17, 0.5, 1, 2, 4, 6, 8, 12 and 24 h; rats, 0.17, 0.5, 1, 2, 3, 5, 8, 12 and 24 h) into Eppendorff tubes containing $20 \mu\text{l}$ saturated solution of ethylenediamine tetra-acetic acid. Plasma was obtained by centrifuging the blood using a biofuge. The obtained plasma was stored at -20°C until analysis.

Analytical method

An HPLC method was developed for the quantitation of linezolid in plasma. To a $100 \mu\text{l}$ aliquot of plasma taken into a clean and dry centrifuge tube, 3 ml dichloromethane/ethyl acetate (1:2, v/v) mixture was added and the contents vortexed for 2 min on a cyclomixer followed by centrifugation for 10 min at $1760 g$ on a table-top centrifuge (all Remi Instruments, Mumbai, India). The clear organic layer (2.7 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 mobile phase and analysed by C-18 reverse-phase HPLC (Intersil ODS-2, $4.6 \times 250 \text{ mm}$, $5 \mu\text{m}$; GL Sciences, Inc., Tokyo, Japan) column. The mobile phase (70% 0.01 M KH_2PO_4 buffer, pH 3.2:10% methanol:20% acetonitrile mixture, v/v) was delivered at 1 ml min^{-1} , and the effluent was monitored at 253 nm. The standard curve for linezolid was linear ($r=0.99$) over the range $0.1\text{--}50.0 \mu\text{g ml}^{-1}$ in plasma. The accuracy and precision of the quality control samples were within 15% of deviation and of 15% relative standard deviation, respectively.

Data analysis

The plasma concentration–time data generated in rats and mice in the present authors' laboratories were subjected to one-compartmental modelling using WinNonlin (v.2.1; Pharsight, CA, USA) to obtain clearance (CL), the volume of distribution (V_{ss}) and the elimination rate constant (K_{10}).

Allometric scaling and calculations

Parameters such as CL, V_{ss} and first-order rate constants were subjected to simple allometry scaling using the following relationship:

$$Y = aW^b,$$

where Y is the relevant pharmacokinetic parameter, W is body weight, and a and b are the coefficient and exponent of allometric equation, respectively. Following allometric scaling, human half-lives ($t_{1/2}$) were obtained using the relationship:

$$t_{1/2} = 0.693/K_{10}$$

Pharmacokinetic modelling/simulation of human plasma curves

The obtained V_{ss} and K_{10} parameters from allometric prediction were incorporated in a one-compartmental model, and human plasma data of linezolid were predicted at a clinical dose (600 mg, single dose).

Results

The observed plasma concentration–time profiles for linezolid at an i.v. dose of 10 mg kg^{-1} in mice and rats are shown in figure 1. Regardless of species, the i.v. plasma curves were almost parallel to each other and were best described by fitting to a one-compartmental model. The pharmacokinetic parameters of linezolid in both mice and rats are given in table 1. The pharmacokinetic parameters generated for linezolid (shown in parenthesis) were very close to those reported in the literature (Slatter *et al.* 2001). The use of literature parameter values for CL, V_{ss}

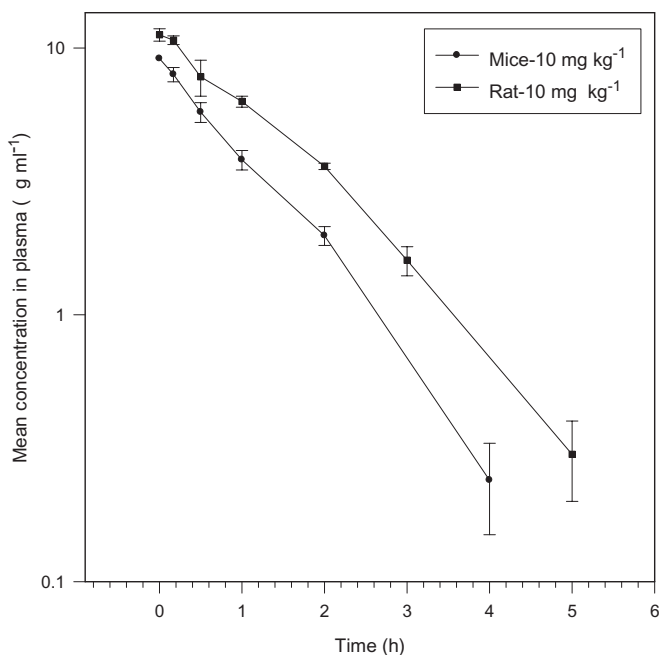


Figure 1. Intravenous plasma versus time curves of linezolid in SAM and Wistar rats.

Table 1. Literature pharmacokinetic parameters (Slatter *et al.* 2002) for linezolid in mouse, rat and dog.

Species	Mouse	Rat	Dog
Dose (mg kg ⁻¹)	10	10	25
K_{10} (h ⁻¹)	1.26 (0.94)	0.69 ± 0.06 (0.84 ± 0.07)	0.18 ± 0.01
V_{ss} (litres)	0.024 (0.027)	0.15 ± 0.02 (0.16 ± 0.008)	6.3 ± 0.05
CL (lh ⁻¹)	0.03 (0.024)	0.12 ± 0.05 (0.10 ± 0.005)	1.19 ± 0.02

Values in parentheses were generated in the present authors' laboratories.

and K_{10} in mice, rats and dogs, along with the in-house generated values provided a perfect allometric fit when scale up was performed ($r = 0.98$) (figures 2–4). The allometric scaling equations for CL, V_{ss} and K_{10} were $-0.5465W^{0.6595}$, $-0.1369W^{0.9246}$ and $-0.4117W^{-0.3139}$, respectively (table 2). The human predicted values from the respective allometric equations for CL, V_{ss} and K_{10} were generally in agreement with the corresponding values reported in the literature (table 3). Based on a one-compartmental model, a human plasma concentration–time curve was generated at an i.v. dose of 600 mg (figure 5). Both predicted human exposure (AUCs) and $t_{1/2}$ values using an allometric approach provided values in close agreement with those observed in the clinic for linezolid (Pharmacia and Upjohn 2000).

Discussion

Interspecies scaling via an allometric approach has been used extensively to predict human pharmacokinetics (Boxenbaum 1984, Aviles *et al.* 2001,

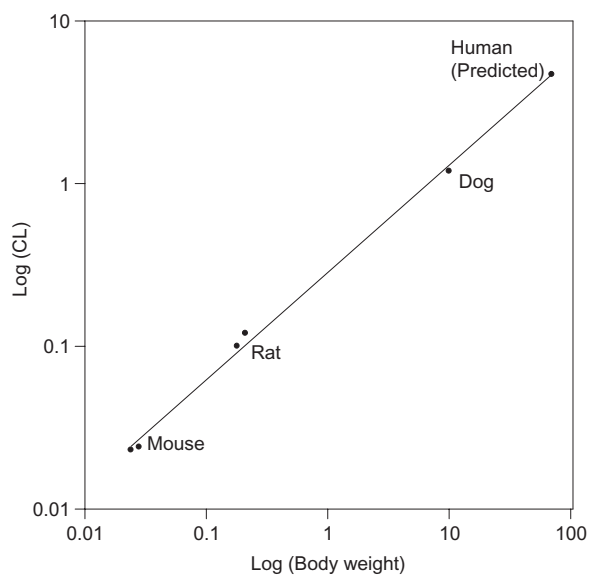


Figure 2. Allometric scaling of linezolid clearance data generated in mouse, rat and dog.

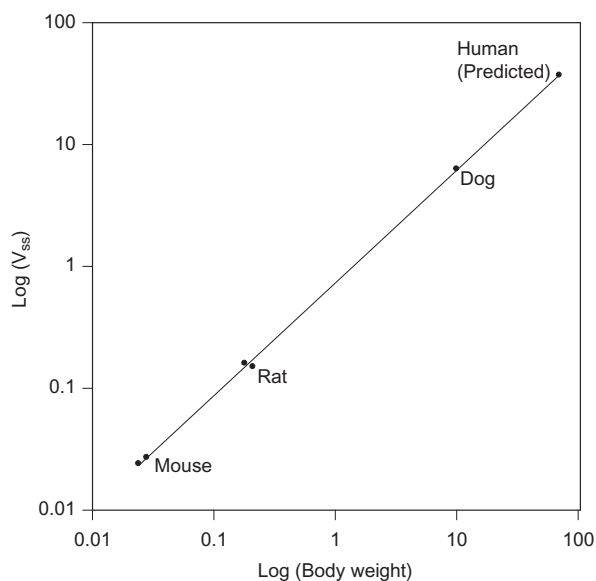


Figure 3. Allometric scaling of linezolid V_{ss} data generated in mouse, rat and dogs.

Sukbuntherng *et al.* 2001, Mahmood 2002, Ward *et al.* 2002). There have been several instances where allometric predictions have served as a guide for fixing the initial doses to support first-in-human single-dose escalation studies (Khor *et al.* 2000, Mahmood 2001a) and/or designing early pharmacokinetic studies (Shin *et al.* 2003). Within the field of allometry, several variants of allometric predictions can be attempted to obtain a better correlation and increase the confidence level of prediction of human data (Mahmood 2001b). For example, clearance prediction in simple allometry can be made merely by using the observed clearances versus body

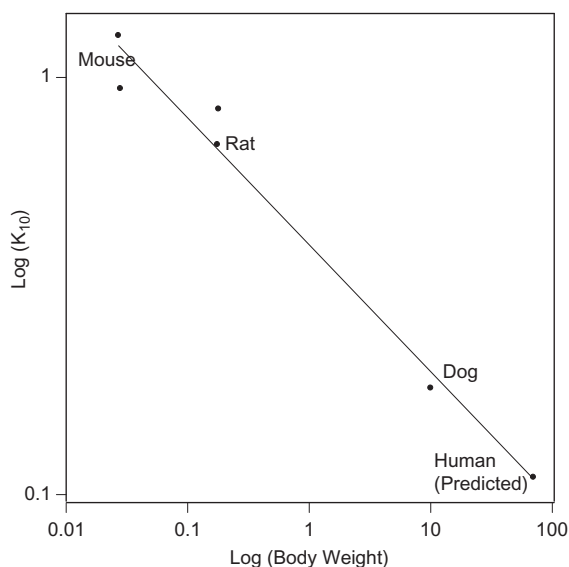


Figure 4. Allometric scaling of the elimination constant of linezolid from mouse, rat and dog.

Table 2. Exponents and correlation coefficients of the pharmacokinetic parameters for linezolid.

Parameter	CL	V_{ss}	K_{10}
Exponent	0.6595	0.9246	-0.3139
Coefficient	-0.5495	-0.1369	-0.4117

Table 3. Literature values (Pharmacia and Upjohn 2000) versus allometric data.

Parameter	Literature quoted	Obtained from allometry
K_{el} (h^{-1})	0.09–0.30	0.10
V_{ss} (litres)	21–53	37.07
CL (lh^{-1})	4.0–10.5	4.68

weights, whereas other variants of allometric approaches for similar predictions include (1) predictions based on the product of clearance and maximum life-span potential versus body weight and (2) predictions based on the product of clearance and brain weight versus body weight. Shin *et al* (2003) also predicted the human pharmacokinetics of SJ-8029, a novel anticancer agent possessing microtubule and topoisomerase inhibiting activities, using species-invariant time transformations involving kallynochron, apolysichron and dienetichron times. Interestingly, the predictions based on kallynochron and apolysichron transformations suggested comparable values for CL, V_{ss} and $t_{1/2}$ parameters. Therefore, in view of the large body of literature evidence on the continuous use and usefulness of allometric scaling methods, the present paper attempted retrospectively to predict the human pharmacokinetic parameters of linezolid (CL, V_{ss} , half-life) using a simple allometric approach and as well as simulating human plasma concentration–time curves using a one-compartmental model (using VSS and K_{10}).

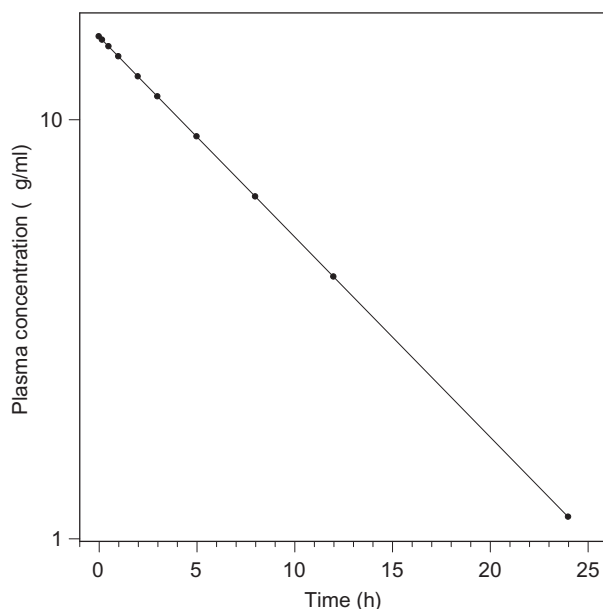


Figure 5. Simulated single dose human profile of linezolid at a 600-mg dose.

The closeness of the prediction of human linezolid data using animal data was somewhat expected. First, linezolid is not reported to be a substrate for all major CYPs and consistent absorption, distribution, metabolism and excretion profiles have been reported across several animal species including low protein binding of less than 35% (Slatter *et al.* 2002). Second, the oxidative metabolism of linezolid that accounts for a major portion of non-renal clearance is mediated via a non-enzymatic process. This process involves ubiquitous reactive oxygen species resulted in morpholine ring oxidation to form a major metabolite of linezolid (Wienkers *et al.* 1999, Wynalda *et al.* 2000). Third, renal clearance of the parent drug alone accounts for almost 33% of the total clearance of linezolid (Pharmacia and Upjohn 2000). Therefore, based on excellent correlations of parameters such as CL , V_{ss} and K_{el} ($r \geq 0.98$), it was clear that the allometric scaling approach would work out for humans. In addition, the allometric exponent for clearance (0.662) deviated within 15% from the suggested value of 0.75 to have good interspecies predictability. Similarly, the allometric exponent for volume of distribution (0.973) was within 5% of the suggested value of 1.000 assuring confidence in the prediction of this parameter (table 3). In view of the above, further improvements in allometric correlation/predictability using other factors such as liver blood flow, longevity, brain weight and renal excretion rate were not attempted for linezolid. However, if new oxazolidinone(s) do not confirm to the ideal physicochemical properties of linezolid, it might become necessary to use correction factors in the scaling method, as discussed above.

Based on the simple allometric predictions, the present authors were encouraged to simulate human plasma profiles using a one-compartmental model for linezolid. The exercise predicted the human systemic exposure and terminal half-life, which were in close agreement with the values observed for linezolid in the clinic.

As it has been demonstrated in a retrospective manner when using linezolid as an example that it appears quite feasible to undertake prospective allometric evaluation

for novel oxazolidinones structurally related to linezolid. In general, a knowledge of *in vitro* potency, post-antibiotic effect and the sub-MIC effect of the oxazolidinone makes it possible to compute the duration of activity of a single (first) dose in relation to the simulated human plasma concentration–time profile (based on allometric approach) to determine the need or appropriate time for the second dose. Further, inclusion of the terminal half-life parameter in this assessment would aid in the evaluation and/or validation of the feasibility of b.i.d. versus once-a-day dosing. The magnitude of dose for the clinical use of the oxazolidinone can be arrived at rationally using the effective da_{50} values in *in vivo* preclinical efficacy models (systemic infection models, lung infection models, thigh infection models, etc.) and the scaled drug kinetic parameters to humans (bioavailability, CL, V_{ss} , terminal half-life, etc.). Subsequent human plasma profile simulations would provide additional information for dose(s) required to target other pathogens that require a different range of MICs. In addition, one could simulate human plasma profiles using C_{max}/MIC and/or AUC/MIC as a guiding factor to arrive at therapeutically effective dose ranges for the oxazolidinone. The present authors believe that such an exercise of simulating human plasma–time curves and generating human pharmacokinetic parameters a priori for the oxazolidinone under consideration would benefit greatly the design of the corresponding toxicology program (b.i.d. or once daily dosing; dose selection; toxicity assessment using C_{max}/MIC and/or AUC/MIC or time above MIC threshold, etc.). As is often observed, surprises in drug development should be carefully monitored with the estimates of safety margins being re-evaluated whenever an opportunity is presented with new evidence becoming available and used as a guide for drug development.

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Pharmacokinetics of two major hydroxylated polychlorinated biphenyl metabolites with specific retention in rat blood

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1. Hydroxylated metabolites of polychlorinated biphenyls (OH-PCBs) are, depending on their structure, strongly retained in mammalian, fish and bird blood. This is due to strong, though reversible, binding to the thyroxine binding and transporting protein transthyretin. 2,3,3',4',5-Pentachloro-4-biphenylol (4-OH-CB 107) and 2,2',3,4',5,5',6-heptachloro-4-biphenylol (4-OH-CB 187) are two of five major OH-PCB congeners in human plasma.

2. The relative amounts of OH-PCB congeners vary between species and also between human populations, in spite of similar PCB congener patterns, and may depend on different pharmacokinetic parameters of the OH-PCBs. In the present study, the pharmacokinetic parameters of 4-OH-CB 107 and 4-OH-CB 187 were determined in the rat after a single intravenous dose of $1 \mu\text{mol kg}^{-1}$. Plasma samples were analysed by gas chromatography/mass spectrometry.

3. 4-OH-CB 107 had a half-life of 3.8 days; 4-OH-CB 187 had a half-life of 15 days. Volumes of distribution were 0.07 and 0.11 l kg^{-1} , respectively; clearances (ml h^{-1}) were 0.67 and 0.22 , respectively; and the areas under the curve were estimated as approximately 1500 and $4450 \text{ nmol h ml}^{-1}$.

4. The pharmacokinetic parameters thus determined help to explain the observed differences in the relative amounts of OH-PCBs in humans and other mammals exposed to environmental PCBs.

Introduction

The strong retention of a hydroxylated metabolite of 3,3',4,4'-tetrachlorobiphenyl (CB-77) was first observed in the blood of laboratory mice (Darnerud *et al.* 1986, Klasson Wehler *et al.* 1989). Subsequently, a large number of polychlorobiphenyls (OH-PCBs) were determined in rats dosed with a commercial PCB mixture and in humans and grey seals exposed to environmental PCBs (Bergman *et al.* 1994). Several of the retained OH-PCB congeners have been shown to bind to the thyroxine-binding protein transthyretin (TTR) when tested *in vitro* for their potential to compete with the natural ligand (Lans *et al.* 1994, Brouwer *et al.* 1998). The TTR-binding OH-PCB congeners all have structural similarities to thyroxine, with chlorine atoms in both *ortho* positions to the hydroxyl group (Letcher *et al.* 2000).

Following the synthesis of numerous authentic reference standard OH-PCBs, it was possible to identify and suggest structures for 38 individual OH-PCB

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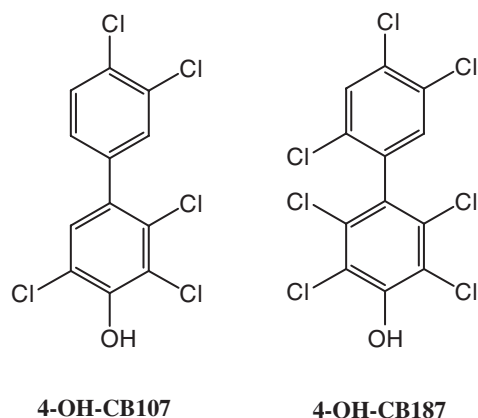


Figure 1. Chemical structures of 2,3,3',4',5-pentachloro-4-biphenylol (4-OH-CB-107) and 2,2',3,4',5,5',6-heptachloro-4-biphenylol (4-OH-CB-187).

congeners present in human blood (Hovander *et al.* 2002). However, the predominant PCB metabolites being retained varies between two and five OH-PCB congeners in blood from mammals, birds and fish (Bergman *et al.* 1994, Klasson Wehler *et al.* 1998, Sandau *et al.* 2000, Sandau 2001, Hovander *et al.* 2002, Campbell *et al.* 2003, Li *et al.* 2003, Fängström *et al.* 2004). The most abundant OH-PCBs (the nomenclature is as explained in the Materials and methods) in human blood are 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB-107, figure 1), 4-hydroxy-2,2',3,4',5,5'-hexachlorobiphenyl (4-OH-CB-146) and 4-hydroxy-2,2',3,4',5,5',6-heptachlorobiphenyl (4-OH-CB-187, figure 1), which are metabolites of CB-105/CB-118, CB-138/CB-153 and CB-183/CB-187, respectively (Sjödén *et al.* 1998). These OH-PCBs have been reported to be present in high concentrations, comparable with PCB levels, in humans (Sandau *et al.* 2000, Sjödén *et al.* 2000, Fängström *et al.* 2002).

The OH-PCBs retained in plasma are formed by a cytochrome P450-catalysed oxidation, in most cases to an arene oxide followed by a 1,2-shift of a chlorine atom, resulting in a *para*-hydroxylation, e.g. CB-105 (Haraguchi *et al.* 1998) CB-118 (Klasson Wehler *et al.* 1993, Haraguchi *et al.* 1998), CB-138 and CB-153 (Sjödén *et al.* 1998). Direct hydroxylation of the PCB congener may also occur and is one explanation for the formation of 4-OH-CB-187, as this metabolite has been shown to be formed from CB-187 (Sjödén *et al.* 1998). 4-OH-CB-187 can also be formed from CB-183 via an arene oxide and a 1,2-shift, although no evidence for this route has been reported yet.

The PCB gas chromatographic peak pattern is fairly similar in higher animals, e.g. seals, albatrosses and humans, whereas the OH-PCB pattern varies between different human populations (Sandau *et al.* 2000, Sjödén *et al.* 2000, Fängström *et al.* 2002). These differences may be explained by variations in exposure and metabolism. As yet, there are only limited data on the pharmacokinetics of individual PCB congeners. Following oral administration, half-lives in rat tissues have been determined for a set of PCB congeners, including CB-105 and CB-118 but not CB-183 and CB-187 (Öberg *et al.* 2002). CB-153, which is considered to be one of the most persistent PCB congeners, was reported by Öberg *et al.* to have a half-life of 100 days in plasma, although in another study this was estimated to be

478 days (Wyss *et al.* 1986). PCB persistence is related to the number of chlorine atoms but is also influenced by their substitution pattern, i.e. lack of vicinal unsubstituted *meta-para* positions (Letcher *et al.* 2000, Öberg *et al.* 2002). The pentaCBs, CB-118 and CB-105, are present only in low concentrations relative to CB-153 in humans and wildlife at high trophic levels, whereas CB-183 and CB-187 are extensively bioaccumulated. The presence of OH-PCBs in plasma is dependent on the metabolic rate of formation from their parent CBs, although the apparent half-lives of CBs does not explain the observed differences in OH-PCB patterns. The OH-PCB congeners chosen in the current study represent two congeners whose relationships to the parent PCB differ. Thus, 4-OH-CB-107 is often a major metabolite in plasma formed from CB-118 and CB-105, which have intermediate persistency (Bergman *et al.* 1992). The parent PCBs of 4-OH-CB-187, CB-187 and/or CB-183, are both highly persistent PCB congeners. This implies other differences in pharmacokinetic parameters that result in different retentions of the two OH-PCB metabolites.

The aim of the present work was to determine the pharmacokinetic parameters of 4-OH-CB-107 and 4-OH-CB-187 (figure 1), the two dominating OH-PCB congeners present in the blood of humans and wildlife, as determined in the rat after intravenous administration.

Material and methods

Chemicals

2,3,3',4'-Pentachloro-4-biphenylol (4-OH-CB-107) — abbreviations according to Ballschmitter *et al.* (1993) and Letcher *et al.* (2000) — was synthesized from 2,3,5-trichloroanisidine and 1,2-dichlorobenzene by the Cadogan (1962) diaryl coupling method. 2,2',3,4',5,5',6-Heptachloro-4-biphenylol (4-OH-CB-187) was prepared from 4-iodo-2,3,5,6-tetrachloroanisole and 2,4,5-trichloroiodobenzene using the Ullmann diaryl coupling method (Fanta 1964). The purity of the compounds was >98% according to gas chromatography. The synthesis of starting materials and couplings methods for the preparation of 4-OH-CB-107 and 4-OH-CB-187 are described in detail elsewhere (Bergman *et al.* 1995, Hovander *et al.* 2002). The internal surrogate standard 4-hydroxy-2,3,3',4',5,5',6-heptachlorobiphenyl (4-OH-CB-193) was synthesized in-house (Bergman *et al.* 1995) as well as 2,3,3',4',5,5',6-heptachlorobiphenyl (CB-189), which was used as the recovery standard (Sundström *et al.* 1975). Diazomethane was prepared as described by Fieser and Fieser (1967). All other chemicals and solvents were of pesticide or analytical grade and obtained commercially.

Each compound, 4-OH-CB-107 (10.6 mg, 30 µmol) and 4-OH-CB-187 (12.7 mg, 30 µmol), were dissolved in dimethylamide (DMA) (12 ml) and mixed with polyethylene glycol (PEG) (12 ml). Water (6 ml) was added dropwise to the DMA/PEG solution with vigorous stirring. The final concentrations were 0.35 mg (1.0 µmol) ml⁻¹ 4-OH-CB-107 and 0.42 mg (1.0 µmol) ml⁻¹ 4-OH-CB-187.

Instruments

Gas chromatography was performed on a Varian 3400 gas chromatograph equipped with a CP-Sil 8-column (25 × 0.15 mm i.d. and 0.12 µm film thickness; Chrompack, EA Middelburg, the Netherlands) and an electron-capture detector. Injections were made in the splitless mode. Hydrogen was used as the carrier gas and nitrogen as the make-up gas. The column temperature was programmed as follows: 80°C (2 min), 10°C min⁻¹ up to 300°C (7 min). The injector and detector temperatures were held at 280 and 360°C, respectively. Data were collected and processed using ELDSPRO v1.0 from Chromatographic Data System AB (Stockholm, Sweden).

Animals

Twenty-four male Sprague–Dawley rats (270–340 g; B&B, Sollentuna, Sweden) were kept in cages (three per cage) under a 12/12 h light/dark cycle with free access to food (R70 Lactamin from Lab For, Sigtuna, Sweden) and water. The study was performed under licence S1/02.

Experimental design

Twelve rats were divided into four groups of three for each compound under study. The dose of each compound was 1 µmol kg⁻¹ body weight and the total volume given to each rat was 1 ml kg⁻¹. The compounds were administered intravenously in the tail vein. Blood samples were taken at 6 h and

28 days after dosing for group 1, at 24 h for group 2, at 72 h for group 3 and at 7 days for group 4. A blood sample (1 ml) for group 1 at 6 h was taken from the orbital venous plexus under light inhalation anaesthesia. The remaining blood samples (maximum volume) were taken by heart puncture under anaesthesia. The blood samples, collected into tubes, were immediately placed on ice and centrifuged to obtain plasma. Plasma samples were stored at -18°C until taken out for cleanup and analysis.

Chemical analysis

To limit the number of samples for analysis, one sample from a 4-OH-CB-107 dosed rat was combined with a sample from a 4-OH-CB-187 dosed rat, in all cases with the exception of samples from the last time point (28 days), when low levels of the analytes were expected. A previously described method was used for extraction and cleanup, with a few modifications (Hovander *et al.* 2000). Briefly, plasma (2.5 g or 2×1.25 g) was transferred to screw-capped tubes and diluted with potassium chloride (1%) if less than 2.5 g. The internal standard, 4-OH-CB-193 (2 μg , exception; 0.1 μg at time point 672 h for 4-OH-CB-107 samples) was added to the samples. The proteins were denatured with isopropanol and hydrochloric acid (6 M) and the substances were extracted and re-extracted from plasma with *n*-hexane:methyl *tert*-butyl ether. The combined extracts were washed with a potassium chloride solution (1%). To methylate the phenolic group, samples and external standards (of known concentrations) were treated with an excess of diazomethane at room temperature (3 h) and protected from light. Afterwards the remaining ethereal diazomethane was evaporated (with caution) under a gentle stream of nitrogen. The methylated analytes were purified on a silica gel/sulfuric acid column (2:1 by weight, 0.5 g), with dichloromethane (10 ml) as mobile phase. CB-189 was added as a recovery standard to all samples before analysis by means of gas chromatography–electron-capture detector. The recoveries of 4-OH-CB-193 were in accordance with the results obtained in the methodology development study (95–100%) (Hovander *et al.* 2000). For the pharmacokinetic calculations, use was made of the approximation: 1 g plasma equals 1 ml plasma.

Pharmacokinetic calculations

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 analyses were performed. The terminal half-life ($t_{1/2z}$) was calculated by use of the linear trapezoidal rule up to the last data point plus the residual area up to infinity calculated by integration. The percentage of the total area under the curve (AUC) extrapolated was also determined. For 4-OH-CB-107, the part of the AUC that was extrapolated from the last sample point to infinity was very low (below 1%) and was quite acceptable for 4-OH-CB-187 (26%), indicating that the sample points chosen were appropriate for the calculation of the pharmacokinetic parameters in this study. Total plasma clearance (CL) was calculated from:

$$\text{CL} = \text{dose}/\text{AUC}_{\text{inf}}$$

and the apparent volume of distribution at steady state from:

$$V_{\text{ss}} = (\text{dose} * \text{AUMC}_{\text{inf}})/(\text{AUC}_{\text{inf}})^2$$

AUMC_{inf} is the area under the first moment curve calculated up to the last data point by the trapezoidal rule and then to infinity by means of integration.

Results

Measured plasma sample concentrations of 4-OH-CB-107 and 4-OH-CB-187 are given at the five time points (6 h–28 days) when samples were taken from the rats (table 1). The mean plasma concentrations of both 4-OH-CB-107 and 4-OH-CB-187 are shown in figure 2.

The elimination half-lives ($t_{1/2}$) for 4-OH-CB-107 and 4-OH-CB-187 were calculated as 3.8 days (91 h) and 14.9 days (358 h). The calculated pharmacokinetic parameters are presented in table 2. One plasma sample showed a very low concentration of 4-OH-CB-107 (0.2 nmol g^{-1} , 168 h), not comparable with the other two samples and assessed as an outlier. It was therefore excluded from the pharmacokinetic calculations.

Table 1. Concentrations [nmol (mg) g⁻¹ plasma] of 4-OH-CB 107 and 4-OH-CB 187 in rat plasma following intravenous dosing.

Time (h)	4-OH-CB 107 [nmol (mg) g ⁻¹ plasma]	4-OH-CB 187
6	14.2 (4.9)	10.7 (4.4)
	14.3 (4.9)	13.3 (5.5)
	15.3 (5.2)	12.3 (5.1)
24	11.5 (3.9)	10.9 (4.5)
	11.4 (3.9)	9.4 (3.9)
	10.4 (3.6)	9.7 (4.0)
72	6.4 (2.2)	9.5 (3.9)
	7.8 (2.7)	8.8 (3.6)
	8.2 (2.8)	7.5 (3.1)
168	3.3 (1.1)	5.5 (2.3)
	3.4 (1.2)	5.9 (2.4)
	0.2 (0.07)†	6.3 (2.6)
672	0.05 (0.02)	2.1 (0.9)
	0.02 (0.01)	2.6 (1.1)
	0.09 (0.03)	2.2 (0.9)

†Excluded from the pharmacokinetic calculation.

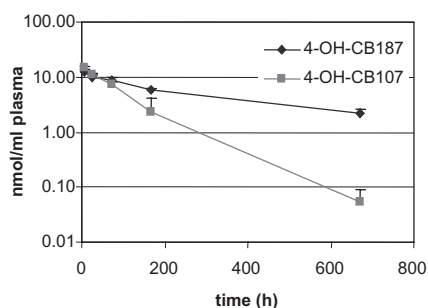
Figure 2. Time-related mean plasma concentration and standard deviations (nmol g⁻¹ fresh weight) in rats (mean of three rats plus error bars) dosed with 4-OH-CB-107 and 4-OH-CB-187.

Table 2. Pharmacokinetic parameters for 4-OH-CB-107 and 4-OH-CB-187 in rats.

	4-OH-CB-107	4-OH-CB-187
Elimination half-life (h)	91	358
AUC _{inf} (nmol h ml ⁻¹)	1487	4452
Cl (ml h ⁻¹)	0.67	0.22
V _{ss} (l kg ⁻¹)	0.07	0.11

Discussion

The plasma half-lives of 4-OH-CB-107 and 4-OH-CB-187 were calculated as approximately 4 and 15 days, respectively. The longer half-life of 4-OH-CB-187 is in agreement with the observed dominance of this metabolite in OH-PCB patterns in environmentally exposed species. The fourfold difference in half-life between the two congeners may be due to differences in their chemical properties, protein

binding, and tendency to undergo further metabolism and excretion. However, the two OH-PCB congeners are structurally similar, the major difference being two additional chlorine atoms in 4-OH-CB-187. In both cases their lipophilicity is high, with octanol/water partitioning coefficients ($\log K_{ow}$) calculated as 5.9 ± 0.4 and 6.9 ± 0.4 , respectively, for 4-OH-CB-107 and 4-OH-CB-187 (ACD/labs softwareTM, version 5.0). The acidity of 4-OH-CB-107 is somewhat lower than that of 4-OH-CB-187, with pK_a values of 5.4 ± 0.2 and 4.1 ± 0.2 , respectively (ACD/labs softwareTM, version 4.5), although quite a similar fraction of both compounds will be ionized *in vivo* (physiological pH 7.4). Thus, these physico-chemical parameters should not in themselves be the reason for the differences in half-lives of the two OH-PCB metabolites.

An important factor for the retention of OH-PCBs in blood is their affinity for TTR. TTR is a well-conserved protein between species (Power *et al.* 2000). The affinities of 4-OH-CB-107 and 4-OH-CB-187 for TTR relative to thyroxine are 5.7 and 5.3, respectively (Legler *et al.* 2002). These affinities are at the higher end of OH-PCBs and also comparable with the affinity of other ligands (Sandau 2001). Due to the similar binding of 4-OH-CB-107 and 4-OH-CB-187 to TTR, it is reasonable to suppose that this does not have any major effect on the half-lives of the compounds. However, OH-PCBs are most probably also bound to other plasma proteins, e.g. albumin. Unfortunately, there are no data available on protein binding for OH-PCBs, although differences in protein binding would affect the half-life. In the case of another chlorinated phenol, pentachlorophenol (PCP), protein binding was determined to be almost 95%, mainly (but not only) to albumin, and considered to be the major factor contributing to the observed plasma half-life of 40 h (β phase) after an oral dose of $38 \mu\text{mol kg}^{-1}$ to rats (Braun *et al.* 1977). PCP also has an affinity for TTR, albeit relatively low — 1.7 compared with thyroxine (Van den Berg 1990).

Both 4-OH-CB-107 and 4-OH-CB-187 have low volumes of distribution, 0.07 and 0.11 l kg^{-1} , respectively (table 2) which are similar to the volume previously reported for PCP (0.13 l kg^{-1}) (Braun *et al.* 1977). This is in accordance with the dominating retention of these PCB metabolites and of PCP in blood from experimental animals and in, for example, humans (Bergman *et al.* 1994, Hovander *et al.* 2002, Meironyté Guvenius *et al.* 2002).

Binding to plasma proteins is likely a major contributory factor for the comparatively long half-lives of the two OH-PCBs, and their physicochemical properties (high lipophilicity) also contribute to this. A difference in further metabolism, into conjugates or further oxidation is probably another important factor contributing to the observed differences in their half-lives. It is likely, though not proven, that further metabolism of 4-OH-CB-107 is more rapid than that of 4-OH-CB-187. Metabolism of PCB congeners is highly dependent on the number of chlorine atoms and particularly on the absence of vicinal unsubstituted carbon atoms in the *meta* and *para* positions (Letcher *et al.* 2000, Öberg *et al.* 2002). 4-OH-CB-107 can be further oxidized in the non-hydroxylated phenyl ring, resulting in a di-hydroxylated pentaCB. A di-OH pentaCB has been observed as a metabolite in mink after administration of CB-105 (Klasson Wehler *et al.* 1993). Two diOH-penta CBs (and also diOH-heptaCBs) have been identified in human plasma (Hovander *et al.* 2002).

Furthermore, phenolic metabolites are commonly conjugated to sulfate or glucuronic acid before excretion. Conjugated PCB metabolites have been reported

in urine and bile from rats after administration of parent PCB congeners (Bakke *et al.* 1982, 1983, Bergman *et al.* 1982). Inhibition of conjugation or a low conjugation rate would considerably slow down the excretion rate. *Meta*- and *para*-substituted OH-PCBs inhibit the diiodothyronine (T₂) sulfotransferase, apparently irrespective of the number of chlorine atoms, with IC₅₀ values ranging from 0.2 to 3.8 M (4-OH-CB-107 0.6 M) (Schuur *et al.* 1998b). It is not known whether the hydroxy metabolites inhibit their own sulfation in mammals, as far as we are aware. OH-PCBs are poor substrates for uridine diphosphate glucuronosyl transferases (Tampal *et al.* 2002). The rate of glucuronidation of the 15 studied OH-PCBs decreased with the number of chlorine atoms and 4-OH-CB-187 has the slowest rate of glucuronidation of all the OH-PCBs tested (4-OH-CB-107 not tested). These data could partly explain why 4-OH-CB-187 has a longer retention time than 4-OH-CB-107.

In the present study, the plasma half-life of 4-OH-CB-107 was calculated to be approximately 4 days, which is 20 times shorter than the half-life recently published for 4-OH-CB-107 in rats dosed with a mixture of 8 PCB congeners, including both precursors for this metabolite, CB-105 and CB-118 (Öberg *et al.* 2002). This divergent result may be explained by differences in experimental design. In Öberg *et al.*, the parent compounds were given to the animals, whereas the metabolite itself was administered in the present study. The fact that the metabolite is more hydrophilic means that it will have a shorter half-life than its parent compounds (the plasma $t_{1/2}$ was 98 days for CB-105 and 117 days for CB-118). When the parent compounds are administered, the formation of 4-OH-CB-107 (and other metabolites) will continue and a true elimination phase for 4-OH-CB-107 can only be reached after complete metabolism or elimination of the parent compounds. Furthermore, there are other differences in experimental set-up: parent PCBs administered as a cassette dose as well as different doses and administration routes (eight PCB congeners at a total oral dose of 0.24 versus 1.0 $\mu\text{mol kg}^{-1}$ intravenously). The cassette dosing used by Öberg *et al.* will have resulted in formation of several OH-PCBs and therefore also potential competition for the TTR binding site as well as the potential for inhibition of metabolism (Van den Hurk *et al.* 2002).

The retention of certain OH-PCBs in plasma is a combination of the bioaccumulation of persistent parent PCB congeners in tissues and the affinity of the metabolites for proteins, TTR and other proteins. The different patterns of OH-PCBs observed in plasma both in different species and in different human populations may, of course, have several explanations related to species-specific proteins and metabolism. The present study has shown that the two major OH-PCBs, 4-OH-CB-107 and 4-OH-CB-187, have different pharmacokinetic parameters in the rat. The longer half-life of 4-OH-CB-187 is in accordance with the dominance of this OH-PCB in plasma profiles from several species environmentally exposed to PCB. Considering the very long half-lives of many PCB congeners, the exposure of humans and wildlife to certain OH-PCB congeners is high. The AUC for 4-OH-CB-187 has been determined to be four times higher than that for 4-OH-CB-107. OH-PCBs have been shown to have endocrine mimicking effects (Brouwer *et al.* 1998), and to influence the metabolism of, for example, thyroid hormones (Schuur *et al.* 1998a, c) and the pharmacokinetic parameters of individual OH-PCB and PCB congeners can add valuable information to the understanding and assessment of risks related to endocrine effects.

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Metabolism of the antineoplastic and immunosuppressive drug 2-CdA (Leustatin®) in animals and humans

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1. The *in vivo* metabolism of the antineoplastic and immunosuppressive drug 2-CdA (Leustatin®) was investigated in mice, monkeys and humans after a single subcutaneous dose of cladribine 60 mg kg⁻¹ to eight male and eight female mice and 10 mg kg⁻¹ to one male and one female monkey, and an intravenous infusion dose of cladribine 22–45 mg⁻¹ per subject to 12 male patients.

2. Plasma (1 h), red blood cells (1 h) and faecal samples (0–24 h) were obtained from mice and monkeys, and urine samples (0–24 h) were obtained from these species and humans.

3. Unchanged cladribine (urine: 47% of the sample in human; 60% of the sample in mouse; 73% of the sample in monkey) and 10 metabolites, consisting of four phase I metabolites (M1–3, M7) and six phase II metabolites—five glucuronides (M4, M6, M8–10) and one sulfate (M5) — were profiled, characterized and tentatively identified in plasma, red blood cells, and faecal and urine samples on the basis of API ionspray-mass spectrometry (MS) and MS/MS data.

4. Metabolites were formed via the following three metabolic pathways: oxidative cleavage at the adenosine and deoxyribose linkage (A); oxidation at adenosine/deoxyribose (B); and conjugation (C).

5. Pathways A and B appear to be major steps, forming four oxidative/cleavage metabolites (M1–3, M7) (each 3–20% of the sample).

6. Pathway C along or in conjunction with pathways A and B produced cladribine glucuronide, cladribine sulfate and four glucuronides of oxidative/cleavage metabolites in minor/trace quantities (each ≤ 5% of the sample).

7. In addition, the *in vitro* metabolism of cladribine was conducted using rat and human liver microsomal fractions in the presence of an β -nicotinamide adenine dinucleotide phosphate-generating system. Unchanged cladribine (≥ 90% of the sample) plus three minor metabolites, M1–3 (each < 8% of the sample), were profiled and tentatively identified by thin-layer chromatography and MS data.

8. Cladribine is not extensively metabolized *in vitro* and *in vivo* in all species. However, humans appear to metabolize cladribine to a greater extent than other animals.

Introduction

Cladribine (2-CdA) (Leustatin®), 2-chloro-2'-deoxy- β -D-adenosine (figure 1), is a novel antineoplastic, immunosuppressive and anti-inflammatory agent (Carson *et al.* 1977, 1983, 1984, 1992, Riscoe *et al.* 1989, Bentler *et al.* 1991, 1992, 1994,

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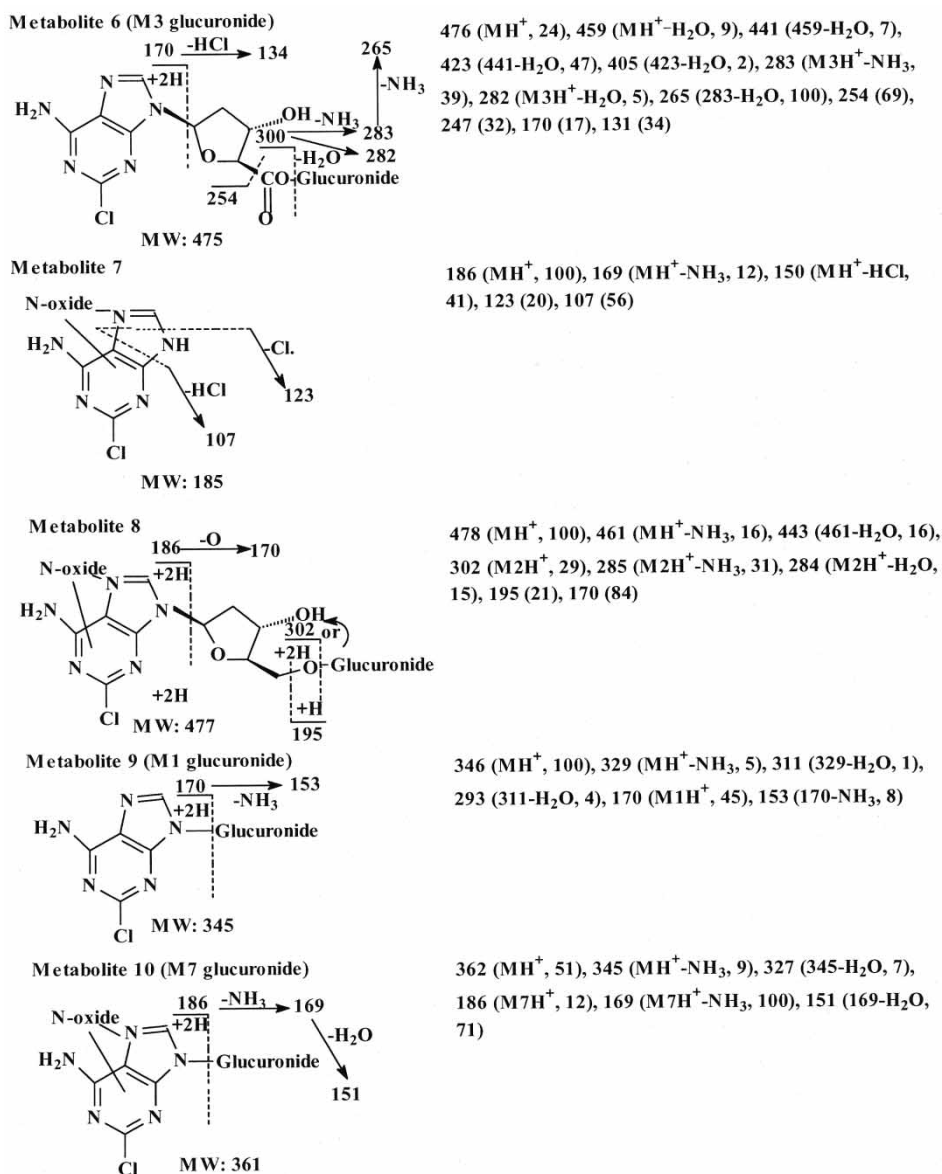


Figure 1. Structures and mass spectral data for cladribine and metabolites.

Saven and Piro 1992, Sipe *et al.* 1994, Chabner *et al.* 2001). Leustatin injection is currently being marketed worldwide as a treatment for hairy cell leukaemia, a rare formed of cancer. The National Cancer Institute has designated Leustatin the drug of choice for treating this disorder, which has historically been resistant to chemotherapy. Abnormal growth or function of lymphoid cells has caused a variety of diseases, such as lymphomas, chronic lymphocytic leukaemia, hairy cell leukaemia and autoimmune disorders such as multiple sclerosis (Seto *et al.* 1985, Bentler *et al.* 1992, 1994, Sipe *et al.* 1994, Rice *et al.* 2000). Numerous cytotoxic drugs have been developed to destroy lymphocytes without causing

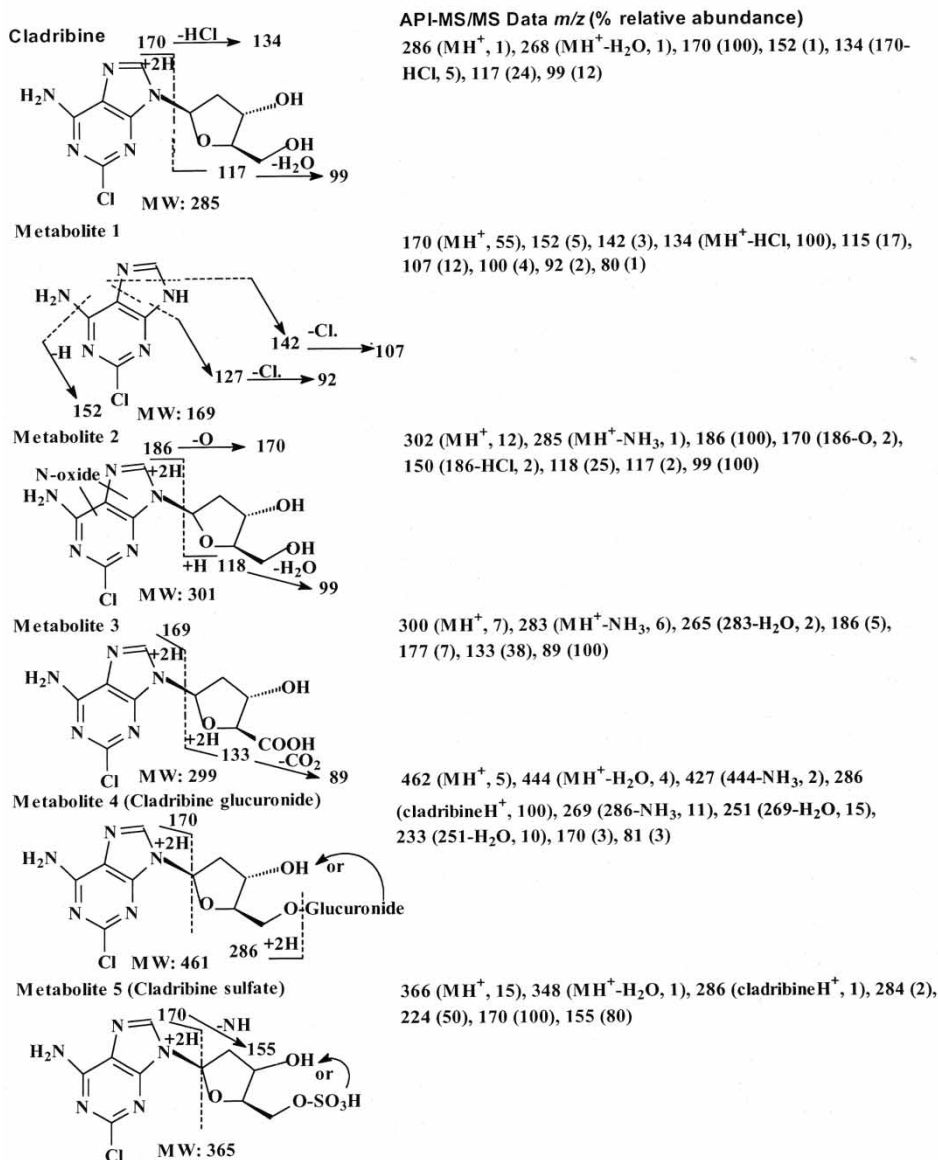


Figure 1. Continued.

damage to normal body cells. However, some of drugs, including glucocorticoids and α -interferon, target lymphoid cells and destroy rapidly not only proliferating cells of tissues, in particular bone marrow, but also normal tissues (Seto *et al.* 1985, Bentler *et al.* 1992, 1994). In the study of inherited enzyme defects, it often extends well beyond the disorder of adenosine deaminase deficiency. An understanding of the special role of adenosine deaminase in lymphocytes has resulted in the development of the antineoplastic and immunosuppressive drug, 2-CdA (Carson *et al.* 1983, 1984, 1992, Bentler, 1992), which has been the most remarkable therapeutic ratio resistant to deamination by adenosine deaminase (Carson *et al.* 1983, 1984, Bentler, 1992). The most successful therapeutic results

have been obtained in patients with hairy cell leukaemia (Bentler 1992, Carson *et al.* 1992). 2-CdA is effective in patients with chronic lymphocytic leukaemia, even when they are resistant to other therapeutic agents (Bentler, 1992, Carson *et al.* 1992). It appears to be moderately effective in patients with progressive multiple sclerosis (Rice *et al.* 2000).

The mechanism of action, pharmacokinetics, clinical attributes and bioanalytical assay of 2-CdA have been reported previously (Liliemark and Juliusson 1991, Liliemark *et al.* 1992, Saven *et al.* 1996, Moyer *et al.* 1998). The excretion and metabolism of 2-CdA in animals and humans has not been documented. Preliminary results from the present authors' laboratories on the *in vivo* metabolism of 2-CdA in mice and monkeys and humans describing the identification of 10 metabolites have been reported in abstract form (Wu *et al.* 1999). The present paper describes in more detail the profiling, characterization and identification of unchanged 2-CdA and its 10 metabolites in the rat and human liver microsomal incubations, and mouse, monkey and human biological samples. Metabolic pathways for 2-CdA have been proposed.

Materials and methods

Chemicals and materials

Cladribine (^3H and unlabelled), 2-chloro-2'-deoxy- β -D-adenosine ($8\text{-}^3\text{H}$) (2-CdA) and 2-chloroadenine (2-CAD, M1) (figure 1) were supplied by Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Spring House, PA, USA) with a chemical purity > 97% for 2-CdA and 2-CAD, and a radiochemical purity > 97% for ^3H -2-CdA (16.8 Ci mM^{-1}) (as assessed by radio-TLC, HPLC, LC/MS). *Meta*-chloroperoxybenzoic acid (MCPBA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA), and glass-distilled solvents were purchased from Burdick and Jackson Laboratories (Muskegon, MI, USA). The incubation components for rat and human liver microsomes, Tris, potassium chloride, magnesium chloride, NADP^+ , NADPH and glucose 6-phosphate, were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Liver microsomal fraction

The human liver microsomal fraction was purchased from XenoTech (Kansas City, KS, USA), and the rat liver microsomal fraction was obtained from In Vitro Tech (Baltimore, MA, USA).

Incubation conditions

Chilled, freshly made components were added to each flask (on ice) in the following order: 1.15% KCl in 0.05 M Tris buffer (pH 7.4), 5 mM MgCl_2 , 5 mM glucose 6-phosphate, 0.5 mM NADPH, rat or human liver microsomes (about 20 mg mL^{-1} protein), and (^3H for rat or unlabelled for human) 2-CdA solution ($100\text{ }\mu\text{g mL}^{-1}$ in methanol/water, 1/1, v/v) spike, to obtain a final volume of 5 ml and a 2-CdA concentrations of 50 and 100 ng mL^{-1} . After the addition of the last component, each flask was incubated in a Dubnoff Metabolic Shaker Incubator (Precision Scientific, Chicago, IL, USA) at 37°C . Samples were removed at 0, 30 and 60 min. Control flasks were incubated without rat and human liver microsomal fractions to determine drug stability under incubation conditions. The incubates were placed into prelabelled tubes, and 1 ml acetonitrile was added to terminate the reaction on an acetone-dry ice bath. The samples were stored at -20°C pending analysis.

Animal experiments

2-CdA was administered subcutaneously to eight mice (four males and four females, 60 mg kg^{-1} dose) and two monkeys (one male and one female, 10 mg kg^{-1} dose). Blood samples were obtained at 1 h from each mouse (two males and two females) and monkey via cardiac puncture and from the femoral vein, respectively. Plasma and red blood cells samples were separated by centrifugation. Urine and faecal samples (0–24 h) were collected from each of four mice (two males and two females) and two monkeys (one male and one female). Faecal samples were homogenized with methanol using a Polytron tissue homogenizer (Brinkman Instrument, Westbury, New York, USA). Faecal extracts were obtained by filtering the homogenates. The resultant extracts were combined. Combined male and female sample pools from red cells (1 h), plasma (1 h), urine (0–24 h) and faecal extract (0–24 h) were obtained for metabolite profiling, isolation and identification. All sampled pools were stored at -20°C pending analysis.

Human urine

2-CdA (22–45 mg⁻¹ per subject) was administered as a 1-h intravenous infusion to each of 12 male patients. Urine samples were obtained 0–24 h post-dose (Saven *et al.* 1996).

Analytical equipment

Thin-layer radiochemical analyses were conducted using 5 × 20 cm silica gel GF plates (250 µm gel thickness; Anatech, Inc., Newark, DE, USA). TLC plates were analysed using a Radiochromatogram Imaging System (SID 100) (Bioscan, Inc., Washington, DC, USA). API-ionspray MS and MS/MS spectra were obtained in a PE Sciex API-III Plus (Perkin-Elmer Sciex, Thornhill, Ontario, Canada), a triple quadrupole mass spectrometer interfaced to a Hitachi HPLC solvent delivery system (L-6200A, Intelligent Pump, Hitachi Co., Tokyo, Japan) via an ionsprayer using nitrogen as a curtain and nebulizing gas and argon as a collision gas for MS/MS analysis.

Sample preparation

For the sample preparation of plasma, red cells and urine, each pooled sample (5–10 ml) was extracted with Amberlite® XAD-2 resin twice. The combined XAD-2 resin was washed with water and hexane followed by methanol extraction. Each methanol extract residue was reconstituted in a small volume of methanol and further analysed using LC/ionspray-MS and MS/MS techniques.

Metabolite profiling, isolation and identification

Aliquots of each microsomal incubation were evaporated to dryness under nitrogen and reconstituted in methanol before analysis. The TLC analysis of all samples was performed using the following solvent system: dichloromethane:methanol:ammonium hydroxide (80:20:2, v/v/v). Each concentrate was applied as a spot on a 5 × 20 cm TLC plate, developed to a height 14 cm above the spot, air dried and analysed by radiochromatography using a Bioscanner. All unchanged 2-CdA and metabolites separated and isolated by TLC were prepared for MS analysis by extraction from the plate adsorbent with dichloromethane/methanol (1:1, v/v). Each HPLC analysis was conducted using LiChrosorb RP-18 MPLC® guard and analytical column (10 µm, 10 × 4.6 mm i.d.) (Brownlee Laboratories, Inc., Santa Clara, CA, USA) in a linear gradient program, from 2% methanol (98% water) to 25% methanol (75% water) in 10 min, and 25% methanol (75% water) in 15 min with both methanol and water containing 0.2% ammonium acetate at a flow rate of 1 ml min⁻¹. Unchanged 2-CdA and its metabolites were purified and fractionated from the above LC system and analysed using PE Sciex API-ionspray MS and MS/MS techniques. The mobile phase for this system was acetonitrile/water with 2.5 mM ammonium acetate buffer (pH 4.0, 50/50, v/v) at a flow rate of 0.1 ml min⁻¹.

Data analysis

An estimate of the relative percentages of unchanged 2-CdA and each metabolite in a given sample was made using the integrated TLC chromatogram, and the integrated API-Q1 scan MS (TIC, total ion chromatogram).

Synthesis of cladribine-N-oxide

2-CdA-N-oxide, a minor metabolite of 2-CdA, was synthesized using 2-CdA (5 mg) in dichloromethane (5 ml) reacted with MCPBA (*meta*-chloroperoxybenzoic acid, 5 mg) at 0°C for 30 min. The reaction mixture was allowed to return to room temperature and extracted with 1 N NaOH aq. solution (5 ml) to remove an excess amount of unreacted MCPBA and MCBA. The dichloromethane extract was washed with water, dried with anhydrous sodium sulfate and evaporated to dryness to yield a residue, which was reconstituted in methanol and injected onto the LC/MS system.

Results

Unchanged 2-CdA plus 10 metabolites were characterized and tentatively identified from mouse and monkey plasma, red blood cells, urine and faeces, and human urine (table 1). The *in vitro* metabolism of 2-CdA was conducted in rat and human liver microsomal fractions, and unchanged 2-CdA plus three metabolites were profiled and identified in the 60-min incubations (figures 2 and 3). The structures of 2-CdA and each metabolite and their MS data are presented in figure 1. Representative TLC and API-Q1 scan MS profiles are shown in rat and human liver microsomal fractions and monkey urine (figures 2–4). Representative MS/MS spectra for characterizing 2-CdA and its glucuronide (M4), and M1 are

Table 1. *In vivo* metabolism of cladribine in mouse, monkey and human.

		Mouse				Monkey			Human
Analyte		Urine (0–24 h)	Plasma (1 h)	Red cells (1 h)	Faeces (0–24 h)	Urine (0–24 h)	Plasma/red cells (1 h)	Faeces (0–24 h)	Urine (0–24 h)
	Cladribine (2-CdA)	~60	~90	~56	+	~73	~85	+	~47
M1	2-Chloroadenine (2-CAD)	~5	~5	~15	+	~3	~10	+	< 5
M2	Cladribine-N-oxide	~5	–	–	–	~5	–	–	10
M3	Carboxy-cladribine	~10	–	–	–	~4	–	–	20
M4	Cladribine glucuronide	~2	–	–	–	~1	–	–	≤ 5
M5	Cladribine sulfate	~3	–	–	–	< 5	–	–	≤ 5
M6	M3 glucuronide	< 1	–	–	–	< 1	–	–	≤ 5
M7	M1 N-oxide	~4	–	~8	+	~8	–	+	–
M8	M2 glucuronide	–	–	–	–	< 1	–	–	–
M9	2-Chloroadenine glucuronide	–	–	–	–	< 1	–	–	–
M10	M7 glucuronide	–	–	–	–	< 1	–	–	–
Total		~91	~95	~79	–	~100	< 95	–	~97

+, Identified, but not quantifiable, because of low analyte concentration.

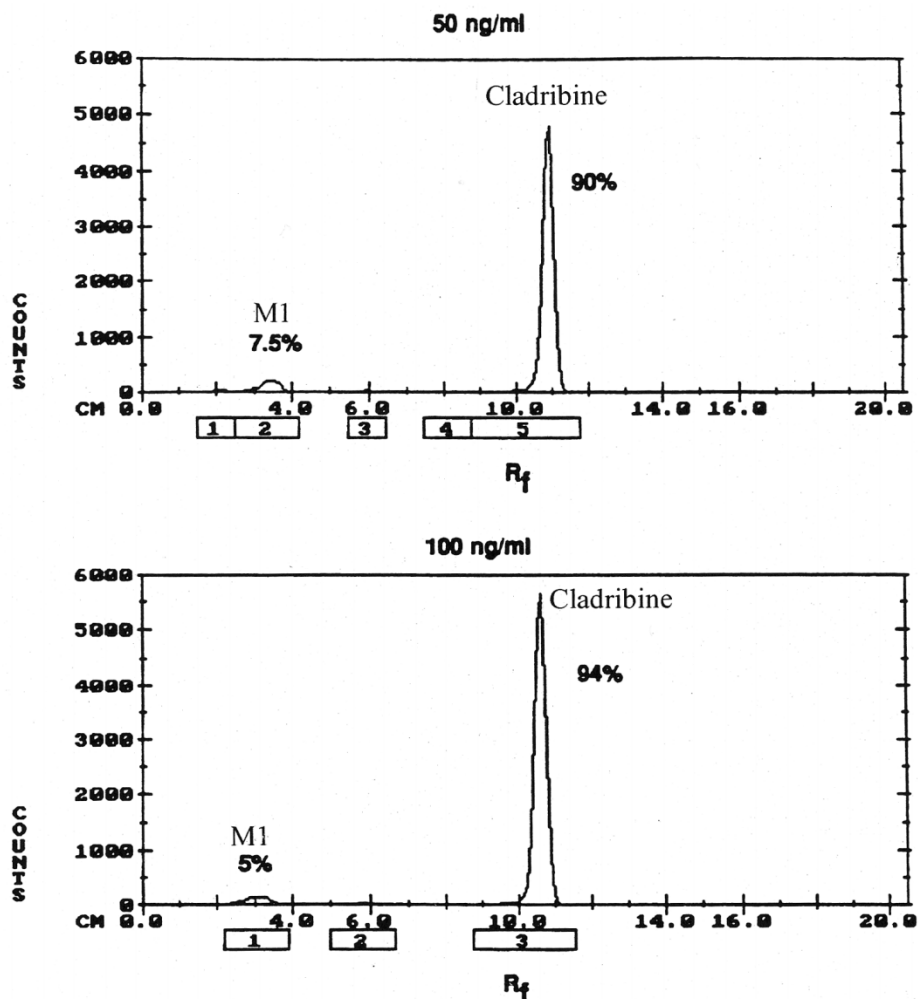


Figure 2. TLC metabolic profile of cladribine in rat liver microsomal incubations (60 min).

presented in figure 5. The characterization and identification of unchanged 2-CdA and each metabolite are discussed as follows.

Unchanged cladribine

Unchanged 2-CdA (47–73% of the sample in urine: > 85% in plasma; > 90% in microsomal incubates) was confirmed as a major component (table 1) in rat and human microsomal incubations (figures 2 and 3), plasma, red blood cells, urine and faeces using API-ionspray-MS and MS/MS techniques, and by comparison with a reference sample. The API-ionspray-MS and MS/MS data are shown in figures 1 and 3–5, which revealed an intense protonated molecule (MH^+) at m/z 286 and MS/MS product ions at m/z 268, 170 (100%), 134 and 117.

Metabolite 1 (M1)

M1 was present in moderate amounts in mouse red blood cells and monkey plasma, and in minor amounts in rat and human liver microsomal incubates, and

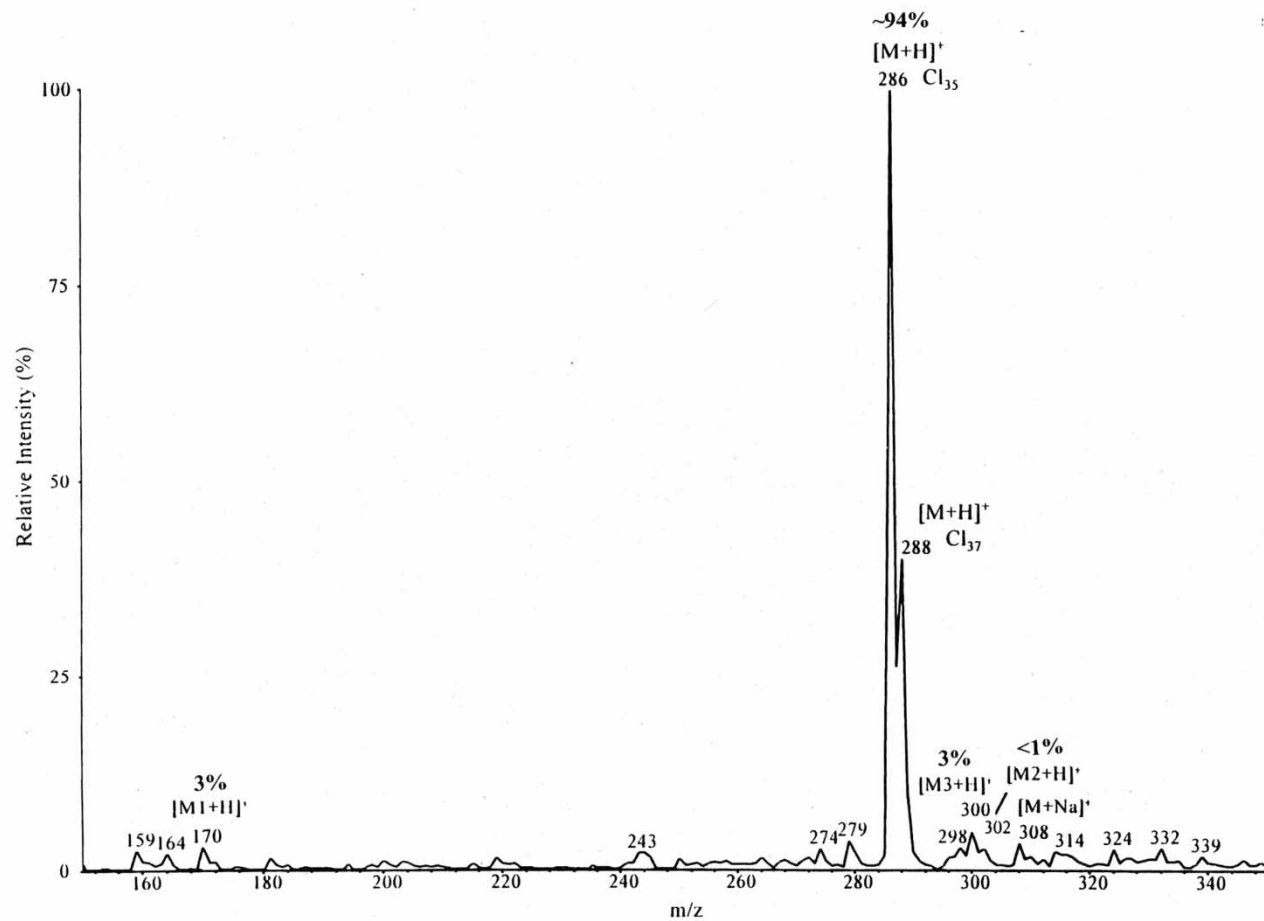


Figure 3. API-MS metabolic profile of cladribine in human liver microsomal incubation (60 min).

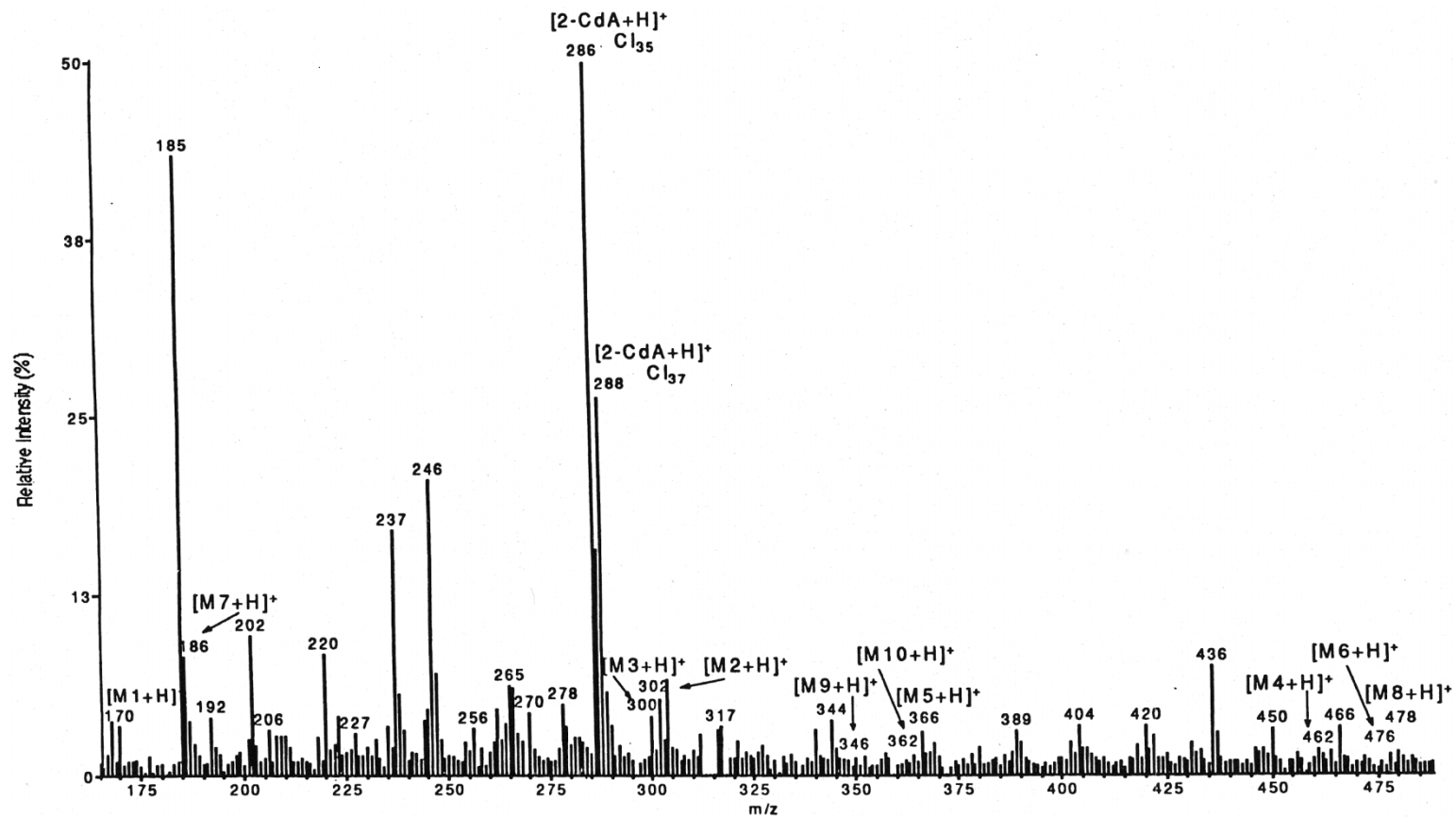


Figure 4. API-MS metabolic profile of cladribine in monkey urine (0-24 h).

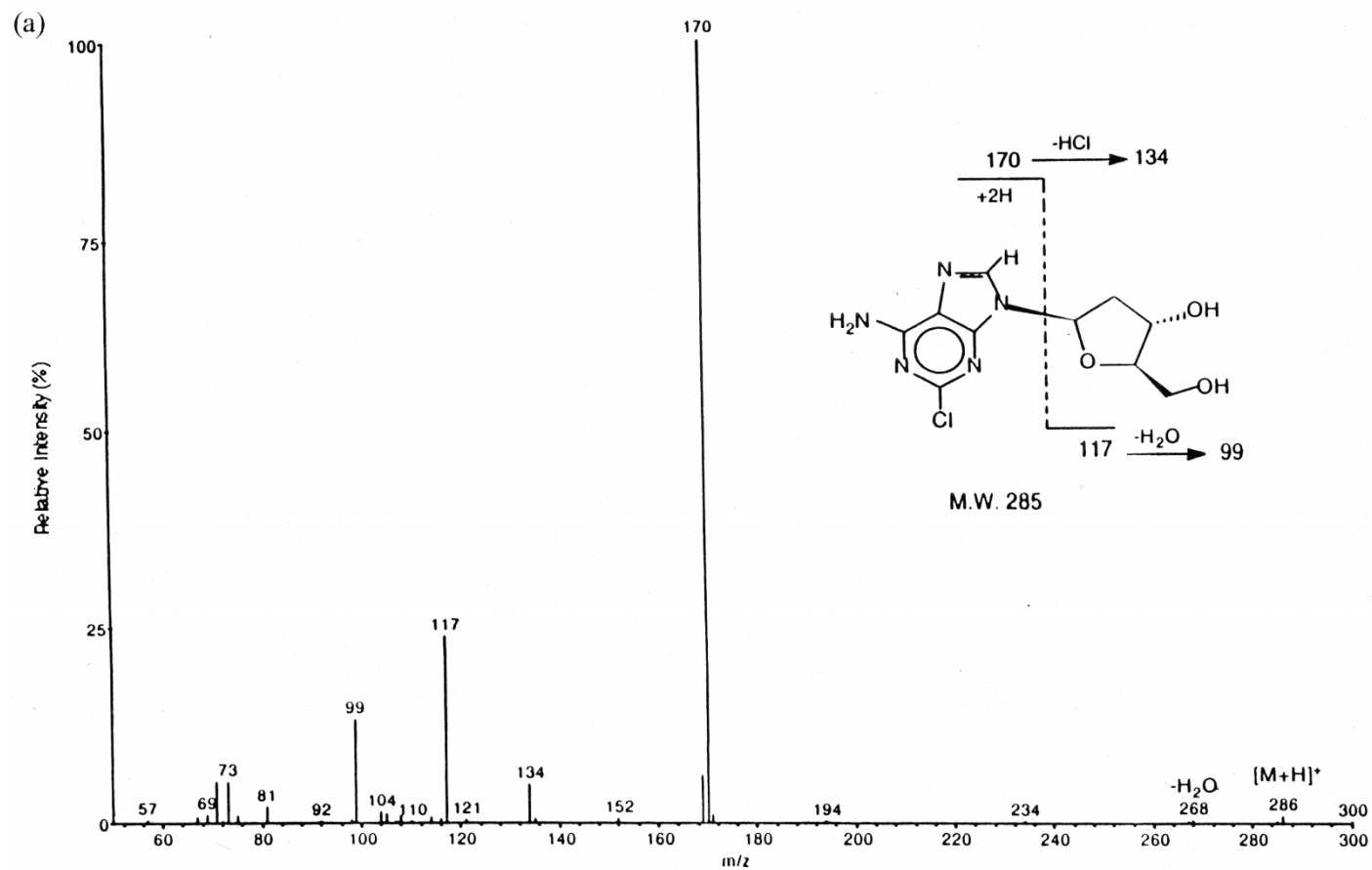


Figure 5. API-MS/MS spectra of cladribine, M1 and M4. (a) Cladribine, (b) M1, (c) M4.

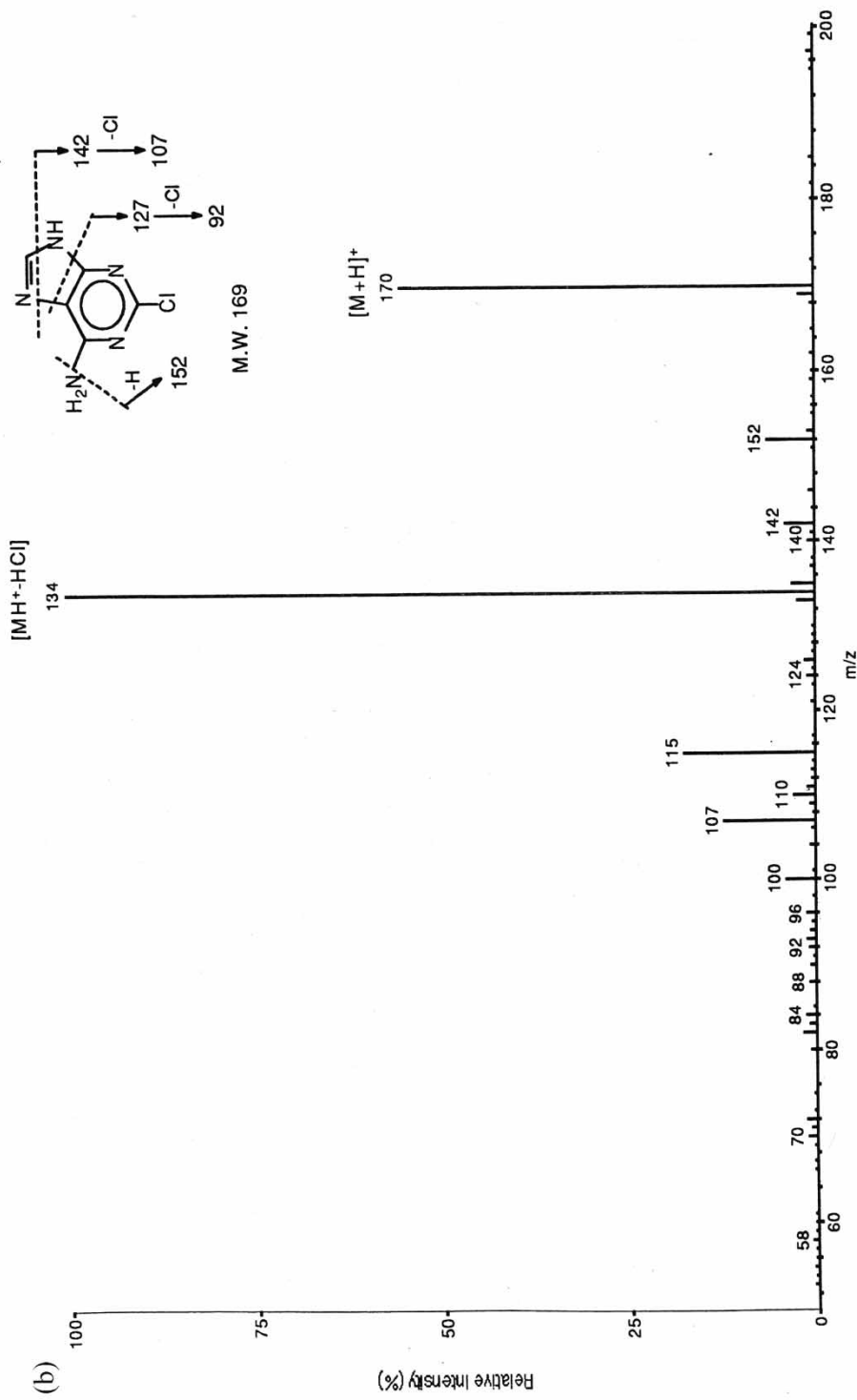


Figure 5. Continued.

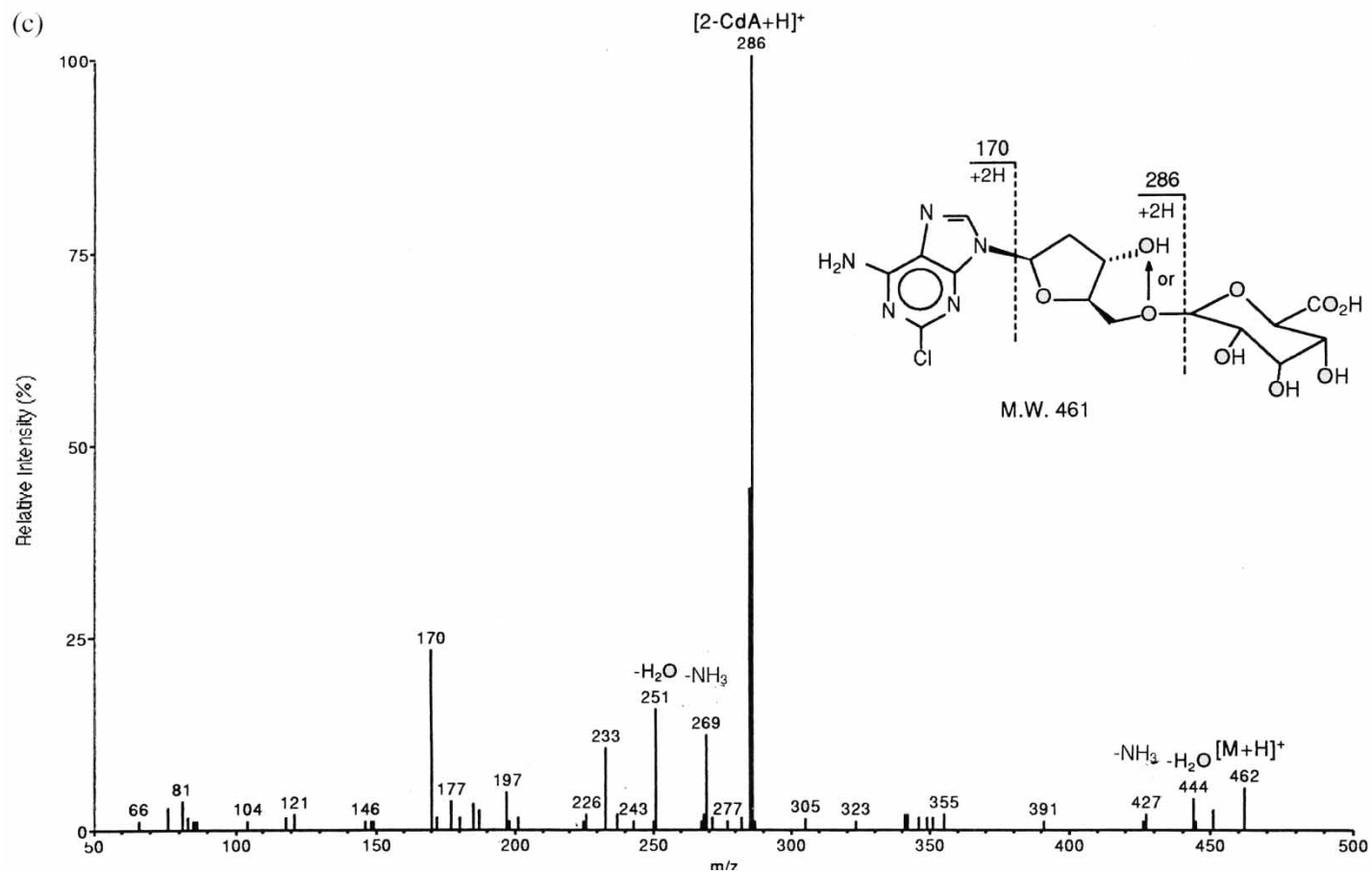


Figure 5. Continued.

mouse, monkey and in moderate amounts in human urine (table 1). The Q1 scan MS analysis of M1 displayed an apparent protonated molecule (MH^+) at m/z 170 (figures 3 and 4), which exhibited informative product ions at m/z 152, 142, 134 ($\text{MH}^+ - \text{HCl}$, 100%), 107 and 92, together with m/z 170 (figures 1 and 5). Based on MS data and by comparison with a reference sample (2-CAD, 2-chloroadenine), M1 was confirmed to be 2-chloroadenine, *N*-desdeoxyribose-2-CdA.

Metabolite 2 (M2)

M2 was identified as a minor oxidized metabolic product in human liver microsomal incubations, and mouse, monkey and human urine (table 1). The MS analysis of M2 revealed an apparent protonated molecule (MH^+) at m/z 302 (figures 1, 3 and 4). The MS/MS analysis of MH^+ showed diagnostic product ions at m/z 186 (100%), 170, 150, 118 and 99, together with m/z 302 (figure 1). Based on MS data and by comparison with a synthetic sample. M2 was identified as 2-CdA-*N*-oxide.

Metabolite 3 (M3)

M3 was present in moderate amounts in mouse and human urine and minor amounts in human liver microsomal incubate and monkey urine (table 1). The Q1 scan MS analysis of M3 displayed a protonated molecule (MH^+) at m/z 300, which exhibited the informative product ions, m/z 283, 265, 133 and 89 (100%) from the MS/MS analysis (figures 1, 3 and 4). M3 was tentatively identified as carboxy-2-CdA.

Metabolites 4 and 5 (M4 and M5)

M4 and M5 were detected in small quantities in mouse, monkey and human urine (table 1). The API-ionspray Q1 scan MS analysis of M4 and M5 showed two protonated molecule ions (MH^+) at m/z 462 and 366, respectively (figures 1, 4 and 5). The MS/MS analysis of m/z 462 (M4H^+) exhibited diagnostic product ions at m/z 286 (protonated 2-CdA, 100%), 269, 251 and 170, along with a weak protonated M4 ion (figures 1 and 5). The MS/MS analysis of protonated M5 indicated the informative product ions at m/z 348, 286 (protonated 2-CdA), 224 and 170 (100%), together with an apparent M5H^+ at m/z 366 (figure 1). M4 and M5 were tentatively characterized as 2-CdA glucuronide and 2-CdA sulfate, respectively.

Metabolite 6 (M6)

M6 was detected in minor quantities in mouse, monkey and human urine (table 1). The Q1 scan MS and MS/MS analysis of M6 revealed a protonated molecule (MH^+) at m/z 476 as well as prominent product ions at m/z 459, 423, 283, 265 (100%), 254, 170 and 131 (figure 1). Based on MS data, M6 was tentatively identified as M3 glucuronide.

Metabolite 7 (M7)

M7 was present in minor quantities in mouse red blood cells, and mouse and monkey urine (table 1). The API-MS and MS/MS analysis of M7 gave an apparent protonated molecule (MH^+) at m/z 186 (100%) and prominent product ions at m/z 169, 150, 123 and 107 (figure 1). The MS data tentatively assigned M7 to be M1-*N*-oxide.

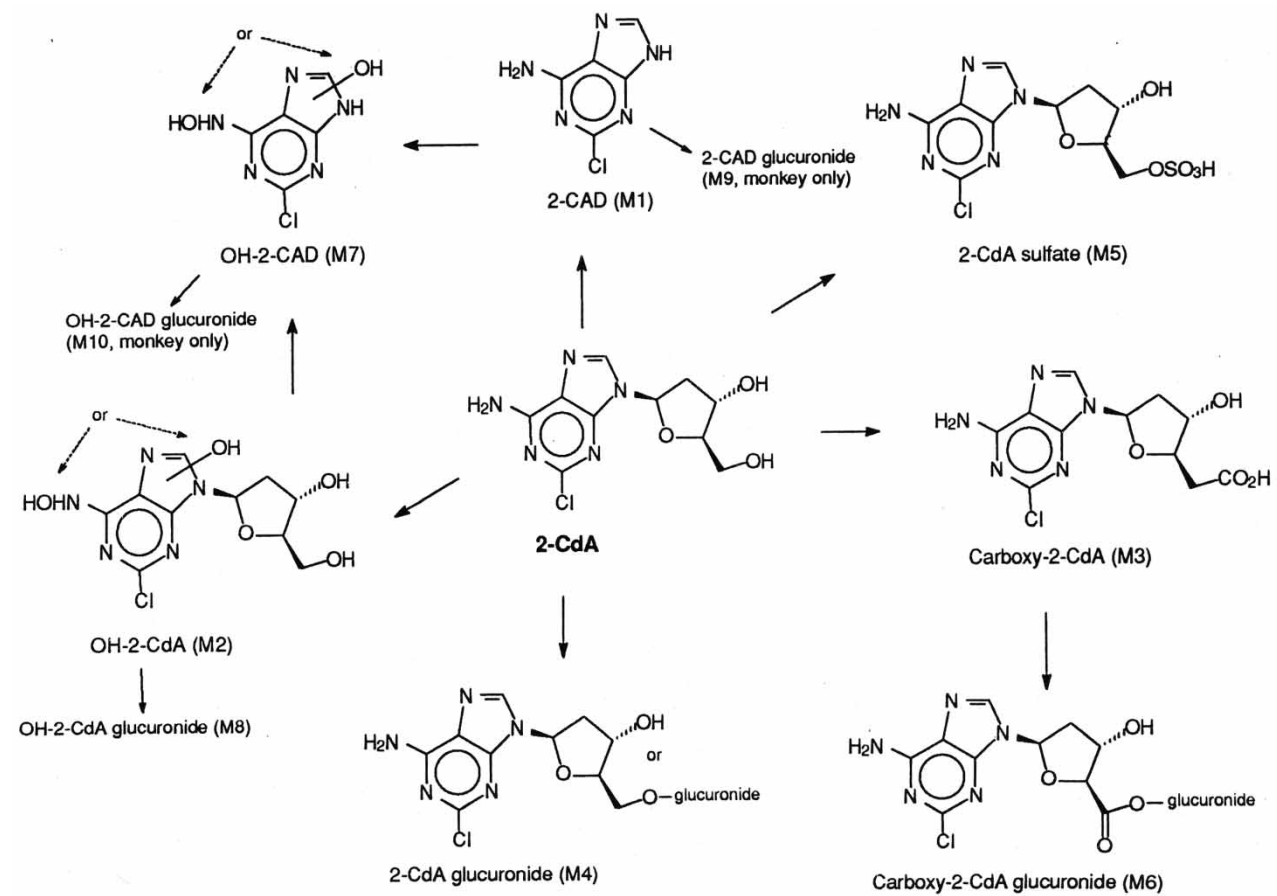


Figure 6. Proposed metabolic pathways for cladribine in mouse, rat, monkey and human.

Metabolites 8–10 (M8–10)

M8–10 were present in trace quantities in mouse and monkey urine (table 1). The MS analysis of M8–10 showed three protonated molecules (MH^+ ions) at m/z 478, 346 and 362 for M8, M9 and M10, respectively (figure 1), and the MS/MS analysis of each protonated metabolite displayed important product ions at m/z 461, 443, 302 ($M2H^+$), 285, 195 and 170, along with $M8H^+$ at m/z 478 (100%) for the structural assignment of M8, m/z 329, 293, 170 ($M1H^+$) and 153, together with $M9H^+$ at m/z 346 (100%) for the structure of M9, and m/z 345, 186 ($M7H^+$), 169 (100%) and 151, along with $M10H^+$ at m/z 362 for the structure of M10 (figure 1). Based on MS and MS/MS data, M8, M9 and M10 were tentatively identified to be M2 glucuronide, M1 glucuronide, and M7 glucuronide, respectively.

Discussion

Unchanged 2-CdA and a total of 10 metabolites consisting of four phase I metabolites (M1–3, M7) and six conjugates (five glucuronides (M4, M6, M8–10), one sulfate (M5)) were characterized and tentatively identified by means of API ionspray-MS and MS/MS data in mouse and monkey plasma, red blood cells and faeces, mouse, monkey and human urine, and rat and human liver microsomal fractions. The API ionspray-MS and MS/MS data revealed apparent protonated molecules (MH^+ ions), and prominent as well as diagnostic product ions for the structural elucidation of 2-CdA and its metabolites. Formation of these metabolites can be explained by three proposed pathways (figure 6): (A) oxidative cleavage at the adenosine-deoxyribose linkage, (B) oxidation at adenine/deoxyribose and (C) conjugation. Pathway A formed a minor metabolite, 2-chloroadenine (M1) *in vitro* and *in vivo* in all species (table 1). Pathway B produced one moderate N-oxidized metabolite, M2, and one moderate oxidized deoxyribose metabolite, M3, in human liver microsomal incubation, and animal and human urine (table 1). Pathway C along, formed two 2-CdA conjugates (glucuronide and sulfate) in minor amounts in animal and human urine (table 1), and in conjunction with pathways A and B, produced four minor or trace glucuronides. In summary, 2-CdA is not extensively metabolized *in vitro* and *in vivo* in animals and humans, however, humans appear to metabolize 2-CdA *in vivo* to a greater extent than animals. Alcohol dehydrogenase and aldehyde dehydrogenase are mainly responsible for metabolizing alcohols to carboxylic acids. Humans appear to metabolize 2-CdA to carboxy 2-CdA in a greater extent than animals. Therefore, humans seem to have more alcohol/aldehyde dehydrogenase activities than animals. The thymidine analogue zidovudine (3-azido-3'-deoxythymidine, AZT, retrovir), an HIV inhibitor, is metabolized to form AZT glucuronide as a minor metabolite in rat urine (De Miranda *et al.* 1990). It is a similar biotransformation pathway to that of 2-CdA.

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Induction of hepatic Cyp2b and Cyp3a subfamily enzymes by nicardipine and nifedipine in mice

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1. Nicardipine (Nic) or nifedipine (Nif) was given to male and female C57BL/6J mice by a single gavage at doses of 100, 200 and 400 $\mu\text{mol kg}^{-1}$, and changes in the levels of mRNA and apoprotein of hepatic cytochrome P450 (P450) enzymes, including Cyp2b9, Cyp2b10, Cyp3a11 and Cyp3a41, were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting, respectively. Furthermore, hepatic microsomal activities for pentoxifyresorufin *O*-dealkylation (PROD) and nifedipine oxidation, which are mainly mediated by Cyp2b and Cyp3a subfamily enzymes, respectively, were measured.

2. Results from RT-PCR analysis revealed that Nic, but not Nif, showed a capacity for activating the *Cyp3a11* gene in either sex of mice and that both chemicals could induce a male-selective activation of *Cyp2b10* gene, although they had no capacity for activating the *Cyp2b9* and *Cyp3a41* genes in either sex.

3. Increased levels of the mRNAs of Cyp2b10 and Cyp3a11 were closely correlated with those of apoprotein and activity of the corresponding P450 subfamily enzymes.

4. The study demonstrated for the first time that Nic, but not Nif, showed the ability to induce Cyp3a11 in both sexes of mice, although both Nif and Nic led to a male-selective induction of Cyp2b10, and that Nic and Nif had no ability to induce Cyp2b9 and Cyp3a41 in either sex.

Introduction

Calcium channel antagonists are widely used for the treatment of hypertension. However, it is known that calcium channel antagonists sometimes give rise to clinical drug–drug interactions (Schlantz *et al.* 1991, Rosenthal and Ezra 1995, Campana *et al.* 1996, Bertz and Granneman 1997). Inhibition of cytochrome P450 (P450) enzymes, especially the CYP3A subfamily, by calcium channel antagonists has been suggested as one possible explanation for such drug–drug interactions (Pichard *et al.* 1990, Ma *et al.* 2000).

Several calcium channel antagonists have been reported to show a capacity for inducing CYP2B and CYP3A subfamily enzymes in the rat liver (Zangar *et al.* 1999, Konno *et al.* 2003) and in human primary cultured hepatocytes (Drocourt *et al.* 2001). Therefore, to understand the drug–drug interactions, studies on the calcium channel antagonist-mediated P450 induction would be important.

The sex- and species-specific inductions of hepatic CYP2B and CYP3A subfamily enzymes have been reported. For example, induction of CYP2B subfamily enzymes by phenobarbital (PB) in the rat liver occurs more preferentially in males than in females (Larsen *et al.* 1994, Shapiro *et al.* 1994, Agrawal *et al.* 1996). Likewise, induction of CYP3A subfamily enzyme(s) by dexamethasone

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(DEX) occurs preferentially in males (Cooper *et al.* 1993). On the other hand, in mice, no clear sex difference is observed in the induction of CYP2B subfamily enzymes by PB (Jarukamjorn *et al.* 1999, 2001, Yamada *et al.* 2002) and in the induction of CYP3A subfamily enzymes by DEX (Sakuma *et al.* 2000). In addition, there is known to be a species specificity in the induction of CYP3A by chemicals including DEX and rifampicin (Xie *et al.* 2000, LeCluyse 2001, Gibson *et al.* 2002). DEX is a potent CYP3A inducer in rats but not in dogs and minipigs (Lu and Li 2001). On the other hand, rifampicin is a potent inducer of CYP3A in humans (Kocarek *et al.* 1995), rabbits (Lange *et al.* 1984), dogs and minipigs but not in rats (Lu and Li 2001).

Recently, the present authors demonstrated that dihydropyridine calcium channel antagonists nicardipine (Nic) and nifedipine (Nif) have the ability to induce the CYP2B and CYP3A subfamily enzymes with different selectivities in the rat liver. Namely, Nic induces CYP3A more than CYP2B subfamily enzymes, whereas Nif does the opposite (Konno *et al.* 2003, 2004, Konno and Degawa 2004). Furthermore, there is a sex difference in P450 induction (Konno *et al.* 2004). For example, Nic shows a capacity for activating the *CYP2B1* and *CYP3A9* genes only in the male rat, but hardly in females, although it shows a capacity for activating the *CYP2B2*, *CYP3A1*, *CYP3A2* and *CYP3A18* genes in either sex. On the other hand, Nif leads to a female-selective induction of the *CYP3A9* gene, although it shows a capacity for activating the *CYP2B1*, *CYP3A1* and *CYP3A2* genes in either sex.

However, studies on the species- and sex differences in calcium channel antagonist-mediated P450 induction have never been reported with the exception of previous findings in the rat (Konno *et al.* 2004). Therefore, the present study examined the induction of hepatic major Cyp2b (Cyp2b9 and Cyp2b10) and Cyp3a (Cyp3a11 and Cyp3a41) enzymes by Nic and Nif in male and female mice. It is demonstrated that Nic and Nif have the ability to induce the Cyp2b and Cyp3a subfamily enzymes with different selectivities, and for the induction of Cyp2b10 by calcium channel antagonists, there was a sex difference.

Materials and Methods

Chemicals

Nicardipine hydrochloride (Nic) and nifedipine (Nif) were purchased from Wako Pure Chemicals (Osaka, Japan). These chemicals (purity >98%) were of the highest grade available.

Treatment of animals

Male and female C57BL/6J mice (6 weeks of age) 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 or Nif dissolved in corn oil (3 ml kg⁻¹) was given to mice by a single gavage at doses of 100, 200 and 400 µmol kg⁻¹, and the mice were killed at the indicated times. In addition, control mice were treated with the vehicle (corn oil) alone.

RT-PCR analysis

Total hepatic RNA was prepared by the method of Chomczynski and Sacchi (1987) and used for the determination of expression levels of P450 genes coding for Cyp2b9, Cyp2b10, Cyp3a11 and Cyp3a41. In addition, the gene expression level of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was measured as an internal control, and no significant change in the gene expression by treatment with Nic or Nif was observed in either sex of mice. Complementary DNA synthesis from total RNA was performed as described (Konno *et al.* 2003). In brief, 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 reaction mixture (25 µl total) 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 amplification conditions used are summarized in table 1. The amount of product generated by each PCR increased linearly in a PCR cycle-dependent manner over the following ranges: 28–34 cycles for Cyp2b9 in male sample; 20–30 cycles for Cyp2b9 in female sample; 26–30 cycles for Cyp2b10; 19–27 cycles for Cyp3a11; 33–40 cycles for Cyp3a41 in male sample; 20–29 cycles for Cyp3a41 in female sample; and 20–30 cycles for GAPDH (data not shown). 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 (Machintosh 1D version 2.02) after visualization with ethidium bromide staining under ultraviolet light, and was normalized to that of the GAPDH.

Microsome preparation

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

Western blot analysis

Western blot analysis was performed by the method of Degawa *et al.* (1989). Briefly, each microsomal preparation (4 and 15 µg protein/lane for Cyp3a and Cyp2b, respectively) was solubilized with sodium dodecyl sulfate (SDS) and then subjected to 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). In addition, goat anti-rat CYP2B1 and rabbit anti-rat CYP3A2 polyclonal antibodies, which are cross-reactive with mouse Cyp2b and Cyp3a subfamily enzymes, respectively, were used in the present experiments. Amounts of the Cyp apoproteins were determined densitometrically with a computer using NIH Image Software (version 1.62).

Microsomal enzyme activities

Hepatic microsomal activity for pentoxyresorufin *O*-dealkylation (PROD) was determined by the method of Burke *et al.* (1994). Briefly, PROD was performed in a reaction medium (495 µl) containing hepatic microsomes (100 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 2 min at 37°C, the reaction was started by the addition of 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 amount of resorufin in the supernatant was measured with a spectrofluorometer at excitation (550 nm) and emission (575 nm).

Nifedipine oxidase activity was determined by the method of Bourrie *et al.* (1996). Briefly, nifedipine oxidation was performed in a 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 20 mM Nif (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 oxidized-nifedipine products in the supernatant were measured by use of an HPLC-UV detection system; column, ODS-2 (5 µm particle size); elution solvent, 60% methanol in water; flow rate, 0.8 ml min⁻¹; UV detection, 235 nm.

Statistical analysis

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

Results

Dose-effects of Nic and Nif on the gene expression of hepatic Cyp2b and Cyp3a subfamily enzymes

Effects of Nic and Nif on the gene expression of Cyp2b9, Cyp2b10, Cyp3a11 and Cyp3a41 in the mouse liver were examined by the RT-PCR with the

Table 1. PCR conditions used in the present experiments.

Target gene		Primer set	Reaction condition			Product size (bp)	Reference
			Denaturation	Annealing	Elongation		
<i>Cyp2b9</i>	forward	5'-GATGACCAGTTCCTTCATCTG-3'	94°C, 30 s	56°C, 40 s	72°C, 1 min	341	Yamada <i>et al.</i> (2002)
	reverse	5'-GCGCTTGTGGTCTCAGTTCC-3'					
<i>Cyp2b10</i>	forward	5'-GACCGTCAGTTCTTGCGCCTG-3'	94°C, 30 s	56°C, 40 s	72°C, 1 min	543	Yamada <i>et al.</i> (2002)
	reverse	5'-GGTGACTCTGTGTGGCACTC-3'					
<i>Cyp3a11</i>	forward	5'-CGCCTCTCCTTGCTGTCACA-3'	94°C, 30 s	55°C, 40 s	72°C, 1 min	260	Yamada <i>et al.</i> (2002)
	reverse	5'-CTTTGCCTTCTGCCTCAAGT-3'					
<i>Cyp3a41</i>	forward	5'-TGCCTCTCTTTGTCATCACG-3'	94°C, 30 s	55°C, 40 s	72°C, 1 min	408	Yamada <i>et al.</i> (2002)
	reverse	5'-AGTCAACTCTTAAAAATCCCT-3'					
<i>GAPDH</i>	forward	5'-TGTGAACGGATTTGGCCGTA-3'	91°C, 1 min	48°C, 1 min	70°C, 2 min	220	Serec <i>et al.</i> (1996)
	reverse	5'-TCGCTCCTGGAAGATGGTGA-3'					

corresponding primer sets (table 1). It was determined that the gene expression levels of hepatic CYP2B1, CYP2B2, CYP3A1 and CYP3A2 increased at 24 h after treatment of male rats with either Nic or Nif (Konno *et al.* 2003). Therefore, the dose-effects of Nic and Nif on hepatic gene expression of the P450s were examined at 24 h after the treatment of mice with the calcium channel antagonist. Representative expression patterns of the P450 genes after the treatments with Nic and Nif are shown in figure 1.

Constitutive expression of the *Cyp2b9* gene was observed in female mice but hardly in males. Nic and Nif showed no capacity for activating the *Cyp2b9* gene in either male or female mice at any doses used. The constitutive expression level of *Cyp2b10* gene was about sevenfold higher in female mice than the males (figure 2). In male mice, the expression level of the *Cyp2b10* gene was significantly increased by the treatment with either Nic or Nif ($200\ \mu\text{mol kg}^{-1}$) and reached six- and eightfold over the control level at a dose of $400\ \mu\text{mol kg}^{-1}$ Nic and Nif, respectively. On the other hand, no significant increase was observed in female mice treated with Nic or Nif at any doses used. In addition, no significant change in the expression of the *GAPDH* gene by Nic and Nif occurred in either sex of mice, and constitutive expression levels of the gene in male and female mice were almost the same.

The constitutive expression level of the *Cyp3a11* gene was slightly higher ($p < 0.05$) in female than in male mice (figure 2). In male and female mice treated with Nic at doses of 100 – $400\ \mu\text{mol kg}^{-1}$, significant increases in the expression level of the *Cyp3a11* gene were observed, and their maximum expression levels were about 1.5–twofold over the sex-matched controls. On the other hand, Nif showed no capacity for activating the *Cyp3a11* gene in either male or female mice. The constitutive expression level of the *Cyp3a41* gene was observed in female mice but hardly in the males. Nic and Nif showed no capacity for activating the *Cyp3a41* gene in either male or female mice.

Time-course for the increased gene expression of Cyp2b and Cyp3a subfamily enzymes by Nic and Nif

Since treatment of mice with either Nic or Nif at a dose of $200\ \mu\text{mol kg}^{-1}$ resulted in changes in expression levels of the *Cyp2b10* and *Cyp3a11* genes (figure 1), we selected the dose of $200\ \mu\text{mol kg}^{-1}$ and further examined the time-dependent changes in the gene expression of the P450 enzymes after the treatment with either Nic or Nif (figure 3).

Treatment with Nic led to a significant increase in expression level of *Cyp2b10* gene at 24 h later in male mice, but not in the females at any time examined. Treatment with Nif also resulted in a male-selective activation of the same gene, and its expression level was increased in a time-dependent manner up to 12 h later. Thereafter, the increased level was gradually decreased, although significant increase was retained up to 48 h later.

Expression levels of the *Cyp3a11* gene in both male and female mice were increased in a time-dependent manner, at least up to 12–24 h after treatment with Nic, whereas no such increase was observed in any experimental groups of the rats treated with Nif. In addition, no significant change in the gene expression of *Cyp2b9* and *Cyp3a41* was observed in any mice treated with either Nic or Nif.

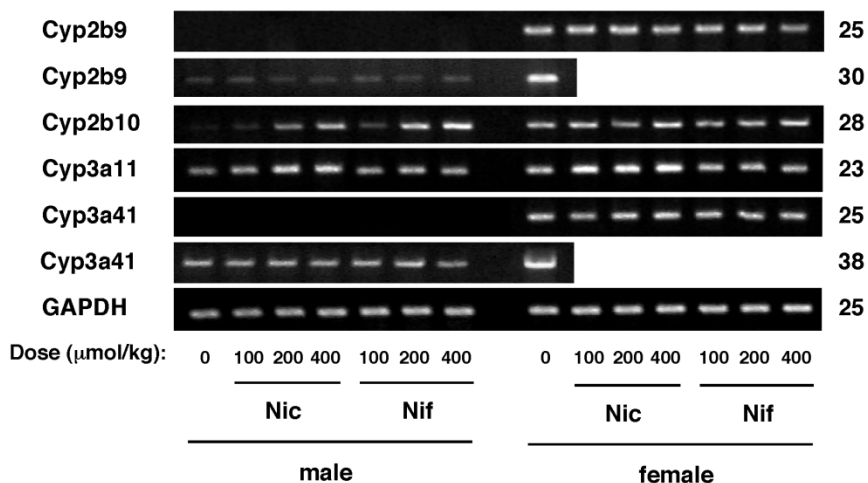


Figure 1. Representative gene expression patterns of hepatic Cyp2b and Cyp3a subfamily enzymes in Nic- and Nif-treated mice. Mice were treated orally with Nic or Nif and killed 24 h later. Total hepatic RNA was extracted from the pooled livers of three mice in each experimental group and used for the RT-PCR analysis. Each number on the right side indicates the number of cycles set used for PCR.

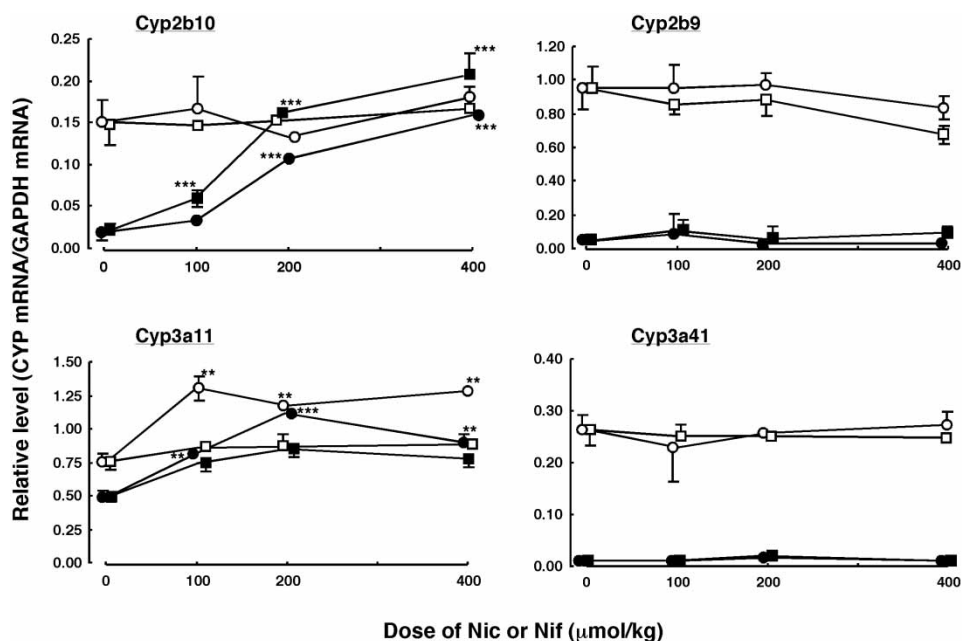


Figure 2. Dose-response effects of Nic and Nif on the gene expression of hepatic Cyp2b and Cyp3a subfamily enzymes. Mice were treated with Nic or Nif at a dose of 100, 200 or 400 $\mu\text{mol kg}^{-1}$ and killed 24 h later. Total hepatic RNA was extracted from three individual mice in each experimental group and used for RT-PCR analysis with PCR cycles as shown in figure 1. Values are represented relative to the level of the corresponding level of GAPDH mRNA; bars show standard deviations. Closed and open symbols represent male and female mice, respectively: closed and open circles, Nic treatment; closed and open squares, Nif treatment; symbol without a bar, standard deviation was included in a symbol. Significant differences from the corresponding sex-matched controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Effects of Nic and Nif on the apoprotein level of Cyp2b and Cyp3a subfamily enzymes

Time-dependent changes in the apoprotein levels of Cyp2b and Cyp3a subfamily enzymes after treatment with either Nic or Nif at a dose of $200 \mu\text{mol kg}^{-1}$ were examined by Western blot analysis, and the representative profile is shown in figure 4. Furthermore, Western blot analyses for three individual samples in each experimental group were performed, and the level of each Cyp apoprotein in each sample was determined by densitometric analysis.

The densitometric analysis revealed that constitutive expression levels of Cyp2b9 and Cyp2b10 apoproteins were 3.3- and 2.4-fold higher, respectively, in female mice than in the males. Treatment with Nic resulted in a male-selective increase in level of Cyp2b10 at 24 h later. Likewise, Nif significantly increased the expression level of Cyp2b10 at 24 h in male mice (4.0-fold increase, $p < 0.01$) and at 12 h in female mice (1.4-fold increase, $p < 0.05$). In addition, results by densitometric analysis revealed that Nic and Nif had no significant effect on the level of hepatic Cyp2b9 at any time examined in either sex.

As for Cyp3a apoprotein(s), constitutive expression level of hepatic Cyp3a apoprotein(s) was almost the same in male and female mice (figure 4). Densitometric analysis revealed that Nic treatment resulted in a significant increase (about 1.5-fold increase, $p < 0.05$) in the level of Cyp3a apoprotein(s) in both sexes of mice

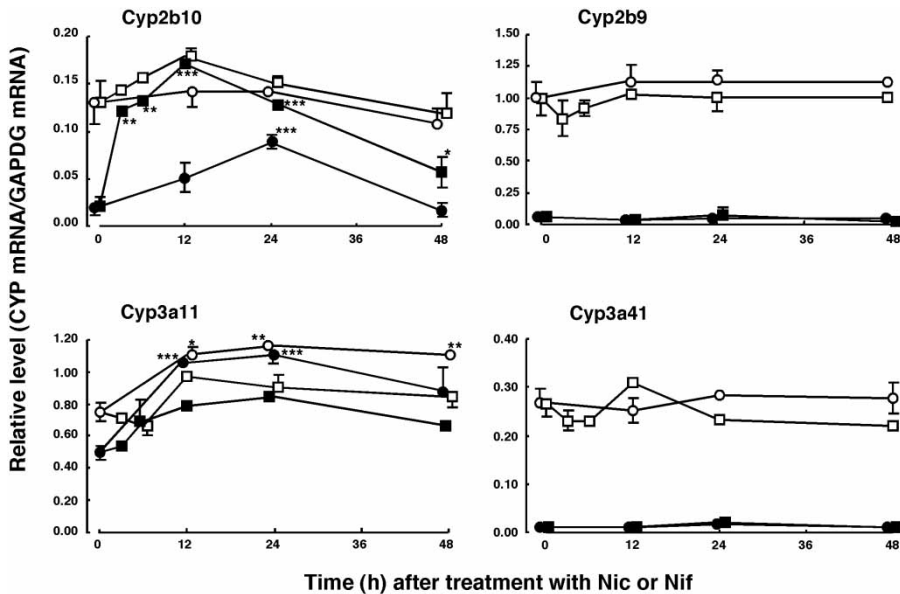


Figure 3. Time-dependent changes in the gene expression of hepatic Cyp2b and Cyp3a subfamily enzymes after the treatment of mice with Nic or Nif. Mice were treated with Nic or Nif ($200 \mu\text{mol kg}^{-1}$) or a vehicle alone (control; time 0) and killed 3, 6, 12, 24 or 48 h later. Total hepatic RNA was extracted from three individual mice in each experimental group and used for RT-PCR analysis with PCR cycles as shown in figure 1. Values are represented relative to the corresponding level of GAPDH mRNA; bars show standard deviations. Closed and open symbols represent male and female mice, respectively; closed and open circles, Nic treatment; closed and open squares, Nif treatment; symbol without a bar, standard deviation was included in a symbol. Significant differences from the corresponding sex-matched controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

at 12–48 h, whereas Nif produced a significant increase (1.4-fold increase, $p < 0.05$) at 24 h in males but hardly in females.

We further examined the dose-effects of Nic and Nif on the apoprotein level of Cyp2b and Cyp3a subfamily enzymes by the method of Western blot analysis, and the representative profile was shown in figure 5. Furthermore, Western blot analyses for three individual samples in each experimental group were performed, and the level of each Cyp apoprotein in each sample was determined by densitometric analysis.

In male mice, the apoprotein level of Cyp2b10 was increased by treatment with either Nic or Nif at doses of 200 and 400 $\mu\text{mol kg}^{-1}$, and the increased level was much higher in Nif-treated mice (about fivefold increase) than in Nic-treated mice (about 2.5-fold increase). In female mice treated with either Nic (400 $\mu\text{mol kg}^{-1}$) or Nif (100, 200 and 400 $\mu\text{mol kg}^{-1}$), about 1.3-fold increase ($p < 0.05$) in Cyp2b10 apoprotein was also observed. In addition, no significant change in apoprotein level of Cyp2b9 was observed in any mice treated with Nic or Nif.

Treatment with Nic at doses of 100, 200 and 400 $\mu\text{mol kg}^{-1}$ resulted in significant increases in the level of Cyp3a apoprotein(s) in both sexes of mice (1.4–1.9-fold increase, $p < 0.05$), and the increased levels were almost the same in male and female mice. In addition, densitometric analysis revealed that Nif showed no significant effect on the level of Cyp3a apoprotein(s) in either sex.

Increases in hepatic microsomal P450 activities by Nic and Nif

In both sexes of mice, levels of the mRNA and apoprotein responsible for Cyp2b and Cyp3a subfamily enzymes were increased by treatment with Nic or Nif at a dose of 200 $\mu\text{mol kg}^{-1}$ (figures 1–5). Therefore, hepatic microsomal activities for PROD and nifedipine oxidation, which are mainly catalysed by Cyp2b and Cyp3a subfamily enzymes, respectively (Burke *et al.* 1994, Bourrie *et al.* 1996), were further examined after treatment with Nic or Nif.

In male rats, hepatic microsomal activities for PROD were increased in a time-dependent manner up to 24 h after treatment with Nic or Nif, and their activities at 24 h later were about twofold (at Nic treatment) and fivefold (at Nif treatment) over the control activity (figure 6). On the other hand, no such increase was

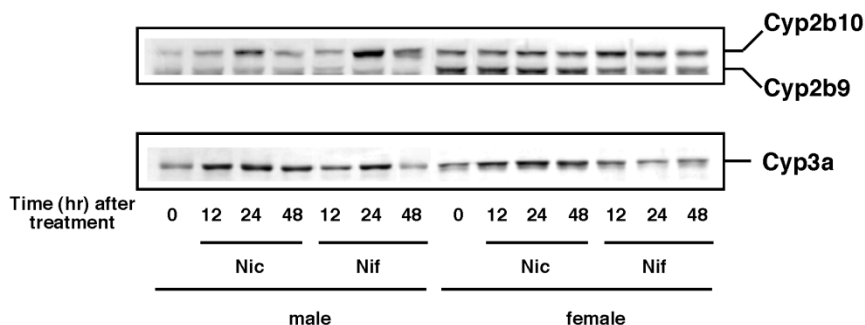


Figure 4. Time-dependent changes in the apoprotein level of hepatic Cyp2b and Cyp3a subfamily enzymes after the treatment of mice with Nic or Nif. Mice were treated with a vehicle alone (control; time 0) or Nic or Nif; 200 $\mu\text{mol kg}^{-1}$, and killed 12, 24 or 48 h later. Hepatic microsomes were prepared from the pooled livers from three mice in each experimental group and used for Western blot analysis.

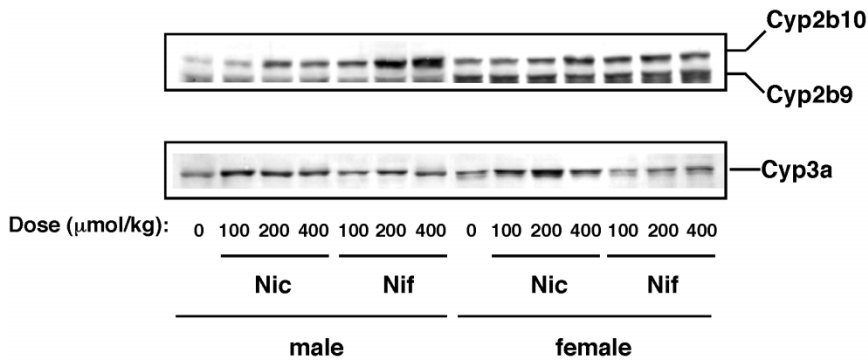


Figure 5. Dose–response effects of Nic and Nif on the apoprotein levels of hepatic Cyp2b and Cyp3a subfamily enzymes. Mice were treated with a vehicle alone or Nic or Nif at doses of 100, 200 or 400 $\mu\text{mol kg}^{-1}$ and killed 24 h later. Hepatic microsomes were prepared from the pooled livers from three mice in each experimental group and used for Western blot analysis.

observed in female mice at any times examined, although constitutive level of PROD activity was about twofold higher in females than in males.

In both sexes of mice, hepatic microsomal activities for nifedipine oxidation were increased in a time-dependent manner up to 24 h after treatment with Nic, and the increased levels were maintained up to 48 h (figure 6). In addition, no such change in the enzyme activity by Nif was observed in either sex of mice at any time examined.

Discussion

The present study demonstrated for the first time that treatment of mice with the dihydropyridine calcium channel antagonists Nic or Nif led to a male-selective activation of the hepatic *Cyp2b10* gene and that Nic also activated the *Cyp3a11* gene in both sexes of mice, whereas Nif showed no such capacity in either sex. In addition, Nic and Nif showed no capacity for activating the *Cyp2b9* and *Cyp3a41* genes. Such changes in expression of the P450 genes were closely correlated with those in the levels of the apoprotein and/or activity of the corresponding P450 subfamily enzymes.

The results from RT-PCR analysis revealed that constitutive expression levels of the *Cyp2b9*, *Cyp2b10* and *Cyp3a41* genes in mice were higher in females than in males. Thus, gender-related gene expression of these P450 enzymes is identical with that in previous reports (Jarukamjorn *et al.* 1999, 2001, Yamada *et al.* 2002). Although the constitutive expression levels of the P450 genes were generally higher in female mice than in the males, activation of the *Cyp2b10* gene by either Nic or Nif occurred in male mice but hardly in female mice. These findings suggest that calcium channel-mediated activation of the *Cyp2b10* gene may occur through a different mechanism from that for its constitutive expression. In addition, Nif showed a higher capacity for activating the *Cyp2b10* gene than Nic. Concerning the activation of the *Cyp3a11* gene, Nic induced the enzyme in both sexes of mice, while Nif did little in either sex. Thus, differences between Nic and Nif in selectivity in the induction of Cyp2b and Cyp3a subfamily enzymes are primarily identified with those in the corresponding P450 subfamily enzymes as previously reported in rats (Konno *et al.* 2003, 2004).

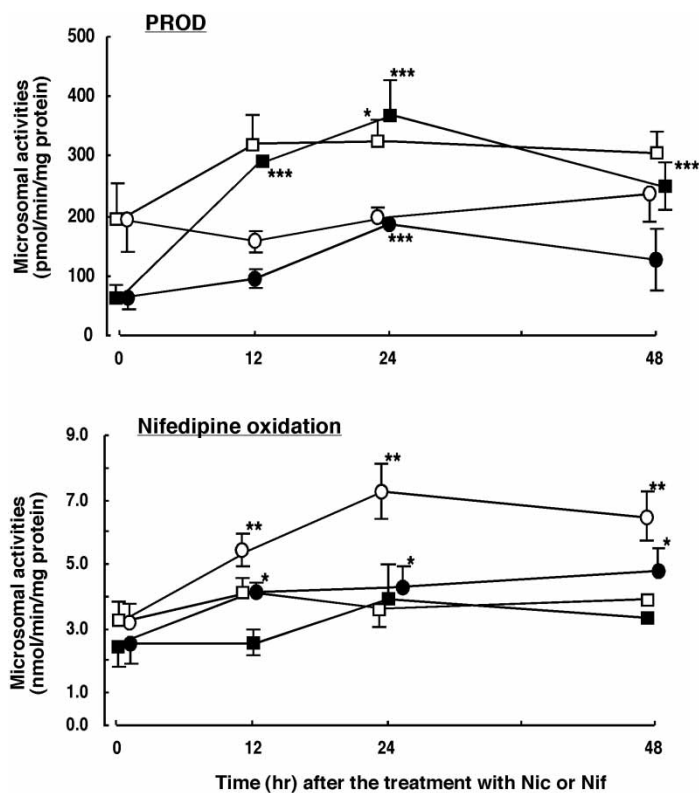


Figure 6. Time-dependent changes in hepatic microsomal P450 activities after the treatment of mice with Nic or Nif. Mice were treated with a vehicle alone (control; time 0) or Nic or Nif; $200 \mu\text{mol kg}^{-1}$ and killed at 12, 24 or 48 h later. Hepatic microsomes were prepared from three individual mice in each experimental group and were used for each enzyme assay. Values and bars represent means and standard deviations, respectively. Closed and open symbols represent male and female mice, respectively: closed and open circles, Nic treatment; closed and open squares, Nif treatment; symbol without a bar, standard deviation was included in a symbol. Significant differences from the corresponding sex-matched controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Since Nic and Nif have been demonstrated to be ligands for the pregnane X receptor (PXR), which directly regulates the transcription of *CYP2B6* and *CYP3A4* genes in human primary cultured hepatocytes (Drocourt *et al.* 2001), a PXR-dependent pathway might be considered, at least in part, as a common mechanism for the activation of the *Cyp2b10* and/or *Cyp3a11* genes by these chemicals. However, the calcium channel antagonist-mediated P450 induction does not seem to occur only through a PXR-dependent pathway, because Nic and Nif led to a male-selective activation of *Cyp2b10* gene and because Nic, but not Nif, could activate the *Cyp3a11* gene. In addition, PB and DEX, which are typical inducers of Cyp2b and Cyp3a subfamily enzymes, respectively, are known to have capacities for inducing hepatic Cyp2b10 and Cyp3a11 enzymes in both sexes of mice (Jarukamjorn *et al.* 1999, 2001, Sakuma *et al.* 2000). Accordingly, mechanisms for the activation of *Cyp2b10* and/or *Cyp3a11* genes by Nic or Nif are not necessarily the same as those for PB- and/or DEX-mediated P450 gene activation, although the mechanisms remain unclear.

In conclusion, it was demonstrated that dihydropyridine calcium channel antagonists, Nic and Nif, induce hepatic Cyp2b and Cyp3a subfamily enzymes with different selectivity and sex dependency in mice, although such calcium channel antagonist-mediated P450 induction in rats has been reported by Konno *et al.* (2003, 2004). To date, calcium channel antagonist-associating drug–drug interactions are primarily thought to occur through inhibition of P450 enzymes, especially CYP3A subfamily enzymes, by a calcium channel antagonist (Guengerich *et al.* 1986, 1991, Pichard *et al.* 1990, Ma *et al.* 2000). However, the present findings suggest that a drug–drug interaction with calcium channel antagonists might occur through not only inhibition but also induction of hepatic P450 enzymes, especially the CYP2B and CYP3A subfamily enzymes.

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Phytochemical-induced changes in gene expression of carcinogen-metabolizing enzymes in cultured human primary hepatocytes

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1. The naturally occurring compounds curcumin (CUR), 3,3'-diindolylmethane (DIM), isoxanthohumol (IXN), 8-prenylnaringenin (8PN), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) protect animals against chemically induced tumours. Putative chemoprotective mechanisms include modulated expression of hepatic biotransformation enzymes. However, few, if any, studies have used human primary cells as test models.

2. The present study investigated the effects of these phytochemicals on the expression of four carcinogenesis-relevant enzymes — cytochrome P450 (CYP)1A1 and 1A2, NAD(P)H:quinone oxidoreductase (NQO1) and glutathione *S*-transferase A1 (GSTA1) — in primary cultures of freshly isolated human hepatocytes.

3. Quantitative RT-PCR analyses demonstrated that CYP1A1 was up-regulated by PEITC and DIM in a dose-dependent manner. CYP1A2 transcription was significantly activated following DIM, IXN, 8PN and PEITC treatments. DIM exhibited a remarkably effective induction response of CYP1A1 (474-, 239- and 87-fold at 50, 25 and 10 µM, respectively) and CYP1A2 (113-, 70- and 31-fold at 50, 25 and 10 µM, respectively), that was semiquantitatively reflected in protein levels. NQO1 expression responded to PEITC (11 × at 25 µM), DIM (4.5 × at 50 µM) and SFN (5 × at 10 µM) treatments. No significant effects on GSTA1 transcription were seen.

4. The findings show novel and unexpected effects of these phytochemicals on the expression of human hepatic biotransformation enzymes that play key roles in chemical-induced carcinogenesis.

Introduction

Diet and its various components are consistently identified as among the most important 'risk factors' for cancer worldwide, yet great uncertainty remains about the relative contribution of nutritive (e.g. vitamins, calories) versus non-nutritive (e.g. phytochemicals, fibre) factors in both cancer prevention and cancer induction. Numerous studies in laboratory animals, and limited human epidemiological data, suggest that a variety of plant-derived compounds (phytochemicals) can lower the risk of developing certain types of cancer (Kelloff *et al.* 2000), although the

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precise mechanisms for such putative chemoprotective effects in humans remain uncertain.

The present study investigated six phytochemicals with putative chemopreventive effects: curcumin (CUR), 3,3'-diindolylmethane (DIM), isoxanthohumol (IXN), 8-prenylnaringenin (8PN), phenethyl isothiocyanate (PEITC), and sulforaphane (SFN). DIM, PEITC and SFN, or more precisely their naturally occurring glucosinolate precursors glucobrassicin, gluconasturtiin and glucoraphanin, respectively, are found in cruciferous vegetables such as broccoli (sprouts), cabbage, cauliflower, Brussels sprouts, turnip and watercress (Kushad *et al.* 1999, Ciska *et al.* 2000). Once the vegetables are mechanically damaged, e.g. during chewing, the biologically active compounds indole-3-carbinol (I3C), PEITC and SFN are released from the glucosinolates by the plant-derived enzyme myrosinase (Shertzer and Senft 2000). During ingestion, particularly in an acidic environment, DIM is formed by non-enzymatic self-condensation of two I3C molecules (De Kruif *et al.* 1991). IXN and 8PN are derived from xanthohumol (XN), a major flavanone in hops (Stevens *et al.* 1999a). Stevens *et al.* (1999b) reported that XN is almost completely converted to its isomer IXN and other prenylated naringenins such as 8PN during the brewing process of beer, thus making the latter compounds more relevant candidates than XN. CUR is a major yellow pigment that has been isolated from the rhizome of the plant *Curcuma longa* (Chen and Fang 1997), and thus occurs in the dietary spices turmeric and curry.

Animal experiments have shown that I3C, SFN, PEITC and CUR exert protective effects towards experimentally induced tumours from a variety of carcinogens, including aflatoxin B₁, nitrosamines, polycyclic aromatic hydrocarbons and heterocyclic aromatic amines (Zhang *et al.* 1994; for reviews, see Hecht 1995, Shertzer and Senft 2000, Lin *et al.* 2001). Furthermore, *in vitro* studies in mammalian or human cell lines demonstrated beneficial effects of all six phytochemicals towards various (geno)toxicological endpoints of several carcinogens, including oestradiol, aflatoxin B₁, benzo[a]pyrene, several nitrosamines and food-related heterocyclic aromatic amines (Hamilton and Teel 1996, Jiao *et al.* 1996, Smith *et al.* 1996, Gerhauser *et al.* 1997, Barcelo *et al.* 1998, Chen *et al.* 1998, Fares *et al.* 1998, Lee *et al.* 1999, Renwick *et al.* 1999, Henderson *et al.* 2000, Miranda *et al.* 2000a, Singletary and McDonald 2000, Bonnesen *et al.* 2001, Sharma *et al.* 2001, Bacon *et al.* 2003). Suggested mechanisms for these chemoprotective effects include (1) inhibitory effects on activation (phase I biotransformation, e.g. cytochromes P450 (CYP)) of the procarcinogen, and/or (2) enhanced detoxification (phase II biotransformation) of ultimate carcinogens (Talalay *et al.* 1995, Manson *et al.* 1997, 2000, Henderson *et al.* 2000, Miranda *et al.* 2000a, b, Lin and Lin-Shia 2001). Indeed, antioxidant response element/electrophile response element (ARE/EpRE)-dependent transcriptional activation of phase II detoxification enzymes, such as glutathione *S*-transferases (GST) and NADPH-quinone oxidoreductase (NQO1), has been implicated as a primary mechanism of chemoprevention, particularly for SFN (Talalay *et al.* 1995), DIM/I3C (Manson *et al.* 2000) and PEITC (Manson *et al.* 1997), at least in animal-derived test systems. On the other hand, inhibition of carcinogen activation may occur with some of these chemicals via direct competitive inhibition of the bioactivating enzyme(s); indeed, inhibitory effects on catalytic activities of several CYPs have been reported for IXN and 8PN (Henderson *et al.* 2000), PEITC (Nakajima *et al.* 2001) and SFN (McCarty 2001). However, repeated exposure may also result in

induction of enzyme activity via transcriptional activation. In particular DIM was found to act as a weak aryl hydrocarbon receptor (AhR) agonist at concentrations in the higher micromolar range (Chen *et al.* 1996), thus resulting in activation of CYP1A1 transcription (Chen *et al.* 1996, Sanderson *et al.* 2001), which is contradictory to the aforementioned key concept of chemoprotection, as CYP1A enzymes are implicated in the activation of numerous dietary carcinogens.

In vitro studies of CUR, DIM, IXN, 8PN, PEITC and SFN on transcriptional activation (induction) of phase I and II biotransformation enzymes have largely been limited to experimental animals and (recombinant) mammalian cell lines, although a few studies have been carried out in human tumour cell lines (Gerhauser *et al.* 1997, van Iersel *et al.* 1997, Chen *et al.* 1998, Lee *et al.* 1999, Wang *et al.* 1999, Miranda *et al.* 2000a, b, Singletary and McDonald 2000, Bonnesen *et al.* 2001, Dahl and Mulcahy, 2001, Sanderson *et al.* 2001). Very few studies have used primary human liver cells. The present authors are aware of only two studies in human hepatocytes and only SFN was examined (Maheo *et al.* 1997, Bacon *et al.* 2003).

It is well known that substrate specificity as well as transcriptional regulation of biotransformation enzymes varies dramatically among different mammalian species (Xu *et al.* 2000, Murray *et al.* 2001, Gibson *et al.* 2002), which makes the extrapolation of chemoprotective effects seen in animal studies to humans extremely difficult. The present study is the first report that uses 'healthy' human liver cells to test the direct effects of these six phytochemicals on the gene expression of selected phase I and II biotransformation enzymes. Isolated hepatocytes in primary culture, derived from five human livers rejected for transplantation, were used to investigate whether CUR, DIM, IXN, 8PN, PEITC and SFN alter the expression of CYPs 1A1 and 1A2 (phase I), as well as NQO1 and GSTA1 (phase II) since these biotransformation enzymes play key roles in the initiation stage of chemical-induced carcinogenesis for a variety of carcinogens.

Materials and methods

Isolation of human hepatocytes, culture and treatment

Human hepatocytes were isolated from five viable human livers rejected for transplantation for various reasons. Information about the hepatocyte donors is given in table 1. All human subjects' protocols were reviewed and approved by the University of Washington and the University of Pittsburgh Institutional Review Boards. Hepatocyte isolation was performed as described by Strom *et al.* (1996). Hepatocyte cultures were maintained at 37°C under 5% CO₂/95% humidified air on a rigid collagen substratum overlaid with Matrigel (Collaborative Biochemicals) in William's E media supplemented with ITS+ reagent (Collaborative Biochemicals), 2 mM L-glutamine, 0.1 µM dexamethasone, 100 µg ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin, 0.25 µg ml⁻¹ amphotericin (Fungizone). Matrigel was used, as previous work in the authors' laboratory demonstrated that Matrigel overlays facilitate the maintenance and induction of xenobiotic-metabolizing enzymes in primary human

Table 1. Hepatocyte donor information.

Donor	Age (years)	Sex	Ethnicity	Causes of death	Smoking
1	12	male	Hispanic	head trauma	non-smoker
2	57	female	African-American	ICB	smoker (45 years)
3	57	male	Caucasian	anoxia	smoker (40 years)
4	37	female	Caucasian	ICB	smoker (20 years)
5	5	male	Caucasian	head trauma	non-smoker

ICB, intracerebral bleeding.

hepatocytes (Gross-Steinmeyer *et al.* 2002), as it does in rat hepatocytes (Sidhu *et al.* 1993). Following a minimum 48 h recovery, hepatocytes were treated with IXN, 8PN and SFN in the concentration range between 4 and 50 μ M; CUR, 10, 25 and 50 μ M; DIM 10, 25 and 50 μ M; PEITC, 4, 10 and 25 μ M. DMSO concentration was 0.1% in vehicle control and treatments. Preliminary experiments established that the dose and exposure periods used were non-cytotoxic, as measured by lactate dehydrogenase leakage using a commercially available kit (Promega). Hepatocytes of donor 5 were additionally treated with concentrations of DIM at 0.1, 1 and 100 μ M; however, 100 μ M was cytotoxic.

mRNA expression analyses

Changes in mRNA expression were determined in hepatocytes from all five donors. The mRNA expression of CYPs 1A1 and 1A2, NQO1 and GSTA1 was determined by real-time RT-PCR. At 0 and 48 h after exposure, the hepatocytes were lysed on the plates, and total RNA was extracted using Trizol reagent (Invitrogen) as recommended by the supplier. The quality of the RNA preparations was assessed electrophoretically via 18S and 28S band intensities. Reverse transcription of 0.1–2 μ g total RNA using oligo-d(T)15 primer and Superscript[®] II RNaseH- (Gibco) was performed according to the manufacturer's instructions. Fluorogenic 5'-nuclease assays (TaqMan[®]) were carried out using an ABI Prism 7700 Sequence detection system (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 20 s and 62°C for 60 s. The gene-specific sequences of primer pairs and probes used in the TaqMan-assays are as follows: CYP1A1 (XM_113272): forward primer, ACCTTCCGACACTCTTCTCTTCG; reverse primer, CAGATGGGTTGACCCATAGCTTCT; probe, CTTCACCATCCCCACAG CACAACAAG. CYP1A2 (NM_000761): forward primer, CCTTCCGACACTCCTCTTCTTGTG; reverse primer, GGGATGTAGAAGCCATTCAGCG; probe, CTTCACCATCCCCACAGCA CAACAAG. NQO1 (NM_000903): forward primer, AGTGGCTCCATGTACTCTCTGCAA; reverse primer, TGGAAGCCACAGAAATGCAGAA; probe, CCACTCTGAATTGGCCAGAG AATGACATTCA. GSTA1 (M21758): forward primer, GACTCCAGTCTTATCTCCAGCTTCC; reverse primer, TGCTTCTTCTAAAGATTTCTCATCCAT; and probe, TGAAGGCCCTG AAAACCAGAATCAGCAA.

Western blot analysis

Changes in apoprotein expression were determined in hepatocytes from donor 5. Following treatment with DIM and 0.1% DMSO for 48 h, SDS-PAGE was performed with S9 fractions using the NuPage Tris/Acetate System (Invitrogen[™]), which allows for the separation of human CYP1A1 and CYP1A2 enzymes. The primary antibody was raised against rat CYP1A1 (Gentest). According to the supplier, the primary antibody is reactive to human CYP1A1 and CYP1A2 proteins, with a considerably higher sensitivity towards human CYP1A2; it does not show any cross-reactivity with other human CYPs. Immunoreactive bands were detected by enhanced chemiluminescence. Normalization was achieved via DNA determination in the initial homogenates before S9 fraction preparation using Hoechst dye #33258.

Determination of 7-ethoxyresorufin-O-deethylase (EROD) and 7-methoxyresorufin-O-demethylase (MROD) activities

Changes in the expression of enzyme activities were determined in hepatocytes from donor 5. Following treatment with DIM and 0.1% DMSO for 48 h, EROD and MROD activities were determined by adopting the procedure of Krovat *et al.* (2000) using S9 fractions of hepatocytes. Normalization was achieved via DNA determination as described above.

Statistical analyses

Statistical significance of relative mRNA expression levels (fold increase over controls) was determined by one-way analysis of variance using Dunnett's post test.

Results

Effects of phytochemicals on CYP1A1 mRNA expression

All six phytochemicals produced a significant induction of CYP1A1 mRNA expression in human hepatocytes (table 2). Both DIM and PEITC showed very extensive dose-related induction responses (on average, 474- and 26-fold at their highest concentrations, respectively). However, the comparison of the expression between the beginning (T0) and end (T48) of incubation (table 3) shows that the 'constitutive' CYP1A1 mRNA levels declined, on average, eightfold during the 48-h culture incubation. Thus, although the large increase in CYP1A1 mRNA

Table 2. Effects of 8PN, IXN, CUR, PEITC, DIM and SFN on mRNA expression in human hepatocytes from five donors following 48-h treatments.

Treatment	Cytotoxicity (%)	CYP1A1	CYP1A2	NQO1	GSTA1
Control (48 h)	0.9–12.4	1.0	1.0	1.0	1.0
8PN 4 μ M	1.2–8.2	1.2 $\pm$ 0.2	1.6 $\pm$ 0.3	0.9 $\pm$ 0.1	1.2 $\pm$ 0.2
8PN 10 μ M	1.1–9.0	1.7 $\pm$ 0.2	2.3 $\pm$ 0.4*	1.6 $\pm$ 0.4	1.0 $\pm$ 0.3
8PN 25 μ M	0.8–8.3	1.8 $\pm$ 0.4*	2.9 $\pm$ 0.3**	0.8 $\pm$ 0.1	1.3 $\pm$ 0.2
8PN 50 μ M	2.4–10.9	2.2 $\pm$ 0.5*	3.0 $\pm$ 0.5**	1.4 $\pm$ 0.2	0.9 $\pm$ 0.2
IXN 4 μ M	1.3–9.0	1.1 $\pm$ 0.1	1.4 $\pm$ 0.2	1.0 $\pm$ 0.1	1.2 $\pm$ 0.2
IXN 10 μ M	1.1–8.9	1.1 $\pm$ 0.1	1.7 $\pm$ 0.2	1.2 $\pm$ 0.2	1.2 $\pm$ 0.1
IXN 25 μ M	1.4–8.9	1.4 $\pm$ 0.2*	3.0 $\pm$ 0.6**	1.1 $\pm$ 0.1	1.2 $\pm$ 0.2
IXN 50 μ M	2.4–11.4	2.1 $\pm$ 0.4**	2.2 $\pm$ 0.4	1.2 $\pm$ 0.1	0.9 $\pm$ 0.2
CUR 10 μ M	0.8–10.6	0.9 $\pm$ 0.1	2.1 $\pm$ 0.6	1.2 $\pm$ 0.1	1.2 $\pm$ 0.3
CUR 25 μ M	1.9–7.7	1.2 $\pm$ 0.2	1.7 $\pm$ 0.2	1.4 $\pm$ 0.2	1.0 $\pm$ 0.1
CUR 50 μ M	1.0–6.4	3.9 $\pm$ 1.2**	2.4 $\pm$ 0.5	2.0 $\pm$ 0.4	1.2 $\pm$ 0.2
PEITC 4 μ M	1.4–5.6	1.9 $\pm$ 0.6	3.3 $\pm$ 0.8	2.1 $\pm$ 0.3	2.1 $\pm$ 0.5
PEITC 10 μ M	1.3–6.0	3.4 $\pm$ 0.8*	4.7 $\pm$ 0.6*	3.1 $\pm$ 0.6*	1.0 $\pm$ 0.4
PEITC 25 μ M	1.4–11.8	26.2 $\pm$ 7.5**	10.4 $\pm$ 3.4**	11.2 $\pm$ 3.6**	1.4 $\pm$ 0.2
DIM 10 μ M	1.0–5.7	87.5 $\pm$ 9.6**	31.1 $\pm$ 2.2**	1.1 $\pm$ 0.1	0.9 $\pm$ 0.3
DIM 25 μ M	1.3–7.4	238.8 $\pm$ 23.7**	70.1 $\pm$ 2.8**	1.9 $\pm$ 0.3	2.1 $\pm$ 0.5
DIM 50 μ M	2.2–9.5	474.1 $\pm$ 87.6**	113.2 $\pm$ 19.0**	4.5 $\pm$ 1.6**	2.3 $\pm$ 0.6
SFN 4 μ M	1.2–7.0	0.8 $\pm$ 0.1	1.7 $\pm$ 0.5	2.7 $\pm$ 0.2	1.7 $\pm$ 0.4
SFN 10 μ M	1.0–6.2	1.0 $\pm$ 0.2	1.2 $\pm$ 0.3	5.0 $\pm$ 0.6**	2.6 $\pm$ 0.7
SFN 25 μ M	1.0–9.2	2.1 $\pm$ 0.5*	0.9 $\pm$ 0.3	4.2 $\pm$ 0.8**	1.7 $\pm$ 0.4
SFN 50 μ M	1.5–12.7	3.0 $\pm$ 0.7**	0.8 $\pm$ 0.2	3.9 $\pm$ 0.9**	0.8 $\pm$ 0.1

The range of cytotoxic effects is expressed as a percentage (100% cytotoxicity is equal to total cellular LDH content after cell lysis). mRNA expression data are means of fold increase values over controls (0.1% DMSO) and corresponding SEM values ($n=5$). * $0.01 < p < 0.05$; ** $p < 0.01$.

Table 3. Effects of 48-h culture period on mRNA expression in human hepatocytes. mRNA expression levels normalized to beta actin mRNA from five hepatocyte preparations (donors 1–5) at the beginning (T0) and at the end (T48) of treatments are expressed as arbitrary values.

Donor	CYP1A1		CYP1A2		NQO1		GSTA1	
	To	T48	To	T48	To	T48	To	T48
1	18	3.2	57	41	0.2	0.7	70	78
2	30	2.9	638	148	4.5	7.1	265	306
3	1.3	0.7	4.0	6.6	0.7	2.3	18	48
4	24	0.9	19	20	1.2	4.0	54	49
5	5.8	0.6	14	25	0.5	1.4	150	300
Mean	16	1.7	146	48	1.4	3.1	111	156
($\pm$ SEM)	(± 5)	(± 0.6)	(± 123)	(± 26)	(± 0.8)	(± 1.1)	(± 44)	(± 60)

following DIM and PEITC must represent true induction effects via transcriptional activation, the modest increase in CYP1A1 mRNA seen following 8PN, IXN, CUR and SFN could have resulted either from induction or from partial stabilization of existing constitutive message.

Effects of phytochemicals on CYP1A2 mRNA expression

Similar to CYP1A1, CYP1A2 transcription was substantially induced in a dose-dependent manner following DIM and PEITC treatments in human hepatocytes, and to a lesser extent, but significantly following IXN and 8PN treatments

(table 2). PEITC demonstrated a substantial effect, with induction responses up to 10-fold at the highest concentration. However, the average effectiveness of CYP1A2 mRNA induction by DIM was even more striking (on average 113-fold at its highest concentration). This magnitude of increase is especially remarkable because, unlike CYP1A1, the basal level of expression of CYP1A2 was substantial and not significantly altered over time in culture (table 3) which further supports the assumption that all observed CYP1A2 induction effects were directly related to phytochemical treatments.

Effects of DIM on CYP1A protein expression and enzyme activity

As mRNA induction responses of CYP1A following DIM treatment were substantial, the effects on protein levels were determined in hepatocytes of one single donor (no. 5). DIM caused a dose-dependent induction of CYP1A2 apoprotein (as shown by Immunoblot) as well as a dose-dependent increase of CYP1A-mediated enzyme activity (as shown by MROD and EROD assays) (figure 1). The induction responses of CYP1A2 apoprotein, EROD activity (mostly attributed to CYP1A1 in humans) and MROD activity (mostly attributed to CYP1A2 in humans) reached 12-, 16- and 36-fold, respectively, at the highest dose of DIM (50 μ M). As shown in figure 2, DIM caused significant induction responses of CYP1A2 mRNA and apoprotein at very low doses in the same donor (4.7- and 7.5-fold at 0.1 and 1 μ M, respectively, on mRNA level, and 4.9-fold at 1 μ M on protein level). Immunoreactive human CYP1A1 protein was not detectable in controls or in DIM-treated cells, possibly due to the markedly lower sensitivity of the antibody towards human CYP1A1 and the substantially lower level of expression of CYP1A1 compared with CYP1A2 at the mRNA level.

Effects of phytochemicals on NQO1 mRNA expression

PEITC, DIM and SFN caused significant and dose-dependent up-regulation of NQO1 mRNA expression in human hepatocytes (table 2). PEITC was the most effective inducer of NQO1 in the study, producing an 11-fold increase of NQO1 expression at 25 μ M. Neither the hops-derived compounds IXN and 8PN nor CUR altered the NQO1 mRNA expression significantly. Table 3 shows that the level of expression of NQO1 in untreated hepatocytes did not decrease after the 48-h culture, suggesting that the increase in NQO1 mRNA seen following PEITC, DIM and SFN treatments represents true up-regulation of the *NQO1* gene.

Effects of phytochemicals on GSTA1 mRNA expression

None of the compounds tested significantly altered the expression of GSTA1 mRNA in human hepatocytes (table 2).

Discussion

The purpose of this study was to test the hypothesis that six putative chemoprotective phytochemicals commonly found in the human diet modulate the expression of genes involved in bioactivation and/or detoxification of chemical carcinogens in a *primary* human hepatic test system. The four target biotransformation genes investigated code for key functions potentially involved in early stages of chemical-induced carcinogenesis: (1) both CYP1A1 and CYP1A2 bioactivate many chemical carcinogens, including aflatoxin B₁, benzo[a]pyrene, food-

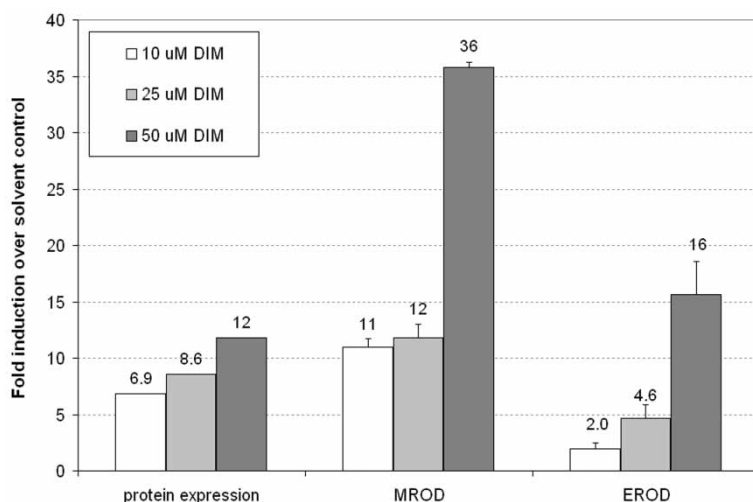
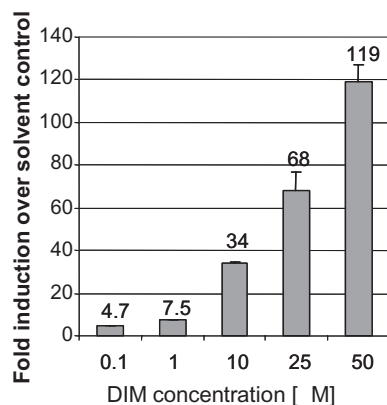


Figure 1. Effects of DIM on normalized CYP1A2 protein expression (Immunoblot) as well as normalized CYP1A-mediated enzyme activities (MROD and EROD) in human hepatocytes of a single donor (no. 5) following 48-h treatments. Values are means of fold increases over solvent controls (two replicates for immunoblots and six replicates for activity assays) and corresponding standard deviations (if $n \geq 3$).

A. CYP1A2 mRNA



B. CYP1A2 apoprotein

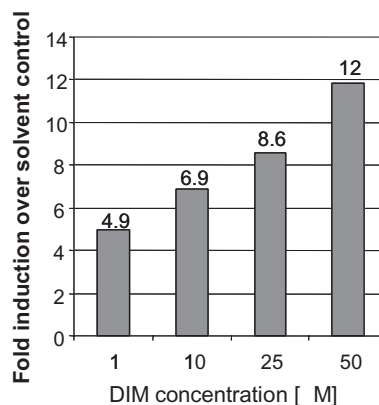


Figure 2. Effects of DIM on (A) normalized CYP1A2 mRNA (real-time RT-PCR) and (B) normalized CYP1A2 apoprotein expression (Immunoblot) in human hepatocytes of a single donor (no. 5) following 48-h treatments in a dose of 0.1–50 μ M. Values are means of fold increases over solvent controls (three replicates for RT-PCR runs and two replicates for immunoblots) and corresponding standard deviations (if $n \geq 3$).

borne heterocyclic aromatic amines, etc., (2) NQO1 is a phase II enzyme that protects cells directly and indirectly against insults of electrophiles and reactive oxygen species (Dinkova-Kostova *et al.* 2000), and (3) GSTA1 is one of the most abundant phase II enzymes in the human liver and is known to deactivate electrophiles derived from several chemical carcinogens (Eaton and Bammler 1999). Consequently, exogenous modulation of the expression levels of these enzymes that would lead to decreased activation and increased deactivation is suggested to be a key concept in chemoprevention.

Modulation of phase I enzymes

Unexpectedly large and consistent induction effects were found for both CYP1A family genes (CYP1A1 and CYP1A2). Most phytochemicals clearly increased mRNA expression of CYP1A1, CYP1A2 or both. Substantial induction responses were also observed following PEITC treatments (26- and 10-fold on average, respectively), though DIM showed even more substantial induction of both CYP1A1 and CYP1A2 in the present study (474- and 113-fold on average, respectively). Whereas the induction of CYP1A1 by DIM has already been described in several mammalian cell systems (Woertelboer *et al.* 1992, Renwick *et al.* 1999) and human cancer cell lines (Chen *et al.* 1996, Bonnesen *et al.* 2001, Sanderson *et al.* 2001), no previous study has reported such an effective up-regulation of human hepatic CYP1A2 by DIM. Lake *et al.* (1998) reported that DIM (at 50 μ M) caused a slight induction of human CYP1A2 protein in cultured human liver slices following 72-h incubation. However, a similar magnitude of CYP1A2 response as seen in the present study, in particular at lower concentrations, was not observed by that research group. In the aforementioned study, MROD activity, a typical marker of CYP1A2 activity, further was not significantly affected, even at the highest DIM concentration (50 μ M). Besides the substantial difference between the two hepatic model systems, one possible explanation for the apparent discrepancy between these two studies could be that after the incubation time of 72 h in Lake *et al.*'s study, CYP1A2 had already passed its peak of induction and, thus, the effectiveness of DIM action was not seen by Lake *et al.* (1998).

Our observation regarding SFN is consistent with Maheo *et al.* (1997), who also found no alteration in transcription levels of CYP1A2 following SFN treatments in cultured human hepatocytes. To the best of our knowledge, no previous studies have examined whether CUR, PEITC, 8PN or IXN alter expression of human CYP1A2. A previous study in murine hepatoma cells found that the hops-derived flavanones, 8PN and IXN, exhibited no induction effects of CYP1A1 (Miranda *et al.* 2000), although we found that 8PN, and to a lesser extent IXN, produced a modest dose-dependent increase in both CYP1A1 and CYP1A2 in human hepatocytes. Ciolino *et al.* (1998) observed a marked accumulation of CYP1A1 mRNA in human breast cancer cells following treatment with CUR. However, CUR caused only a slight increase of CYP1A1 transcript at the highest dose and had no effect on CYP1A2 in our study.

Modulation of phase II enzymes

Three compounds, PEITC, DIM and SFN, induced the phase II gene *NQO1*, coding for a primary cellular function of defence against reactive chemical species, and thus representing a typical marker gene for the identification of chemoprotective candidates that act through ARE-dependent pathways. Interestingly, the hops-derived flavanones 8PN and IXN as well as CUR that were previously shown to induce *NQO1* in murine hepatoma cells (Dinkova-Kostova and Talalay 1999, Miranda *et al.* 2000) did not significantly activate *NQO1* transcription in human hepatocytes. More surprisingly, none of the compounds affected the expression of *hGSTA1* mRNA under the dose and incubation conditions in this study. A previous study in human hepatocytes reported that SFN treatment in the same dose range enhanced transcription of *GSTA* after 72-h incubation (Maheo *et al.* 1997). However, as these authors did not use a specific probe for the *GSTA1*

enzyme, another representative of the human GST alpha-class family might have responded to the SFN treatment in those experiments. Furthermore, our findings were not consistent with other studies in animal models that observed alpha-class GST induction following PEITC and CUR treatments (van Lieshout *et al.* 1996, Sharma *et al.* 2001). Although numerous studies in animal models have found that alpha-class GSTs are highly inducible via ARE-dependent pathways (Pulford and Hayes 1996, Hayes *et al.* 1998), analysis of the 5' sequence of human GSTA1 failed to identify an ARE, which may explain the discrepancy of the present study to those using rodent models. There are no further experimental data yet published about the effects of DIM, 8PN and IXN on human GST expression.

DIM and PEITC act as bifunctional, DIM and SFN as monofunctional inducers

The present study suggests that the phytochemicals DIM and PEITC act as bifunctional inducers in human hepatocytes. Their activation of both phase I and II genes may occur through an AhR-dependent mechanism coupled with the xenobiotic response elements (XRE)-mediated transcription of both CYP1A1/2 and phase II genes such as NQO1 (Dinkova-Kostova and Talalay 2000, Begleiter and Lange 2002). One could think that metabolites of these compounds may have acted as 'monofunctional' inducers such that the transcriptional activation of NQO1 occurred via an ARE/EpRE-dependent mechanism (Wilkinson and Clapper 1997). However, the transcription of the modifier unit of glutamate cysteine ligase, a typical representative for ARE-dependent induction pathways (Erickson *et al.*, 2002), was only significantly activated by DIM, as observed in a different study in the present authors' laboratory (data not shown). Thus, DIM (and also SFN), but not PEITC, acted as monofunctional inducers in human hepatocytes. This type of inducer elevates phase II enzymes (that mostly catalyse detoxification reactions) selectively, usually through ARE/EpRE-related pathways, and, thus, monofunctional inducers are commonly preferred and selected as agents for achieving chemoprotection in humans (Talalay *et al.* 1995).

Relevance of CYP1A2 induction for humans

The flavanones IXN and 8PN showed a modest CYP1A2 induction, but PEITC and particularly DIM showed a remarkably effective up-regulation of CYP1A2 in human primary hepatocytes. As both PEITC and DIM are natural constituents of cruciferous vegetables, these phytochemicals may be responsible for the *in vivo* induction of CYP1A2 enzyme activity observed in humans after consumption of defined diets consisting of selected vegetables such as broccoli (Kall *et al.* 1996), Brussels sprouts and cabbage (Pantuck *et al.* 1997) or a variety of cruciferous vegetables (McDanell *et al.* 1992, Lampe *et al.* 2000). It is still uncertain what concentrations of these phytochemicals might be achieved in the human liver following a typical serving of the corresponding vegetable. Based on the fact that precursor glucosinolates of PEITC as well as of I3C (DIM) occur naturally in parts per million concentrations in cruciferous vegetables (Duke 1992), it is reasonable to expect that hepatic concentrations in the micromolar range could be achieved in humans *in vivo*. It has already been determined that consumption of average portions of appropriate vegetables can result in the release of tens of milligrams of isothiocyanates (Hecht 1995), e.g. a minimum of 12 mg PEITC were released when 2 oz watercress were consumed by human subjects

(Chung *et al.* 1992, Hecht *et al.* 1995). PEITC was further tested in a phase I clinical trial in humans. It should be noted that the parent glucosinolate compounds (e.g. gluconasturtiin, the precursor to PEITC) might have biological effects distinct from the hydrolysed products. For example, Canistro *et al.* (2004) demonstrated that gluconasturtiin itself had genotoxic properties in a yeast mitotic gene conversion assay. They also found that *in vivo* administration of gluconasturtiin to mice substantially inhibited CYP activities toward a variety of marker substrates. What fraction of the effect seen in mice was a result of the hydrolysed product (PEITC) versus possible absorption of the glucosinolate was not determined. Thus, the effects of the glucosinolates in the diet of humans are complex and difficult to predict from *in vitro* studies or animal studies alone (Liebes *et al.* 2001).

The present data suggest that DIM is substantially more potent and effective inducer of CYP1A2 than PEITC, and thus might be more biologically relevant. However, the actual level of exposure of humans towards DIM is particularly difficult to ascertain, as the extent and rate of dimerization of I3C to DIM in the human stomach is unknown and may vary significantly depending upon several factors of each individual (e.g. stomach acid pH level, diet and other physiological factors). Stresser *et al.* (1995) reported that after dosing rats orally with 1 mmole I3C kg⁻¹, hepatic concentrations of DIM reached levels between 1 and 13 µM (concentrations that have been shown to be effective in the present study), although it seems implausible that humans could be exposed to I3C levels of this magnitude (1 mmole I3C kg⁻¹) through vegetable consumption alone. However, after ingestion, I3C spontaneously forms a diversity of acid condensation products in the stomach (Bjeldanes *et al.* 1991) some of which might have similar or even higher biological activities than DIM itself. This also would explain why Leibelt *et al.* (2003) found that I3C had a higher efficacy/potency than DIM regarding the *in vivo* induction of hepatic CYP1A1 and CYP1A2 in rats after 12 months when the molar doses of DIM spanned the range for the percentage of DIM found after acid condensation of I3C *in vivo* or *in vitro*. In that study, the authors presumed that DIM was bioavailable because the compound they used was described as an 'absorbable' formulation by the manufacturer. However, the actual extent of absorption was not determined. These observations, when combined with the potency data provided in the present paper, suggest that human exposure to DIM (and other I3C-derived indoles) following vegetable consumption might be sufficient to exert some modest induction effects on CYP1A2 *in vivo*. However, regular use of DIM or I3C supplements is likely to have substantially greater impacts on hepatic CYP1A2 expression than dietary exposure alone.

In addition to the diet, dietary supplements represent a highly significant route of human exposure to phytochemicals. These so-called 'nutraceuticals' are generally unregulated by the US Food and Drug Administration and are widely available to the general population. Dietary supplements containing pure I3C at 200 mg/tablet are available. An intake of 400 mg I3C supplement daily (as recommended by the supplier) together with moderate consumption of cruciferous vegetables would come much closer to the dose that Stresser *et al.* (1995) used in their animal experiment. There is also a nutraceutical on the market purported to contain pure DIM in an absorbable formulation, with a daily recommended dose of up to 200 and 400 mg for women and men, respectively. Such a high oral intake

of DIM could likely result in hepatic concentrations in the 10–100 μ M range in humans, assuming that at peak absorption 50% of the dose of DIM was distributed to the liver. Taking together all routes of human exposure to significant amounts of PEITC as well as DIM and/or I3C, it is suggested that these phytochemicals may be associated with an increased risk for chemically induced mutagenicity/carcinogenicity by those ultimate carcinogens formed through hepatic CYP1A2 activity in humans (e.g. aflatoxin B₁, nitrosamines, food-related heterocyclic aromatic amines), and thus repeated high doses (e.g. as dietary supplements) of these phytochemicals might be potentially harmful when they are co-consumed with food-related carcinogens.

Conclusion

The findings obtained from a 'healthy' human hepatic cell system provide relevant and novel insights into the unexpected effects of chemoprotective phytochemicals on human biotransformation enzymes that play key roles in early stages of chemical-induced carcinogenesis. In particular, the substantial induction response of CYP1A2, an enzyme involved in the bioactivation of numerous human carcinogens, which was mediated by DIM, raises safety risks involved in consuming dietary supplements of DIM and/or I3C. Furthermore, as there are numerous differences observed when comparing the present findings with published data from animal-derived test models as well as human tumour-derived cell systems, the present study emphasizes the importance of using *primary* human-derived test systems in the evaluation of potential chemoprotective mechanisms of relevance to humans.

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Cross-talk between xenobiotic detoxication and other signalling pathways: clinical and toxicological consequences

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1. Recent investigations on nuclear receptors and other transcription factors involved in the regulation of genes encoding xenobiotic metabolizing and transport systems reveal that xenobiotic-dependent signalling pathways are embedded in, and establish functional interactions with, a tangle of regulatory networks involving the glucocorticoid and oestrogen receptors, the hypoxia-inducible factor, the vitamin D receptor and other transcription factors/nuclear receptors controlling cholesterol/bile salt homeostasis and liver differentiation.

2. Such functional interferences provide new insight, first for understanding how xenobiotics might exert adverse effects, and second how physiopathological stimuli affect xenobiotic metabolism.

Introduction

During the evolution of species, an arsenal of gene superfamilies has been set-up in order to cope with the constant risks presented by xenobiotics. This arsenal comprises two main groups of genes. First, those encoding xenobiotic metabolizing and transport systems (XMTS), including notably the superfamily of cytochrome (CYP) P450s (CYP1–CYP3), conjugation enzymes and transporter proteins (Parkinson 1995, Stieger and Meier 1998, Muller 2000, Faber *et al.* 2003, Lee *et al.* 2003). Second, those encoding specific receptors capable of controlling the coordinated expression of XMTS in response to xenobiotics, including notably the aryl hydrocarbon receptor (AhR), a member of the Per/ARNT/Sim (PAS) family of transcription factors (Nebert 1989, Okey *et al.* 1994, Hankinson 1995), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), members of the nuclear receptor superfamily (Forman *et al.* 1998, Honkakoski *et al.* 1998, Kliewer *et al.* 1999, Waxman 1999, Zelko and Negishi 2000, Moore *et al.* 2002). Because these receptors can interact with and be activated by intracellular xenobiotics, they are often designated as xenosensors. Since PXR and CAR share common ancestors with other nuclear receptors that control endogenous metabolism including vitamin D, thyroid and steroid receptors, they have conserved the capability of interfering functionally either directly or indirectly with other signalling pathways. Similarly, the AhR can interact with other receptors or

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interfere with other signalling pathways. Thus, the xenobiotic signalling pathways appear to be embedded within a tangle of regulatory networks. Consequently, the expression of XMTS-encoding genes is regulated not only by xenobiotics, but also by numerous pathophysiological stimuli (corticoids, hormones, cytokines, bile salts, etc.). Conversely, xenobiotics appear to affect the expression of genes controlling endogenous signalling pathways. The aim of this review is to focus on the interference and cross-talk between xenosensors and transcription factors and/or nuclear receptors, and their physiological and toxicological consequences.

Basic considerations on gene transcription

The expression of eukaryotic protein-coding genes may be regulated at various levels including transcription, pre-mRNA and mRNA processing (splicing, nuclear export, degradation), protein synthesis and post-translational processing (phosphorylation, acetylation, degradation, etc.) (Beelman and Parker 1995, Curtis *et al.* 1995, St Johnston 1995, Goodbourn 1996, Pahl and Baeuerle 1996, Lee and Young 2000, Spector 2003). Regulation at the level of transcription has a substantial impact on gene expression and will therefore be the principal object of this review.

The core promoter found in all protein-coding genes contains generally within the -40 to +40 base pair (bp) region, the transcription start site (noted as +1 for the transcription unit), a nucleotide sequence (usually TATAAAA) called the TATA box and the down-stream promoter element (Lee and Young 2000). TATA box is the site recognized by a multiprotein complex that contains the 12-subunit RNA polymerase II core protein, the general transcription factors (including TBP the TATA box-binding protein, and TFIIA to TFIIH) and several coactivators/mediators which play critical roles in the recruitment of the members of the complex, initiation and elongation of RNA transcripts (Aso *et al.* 1995, Cramer *et al.* 2001, Gnatt *et al.* 2001). This core promoter is effective for basal gene transcription by the RNA polymerase II machinery complex. Upstream of the core promoter, multiple nucleotide sequences (also called response elements) specifically recognized by DNA-binding transcription factors are generally found and form the promoter region of the gene. These notably include the CCAAT box-binding proteins, SP1, members of hepatocyte nuclear factor (HNF) family (liver-enriched transcription factors that play key roles in liver phenotype), and other nuclear receptors. The role of transcription factors is to modulate the expression of genes in response to external or intracellular stimuli (Brivanlou and Darnell 2002).

The nuclear receptor superfamily constitutes a group of ligand-dependent DNA-binding transcription factors (Mangelsdorf *et al.* 1995, Committee 1999, Giguere 1999, Francis *et al.* 2003). They regulate a broad array of physiological processes, by either activating or repressing batteries of genes that harbour specific DNA-binding motifs. In man, the superfamily comprises 48 members (Committee 1999). The activity of these receptors is controlled by the binding of small molecule ligands, generally lipophilic, including hormones, fatty acid derivatives, bile salts, oxysterols, endobiotics and xenobiotics. Nuclear receptors have to carry out at least four specific functions: ligand binding, nuclear translocation, DNA binding, and activation or repression of gene transcription. They all have a similar modular structure in which various domains are involved in the receptor

function: (1) the AF-1 transactivation domain is located in the N-terminal part of the protein; its function is ligand-independent; (2) the DNA-binding domain that consists of two highly conserved zinc finger motifs; (3) the most complex C-terminal part that consists of the ligand-binding domain, the dimerization domain and the AF-2 ligand-dependent transactivation domain. Nuclear receptors are active either as homodimers (as, for example, the glucocorticoid receptor (GR) NR3C1), heterodimers with the retinoic X receptor (RXR, NR2B1, as, for example, PXR, NR1I2) or as monomers (as, for example, LRH-1, NR5A2).

The rate of gene transcription is crucially dependent on the structure of the chromatin in which the genome is packaged (Spector 2003). Chromatin consists of nucleosomes, formed by 160 bp of DNA wrapped into a core of two molecules of each of the four histones H2A, H2B, H3 and H4, which assemble through the linker histone H1. The strong electrostatic interaction between the positively charged histones and the negatively charged DNA backbone accounts for this tight DNA–protein complex. Nucleosomes are further compacted in the chromatin fibre of solenoid shape, each turn of which contains approximately 1000 bp of DNA. In fact, chromatin is under continuous and dynamic remodelling and its physical changes play a direct role in gene expression. It is well established that in the chromosomal context, gene activation occurs mainly in relaxed chromatin (Spector 2003). This is facilitated by acetylation and methylation of the basic N-terminal tail of histones and methylation of DNA which destabilize the nucleosome and chromatin fibre, and eventually render DNA more easily accessible to DNA-binding proteins and to the basic transcriptional machinery (Jenuwein and Allis 2001, Wolffe 2001).

Transcription factors including nuclear receptors interact not only with their DNA response element and partner (i.e. RXR), but also with a number of coregulators that can modulate positively or negatively the rate of gene expression (Belandia and Parker 2003). Nuclear receptors recruit coregulators to target gene promoters, mainly through direct physical interaction with the AF-2 domain, although AF-1 may be also involved, depending on the cellular and environmental context (Hanna-Rose and Hansen 1996, Edwards 1999, Glass and Rosenfeld 2000, Stallcup 2001, Grozinger and Schreiber 2002). The activities of these coregulators include chromatin remodelling, recruitment of other coregulators and recruitment of the basic transcriptional machinery. Coregulators may be classified into two groups: coactivators and co-repressors. The recruitment of coactivators is made through the helix 12 region (AF-2) of nuclear receptors (Brzozowski *et al.* 1997, Bourguet *et al.* 2000). Coactivators possess intrinsic histone acetyltransferase or histone methyltransferase activity, or serve as docking-partners for such proteins, and are accordingly involved in chromatin relaxation, and recruitment of and association with the basic transcriptional machinery. After binding to the activated (agonist-bound) nuclear receptor, coactivators may help in the recruitment of, and act in synergy with additional coactivators, thus contributing to the full activation of expression of the target genes. Coactivators shown to interact with the nuclear receptors include the p160 family (SRC-1, TIF2, GRIP, ACTR), p/CIP and CBP/p300. Co-repressors (such as NcoR and SMRT) preferentially bind to unactivated receptors (absence of ligand, antagonist-bound, reverse agonist-bound) and recruit various forms of histone deacetylases thus leading to chromatin condensation and eventually repression of the target gene expression. Coactivators and co-repressors thus

appear to be integral parts of the signalling pathways in which they are involved (Nishioka and Reinberg 2001).

Thus, the rate of transcription is dependent first on the context of the core promoter sequence and may be modulated at the level of RNA polymerase II complex activity by activators/mediators, and second on the set of transcription factors that upon interaction with their cognate response elements present in the promoter can affect the basal transcription complex activity or recruitment or both. In addition to these response elements found in the promoter, other sequences including enhancers and silencers may influence the rate of gene transcription. These sequences whose influence is location and position independent may be located up to several tenths of kilobases upstream or downstream of the gene core promoter or even within the gene transcription unit itself (Blackwood and Kadonaga 1998).

Different genes may respond to the same transcription factor, although their promoters contain other response elements. Hence, nuclear receptors generally control the expression of a battery of genes that defines a signalling pathway. Examples of signalling pathways include the hypoxia inducible factor (HIF)-, AhR- or vitamin D receptor (VDR)-controlled genes. What turns on a particular battery of genes in a particular cell (phenotype) is therefore the unique combination of promoter response elements and the battery of transcription factors and coregulators which are expressed in that cell.

Transcription factors and nuclear receptors involved in the control of XMTS induction

Since XMTS genes are mainly expressed in the liver (Gonzalez 1992, Shimada *et al.* 1994), their basal tissue-specific regulation is dependent on transcription factors expressed at a high level particularly in the liver such as hepatocyte nuclear factors HNF1/3/4/6, C/EBP and DBP (for a recent review, see Akiyama and Gonzalez 2003). However, the expression of XMTS is known to be substantially affected by numerous xenobiotic inducers including polycyclic aromatic hydrocarbons, barbiturates and macrolid antibiotics (Pascussi *et al.* 2003b). For example, induction of XMTS is known to be responsible for drug–drug interactions and other deleterious effects in man (Lin and Lu 1998, McInnes and Brodie 1988). It is therefore not surprising that xenobiotic-mediated induction of XMTS has been the starting point of numerous and intense efforts towards the understanding of the mechanisms by which these genes are regulated. The implication of the AhR, a member of the PAS family of transcription factors, in the induction of XMTS has been known for more than 20 years (Nebert 1989, Okey *et al.* 1994, Hankinson 1995, Sogawa and Fujii-Kuriyama 1997). In contrast, the role of PXR and CAR in the induction of XMTS has only been clarified more recently (Bertilsson *et al.* 1998, Blumberg *et al.* 1998b, Kliewer *et al.* 1998, Lehmann *et al.* 1998, Sueyoshi *et al.* 1999).

Aryl hydrocarbon receptor (AhR)

AhR and its heterodimeric partner, the AhR nuclear translocator (ARNT), are members of the basic helix–loop helix (HLH) PAS family of transcription factors (Hahn 1998, Mimura and Fujii-Kuriyama 2003). Other members of this family include various genes involved in the circadian rhythm (PER, CLOCK),

the response to hypoxia (HIF1-3), neurogenesis (SIM) and some coactivators of transcription (SRC-1, TIF2). In its inactive form, AhR is located in the cytosol as a multiprotein complex including two heat shock proteins HSP90, the co-chaperone p23 and the hepatitis B virus X-associated protein 2 (Burbach *et al.* 1992, Meyer *et al.* 1998, Kazlauskas *et al.* 2001). These proteins play important roles in the AhR signalling pathway by controlling such steps as ligand binding, cellular localization, translocation to the nucleus, heterodimerization with ARNT, ubiquitination and phosphorylation of the receptor. In contrast, ARNT exhibits a strict nuclear localization, even in the absence of AhR ligands (Eguchi *et al.* 1997). Upon ligand binding, AhR translocates to the nucleus under the control of a bipartite nuclear localization signal (NLS) consisting of two sets of adjacent basic amino acids (13–39) overlapping with the DNA-binding domain of the receptor (Ikuta *et al.* 1998). In the nucleus, AhR associates with ARNT to form a functional transcription factor complex (Hankinson 1995, Sogawa and Fujii-Kuriyama 1997). This complex binds to conserved sequences of DNA termed xenobiotic responsive elements (XRE, canonic sequence: 5'-CACGCNA/T-3'), recruits several coactivators including CBP/P300 and members of the NcoA/SRC-1/pCIP family in a ligand-dependent manner and stimulates the transcription of target genes (Kobayashi *et al.* 1997, Sogawa and Fujii-Kuriyama 1997, Beischlag *et al.* 2002). After transcriptional activation of target genes, AhR is exported from the nucleus and rapidly degraded by the ubiquitin-cytoplasmic proteasome pathway. Inhibition of degradation results in increased target gene induction indicating that AhR protein concentration is an important parameter of the cell's response to the ligands (Ma 2001, Pollenz 2002, Song and Pollenz 2002).

AhR is expressed in every tissue as well as in most cell lines whatever their origin. AhR controls the induction of many XMTS including CYP1A1/2, CYP1B1, UDP-glucuronosyltransferase, glutathione *S*-transferase, aldehyde dehydrogenase, NADPH oxidoreductase, as well as other genes involved in the regulation of the cell cycle and differentiation (Nebert 1989, Nebert and Jones 1989, Hankinson 1995, Whitlock 1999, Shimada *et al.* 2002). A number of genes are also negatively regulated by AhR including the oestrogen receptor (see below) and the tissue plasminogen activator (Sogawa and Fujii-Kuriyama 1997). In addition, AhR controls the expression of the AhR repressor (AhRR) (Baba *et al.* 2001), leading to a negative regulatory loop (see below). Besides TCDD and polycyclic aromatic hydrocarbons, other possible AhR activators have been characterized including benzimidazole derivatives (Diaz *et al.* 1990, Daujat *et al.* 1996, Dzeletovic *et al.* 1997) and various tryptophan derivatives (Wei *et al.* 2000b, Nagy *et al.* 2002). Typical AhR ligands are no longer inducers of XMTS in AhR-null mice that become resistant to chemical carcinogenesis, a point consistent with the fact that CYP1s are potent activators of many procarcinogens (Fernandez-Salguero *et al.* 1997, Mimura *et al.* 1997, Gonzalez and Fernandez-Salguero 1998, Lin *et al.* 2002, Galvan *et al.* 2003). Several other phenotype changes have been reported in these mice including retarded growth, defective development of the liver and immune system, abnormal liver and kidney vascularization, and decreased fertility.

Pregnane X receptor (PXR)

In 1998, Kliewer and co-workers identified a new member of the nuclear hormone receptor family they designated PXR (NR1I3) for the pregnane X

receptor, because it was activated primarily by natural pregnanes. Interestingly, this receptor also appeared to be activated by both synthetic glucocorticoids and antiglucocorticoids, including dexamethasone and pregnenolone 16 α -carbonitrile, two well-known inducers of CYP3As. Simultaneously, two other groups isolated and characterized the same receptor designated by them as SXR (steroid and xenobiotic receptor) and PAR (pregnane activated receptor), respectively (Bertilsson *et al.* 1998, Blumberg *et al.* 1998b). Collectively, these observations established for the first time consistent mechanistic links relating CYP3A induction and a nuclear receptor activated by xenobiotics. Various orthologous forms of PXR have now been isolated from different organisms including man, monkey, pig, dog, rabbit, rodents, fish, chicken and *Xenopus laevis* indicating that the signalling pathway mediated by this xenobiotic-sensing receptor has been preserved during species evolution (Blumberg *et al.* 1998a, Lehmann *et al.* 1998, Nishikawa *et al.* 2000, Handschin *et al.* 2001). Interestingly, the chicken receptor CXR displays almost equal primary sequence identity to both human PXR and CAR (64 and 67% in the DBD and 50 and 48% in the LBD, respectively) (Handschin *et al.* 2001). In contrast to BXR that may have taken on an endogenous benzoate sensing role, CXR might represent the ancestor of genes which diverged to PXR and CAR.

PXR is a class III nuclear receptor that forms functional heterodimers with RXR α . In addition to PXR, the vitamin D receptor (hVDR) and the constitutive androstane receptor (hCAR) that exhibit 68 and 66% amino acid identity, respectively, in the DBD with hPXR, constitute the NR1I subfamily. PXR is expressed mainly in the liver, small intestine and colon as well as in the kidney (Bertilsson *et al.* 1998, Blumberg *et al.* 1998b, Kliewer *et al.* 1998, Lehmann *et al.* 1998), but is not expressed or expressed at very low level in most cell lines. Recently, PXR has been shown to be retained in the hepatocyte cytosol of untreated mice, while it translocates into the nucleus after ligand (pregnenolone 16 α -carbonitrile) administration (Kawana *et al.* 2003). These authors identified a NLS that regulates this process and mapped it to the amino acid residues 66–92 within the DBD of the protein. PXR NLS appears to be a bipartite motif (similar to the one found in the AhR and other receptors and proteins) consisting of two clusters of positively charged amino acids, 17 amino acid residues apart from each other.

In contrast to classical nuclear receptors for which the ligands exhibit very high affinity (K_s in the nM range), PXR binds generally with low affinity (EC_{50} in the micromolar range) a wide variety of structurally diverse exogenous and endogenous compounds including drugs such as rifampicin, phenobarbital, steroid hormones and derivatives, and dietary compounds such as coumestrol and hyperforin (Bertilsson *et al.* 1998, Blumberg *et al.* 1998b, Lehmann *et al.* 1998, Moore *et al.* 2000a). Interestingly, the species-specificity observed in CYP3A inducibility (Maurel 1996) correlates with the species-specificity characterizing the xenobiotic-mediated activation of PXR (LeCluyse 2001). In addition, some agonists of rodent PXR are antagonists of human PXR, notably polychlorinated biphenyls (PCBs). PCBs constitute a family of persistent organic contaminants suspected to cause adverse effects in wild life and man. In rodents, PCBs bind to AhR and PXR and induce the expression of the CYP1A and 3A families. Certain highly chlorinated PCBs can antagonize the human PXR (Tabb *et al.* 2004), so that exposure to such compounds blunts the human xenobiotic response and thus inhibits the detoxication of steroids, bioactive dietary compounds and xenobiotics normally mediated

by PXR. Crystal structure analyses reveal that the ligand-binding domain of human PXR is highly hydrophobic and flexible, allowing lipophilic molecules of different size to bind in multiple orientations (Watkins *et al.* 2001, 2003). Many but not all PXR ligands are CYP3A substrates.

PXR appears to be responsible for the xenobiotic-mediated induction of a battery of genes including CYP3A4, CYP3A7, CYP2B6, CYP2C9, UGT1As, MDR1, BSEP, MRP2 and other genes involved in detoxication, including notably murine 5-aminolevulinic acid synthase ALAS-1 (Bertilsson *et al.* 1998, Blumberg *et al.* 1998b, Lehmann *et al.* 1998, Pascussi *et al.* 1999, Sueyoshi *et al.* 1999, Geick *et al.* 2001, Goodwin *et al.* 2001, Schuetz *et al.* 2001, Synold *et al.* 2001, Gerbal-Chaloin *et al.* 2002, Kast *et al.* 2002, Fraser *et al.* 2003, Rosenfeld *et al.* 2003, Xie *et al.* 2003). Two distinct PXR-responsive elements have been identified in the 5'-flanking region of human CYP3A4. A proximal response element, located at -160 consisting of an everted repeat of the nuclear receptor half-site AGGTCA separated by six nucleotides (ER6) (Lehmann *et al.* 1998), and a distal response element located between -7800 and -7200 (Goodwin *et al.* 1999). A cooperative interaction between both response elements appears to be necessary for full activation of CYP3A4 by PXR. Ligand binding induces the interaction between PXR and the coactivator protein SRC-1 (Kliwer *et al.* 1998). Studies in PXR-null mice revealed that PXR is not only a xenosensor, but also plays a role in the protective mechanism against liver injury produced by secondary bile acids. Indeed, bile acids such as lithocholic acid and 6-ketolithocholic acid are PXR ligands (Moore *et al.* 2002). PXR appears to be involved in the control of cholesterol and bile acid homeostasis in part by down-regulating CYP7A1, the enzyme controlling the rate-limiting step in bile acid biosynthesis (Staudinger *et al.* 2001a, b), and in part by up-regulating CYP3A involved in bile salt biotransformation towards excretion (Staudinger *et al.* 2001a, b, Xie *et al.* 2001). Wild-type mice pretreated with pregnenolone 16 α -carbonitrile (a potent PXR activator in rodents) are protected against lithocholic acid-induced liver injury, whereas PXR-null mice are not. More recently, it has been shown that PXR is a functional receptor of the bile acid precursors 5 β -cholestan-3 α -7 α -12 α -triol and 5 β -cholestan-3 α -7 α -12 α -25-tetrol at physiological concentrations (Dussault *et al.* 2003, Goodwin *et al.* 2003).

It can be hypothesized that PXR polymorphisms contribute to CYP3A4 and other CYP variability. Thirty-eight single nucleotide polymorphisms (SNPs) have been identified in the human PXR gene (Zhang *et al.* 2001). However, none was located in the ligand-binding site and their functional impact remains unclear. On the other hand, two variants resulting from alternative splicing were identified including T2 and T3, which exhibit 39-amino acid N-terminal extension and an internal 37 amino acid deletion, respectively (Bertilsson *et al.* 1998, Dotzlaw *et al.* 1999). The wild-type PXR and both variants are expressed in human liver and intestine and HepG2 cells, while none of the variants is detectable in Caco-2 cells. In transfection assays, T2 was as active as the wild-type PXR in transactivating CYP3A7 and UGT1A1 and 1A3 promoters, while T3 was not active (Gardner-Stephen *et al.* 2004). In addition, several variants of PXR proteins have been identified and their role in CYP3A4 expression were analysed by expression in cell lines by transfection assays (Hustert *et al.* 2001, Koyano *et al.* 2004). Some of them exhibited altered basal and/or induced transactivation of the CYP3A promoter reporter gene, including altered DNA binding. Finally, Dotzlaw *et al.* (1999)

investigated the expression of PXR mRNA and a PXR mRNA variant (T3) in both normal and neoplastic human breast tissues. PXR mRNA levels did not differ between tumours and the adjacent matched normal tissue but did vary among tumours. Interestingly, the level of PXR mRNA was significantly lower in ER+ tumours than in ER- tumours, suggesting that PXR may have a role in human breast tissues. In sum, these data suggest that PXR polymorphism may indeed play a role in the observed interindividual variability of XMTS gene expression and may be involved in rare, atypical responses to drugs or modified sensitivity to carcinogens.

Constitutive androstane receptor (CAR)

CAR (NR1I2) was originally characterized as a transactivator of retinoid acid response elements (RARE), in the absence of ligand (Baes *et al.* 1994). Negishi and co-workers were the first to demonstrate that CAR can transactivate the phenobarbital-responsive element of CYP2B genes (Trottier *et al.* 1995, Park *et al.* 1996, Honkakoski *et al.* 1998). CAR is predominantly expressed in the liver and intestine. In normal untreated hepatocytes, CAR is located in the cytosol (Kawamoto *et al.* 1999, Pascussi *et al.* 2000b). In response to activators such as phenobarbital, CAR translocates to the nucleus where it forms functional heterodimers with RXR α . However, recent work has shown that CAR may also act as a transcriptionally active monomer at the UGT1A1 promoter (Frank *et al.* 2003) suggesting therefore that both CAR-RXR heterodimers and CAR monomers can contribute to the activation of target genes. The molecular mechanism responsible for the nuclear translocation of CAR is still unclear, but is likely to involve specific phosphorylation-sensitive steps, as suggested by the interfering effect of okadaic acid, an inhibitor of protein phosphatases 1 and 2A (Kawamoto *et al.* 1999). Recently, Negishi and co-workers (Kobayashi *et al.* 2003, Yoshinari *et al.* 2003) showed that in the cytoplasm of untreated mouse hepatocytes, CAR exists as a tubulin-associated ternary complex with HSP90 and a newly identified tetrapeptide repeat (TPR) protein, designated cytoplasmic CAR retention protein (CCRP). Interestingly, under the action of phenobarbital, this complex recruits the protein phosphatase 2A. Whether PP2A activity is required for nuclear translocation is not known, although this would be consistent with the inhibition of this process by okadaic acid. A leucine-rich peptide (called xenobiotic response signal, XRS) located at position 313–319 in the C-terminal part of the LBD of the protein in α -helix 10, i.e. outside the AF2 domain, has recently been identified and shown to be involved in the nuclear translocation of CAR (Zelko *et al.* 2001). The XRS sequence present in CAR is different from the bipartite NLS sequence suspected of controlling nuclear translocation of PXR. An amino acid change in the NLS sequence of CAR (lysine 70 to serine in human, rat and mouse) might explain the weakness of this NLS in CAR (Kawana *et al.* 2003). However, the XRS motif present in PXR does not seem to be involved in nuclear translocation. The individual role of NLS and XRS in the nuclear translocation of PXR and CAR, as well as their possible functional interactions with other domains of these proteins or other partners remains therefore to be documented.

In contrast to most other nuclear receptors, CAR has a potent constitutive activity. The LBD-AF2 domain of the protein (including notably the furthest C-terminal helix 12) interacts with various coactivators including SRC-1 in a ligand-independent manner. In the course of searching for other mediators of

CAR activation, it was recently found that the peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) can act as a ligand-independent coactivator of CAR (Shiraki *et al.* 2003). Interaction with CAR involves both the LXXLL motif and the serine/arginine-rich domain (RS domain) of PGC-1 α and is enhanced by RXR binding. In addition, the RS domain of PGC-1 α is required for CAR localization at nuclear speckles. Although the physiological relevance of this activation requires further investigation, it reveals that CAR activation may be controlled by different coactivators. Interestingly, PGC-1 α expression in the liver is induced by starvation or fasting (Shiraki *et al.* 2003).

Some ligands of CAR such as androstenol behave as inverse agonists, i.e. upon binding they affect the protein in such a way that co-repressors instead of coactivators are recruited, and the transcriptional activity of the receptor is thereby decreased (Forman *et al.* 1998). Some of the CAR agonists like TCPOBOP can reverse the inhibition induced by the presence of inverse agonists by inducing the recruitment of the coactivator SRC-1. Interestingly, mutations designed to block ligand binding block not only the inhibitory effect of the androstanes, but also the stimulatory effect of TCPOBOP. Importantly, these mutations do not block the apparently constitutive transactivation by CAR, suggesting that this activity is truly ligand independent (Tzamelis *et al.* 2000). In contrast, oestrogen derivatives exhibit both agonist and antagonist character upon CAR binding by inducing the recruitment of both SRC-1 and NcoR. It can thus be understood why these compounds are weak CAR activators (Makinen *et al.* 2003). Recently, a new agonist of human CAR, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) has been identified (Maglich *et al.* 2003). In contrast to other CAR agonists/activators, this compound preferentially activates CAR (EC_{50} for CAR = 23 nM, compared with EC_{50} for PXR = 3 μ M), so that it may represent a useful tool for discriminating between CAR- and PXR-activated genes (see below). Some CAR activators are not ligands *in vitro*. This is notably the case for phenobarbital, which does not influence CAR-SRC-1 binding. Recent data suggest that PB-induced CAR activation is indirect and likely to trigger nuclear translocation of the receptor (Kobayashi *et al.* 2003, Yoshinari *et al.* 2003). Similarly, bilirubin, an inducer of CAR-target genes including SLC21A6, UGT1A1 and MRP2 in mouse hepatocytes, is apparently not a direct agonist of CAR, but induces the nuclear translocation of the receptor as does phenobarbital (Huang *et al.* 2003). Recently, the same authors reported that 6,7-dimethylesculetin, a compound present in a herbal preparation of Yin Chin (*Artemisia capillaris*) and three other herbs, activates CAR in primary hepatocytes from both wild-type and humanized CAR mice and accelerates bilirubin clearance *in vivo*. They were unable, however, to demonstrate direct binding of this compound to the receptor (Huang *et al.* 2004). On the other hand, the ability of a molecule to act as a ligand (determined by *in vitro* binding assays, for example) is not always sufficient to induce CAR translocation to the nucleus and induction of CAR target genes (Maglich *et al.* 2003). Thus, CAR might be activated through different mechanisms. Like PXR, CAR exhibits pronounced species-specificity of ligands, activators and inverse agonists (Moore *et al.* 2000b).

Recent data show that CAR controls phase I (CYP2B6, CYP2C9, CYP2C19, CYP3A4), phase II (UGT1A1, SULT), and transporter (SLC21A6 and MRP2) genes involved in drug metabolism and bile acid and bilirubin clearance (Sueyoshi *et al.* 1999, Sugatani *et al.* 2001, Gerbal-Chaloin *et al.* 2002, Goodwin *et al.* 2002,

Chen *et al.* 2003, Huang *et al.* 2003, Saini *et al.* 2004), as well as murine 5-aminolevulinic acid synthase ALAS-1 (Fraser *et al.* 2003) and 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (Saini *et al.* 2004). The CAR-response element in rodent CYP2B gene consists of two nuclear receptor binding sites, first termed NR1 and NR2 (Trottier *et al.* 1995, Kawamoto *et al.* 1999, Sueyoshi *et al.* 1999). Both are imperfect DR4 motifs essential for phenobarbital induction of CYP2B genes. In human CYP2B6, this element is located between -1684 and -1733 bp (Goodwin *et al.* 2001). HepG2 or HuH7 cell lines genetically modified in such a way as to constitutively express CAR exhibit measurable expression of CYP2B6 and CYP3A4 (Sueyoshi *et al.* 1999, Pascussi *et al.* 2001). However, the relatively weak response of the PBREM to PXR and CAR activation in transfection assays fails to explain the potent induction observed in primary hepatocyte cultures. Wang *et al.* (2003) identified a novel nuclear receptor response module located -8.5 kilobases in the CYP2B6 regulatory region. This CYP2B6-XREM module contains several potential PXR/CAR binding motifs. Transfection experiments suggested that both PBREM and the distal responsive element cooperate to produce a maximum activation of CYP2B6 transcription.

Finally, *in vivo* disruption of CAR in the mouse completely suppresses phenobarbital- and TCPOBOP-mediated induction of CYP2b10 and CYP3a genes as well as increased liver weight and rate of DNA synthesis in response to phenobarbital, demonstrating the pivotal role of this receptor in these processes (Wei *et al.* 2000a). Interestingly, these CAR-null mice appear to be resistant to acetaminophen toxicity (Zhang *et al.* 2002). In addition, inhibition of CAR activity by administration of the inverse agonist androstanol one hour after acetaminophen treatment blocked hepatotoxicity in wild-type but not in CAR null mice (Zhang *et al.* 2002).

Auerbach *et al.* (2003) described four in-frame splice variants of the human CAR receptor gene. The variant mRNA splice transcripts were expressed in all human livers evaluated. Molecular modelling of the splice variant proteins predicts that the structural effects are localized within the receptor LBD. Assays to assess function indicate that the variant proteins, when compared with the reference protein isoform, exhibit reduced activities with respect to DNA binding, transcriptional activation and coactivator recruitment. In addition to those described for PXR, these data suggest that CAR polymorphism may also contribute to the observed interindividual variability of XMTS gene expression and in rare and atypical responses to drugs or modified sensitivity to carcinogens.

Functional interferences between nuclear receptors and/or transcription factors

There are several possibilities for cross-talk or functional interference between nuclear receptors and/or transcription factors that might affect XMTS gene expression and, ultimately, xenobiotic metabolism and toxicity. This review will focus on the following cases: (1) nuclear receptors share ligands and/or activators; (2) nuclear receptors signal through the same response elements; (3) nuclear receptors share partners; (4) functional interactions between nuclear receptors and/or transcription factors; and (5) nuclear receptor expression is controlled by other nuclear receptors.

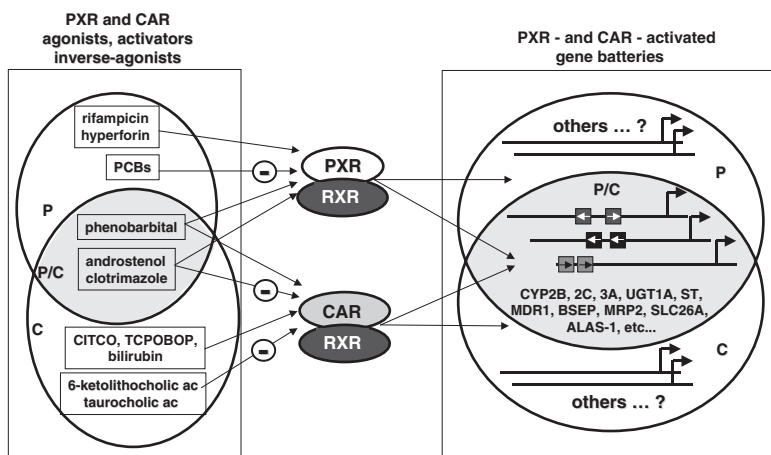


Figure 1. PXR–CAR cross-talk. The sets of PXR and CAR ligands, activators, inverse agonists (left square) and the sets of activated gene batteries (right square) are noted P and C, respectively. The intersection sets are noted P/C. For example, rifampicin belongs to the PXR set as this compound does not affect CAR; CITCO belongs to the CAR set as this compound does not affect PXR (at low concentration); phenobarbital belongs to the P/C intersection as it is an activator of both PXR and CAR. Inverse agonists are noted with a circled minus sign. For example, androstenol and clotrimazole belong to the P/C intersection as they are inverse agonists of CAR but agonists of PXR; bile salts such as 6-ketolithocholic acid or taurocholic acid are inverse agonists of CAR but not ligands of PXR. The same convention applies to the gene battery sets.

Nuclear receptors share ligands and/or activators

This is the notorious case of PXR and CAR (figure 1). PXR and CAR activate distinct but overlapping series of genes including notably CYP2B, CYP2C, CYP3A, UGT, etc. Many compounds can interact with both receptors as agonists, activators and/or inverse agonists (Moore *et al.* 2000b, Ekins *et al.* 2002). For example, phenobarbital and 5 β -pregnane-3,20 dione are activators of both CAR and PXR. On the other hand, PXR activators such as clotrimazole, androstanol and progesterone appear to be inverse agonists of CAR. Similar conclusions were reached with bile salts, a number of them including cholic acid, 12-ketolithocholic acid and 7-ketodeoxycholic acid methyl ester being activators of PXR and suppressors of CAR. It is therefore expected that the final effects of some xenobiotics, bile salts and other receptor activators on XMTS will be complex and dependent on their respective effect on PXR and CAR, and on the respective level of expression of these receptors. In this respect, the use of CITCO (Maglich *et al.* 2003) (see above) could help to differentiate the effects mediated by CAR from those mediated by PXR.

Another interesting example concerns VDR. Although the natural ligands of this receptor are the calcemic hormone vitamin D and derivatives, it was recently demonstrated that secondary bile acids including lithocholic acid and derivatives can bind to and activate VDR (Makishima *et al.* 2002). Lithocholic acid is also a ligand with low affinity for PXR (100 μ M range), whereas its affinity for VDR appears to be one order of magnitude greater (10 μ M range). Lithocholic acid-mediated VDR activation was demonstrated both *in vitro* and *in vivo* in mice by monitoring CYP3A gene expression. The functional significance of this cross-talk is as yet unknown. Similarly, some ligands of the liver X receptor (LXR) such as

5-hydroxycholesterol, 24(*S*),25-epoxycholesterol or N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1(trifluoromethyl)ethyl]-phenyl]-benzenesulfonamide (T0901317), have recently been shown to be capable of activating PXR (Shenoy *et al.* 2004). It is possible that this represents a means of preventing the accumulation of these potentially toxic endogenous molecules.

Nuclear receptors signal through the same response elements

Nuclear receptors generally bind to specific responsive elements (Mangelsdorf and Evans 1995, Mangelsdorf *et al.* 1995) although it appears that some receptors can also bind to and transactivate DNA motifs that are significantly different from their consensus responsive element, generally derived from the comparative analysis of a series of promoters of responsive genes. This has been shown to be the case for various receptors including the glucocorticoid, the oestrogen, the vitamin D and the androgen receptors (Aumais *et al.* 1996, Zhou *et al.* 1997). The apparent ability of a given nuclear receptor to target distinct DNA sequences is believed to result from the flexibility of either the DNA-binding domain, or the hinge domain, or from the association with other transcription factors or the promoter context. From a functional point of view, this versatility leads to the possibility of functional cross-talk between nuclear receptors.

For example, the PXR-response elements found in CYP3A4 are recognized and transcriptionally activated by CAR (Sueyoshi *et al.* 1999, Xie *et al.* 2001). On the other hand, the CAR-response elements of CYP2B6 and CYP2C9 are transactivated by PXR (Goodwin *et al.* 2001, Gerbal-Chaloin *et al.* 2002) (figure 1). Therefore, the differential response of CYPs and other XMTS to a given inducer may originate from either of these receptors or from both, depending on their relative abundance and affinity for the response element. In addition, these receptors compete for a common partner, RXR α , cofactors such as SCR-1, and ligands. Thus, the extent of the cross-talk between CAR and PXR is expected to be dependent on the target gene, the inducer, and on their relative expression levels. The finding that CYP proteins exhibit a broad and overlapping substrate specificity has been recognized for many years. It becomes clear now, more than 20 years later, that the nuclear receptors involved in the control of some XMTS genes similarly exhibit a broad and overlapping ligand/activator and response-element specificity. It is likely that this has represented for living organisms a potential adaptative advantage to increase their ability to detect and respond to a wide variety of potentially harmful xenobiotics.

Another example is the one involving PXR/CAR and vitamin D receptor (VDR, NR1I1) (figure 2). After vitamin D ligand binding, VDR forms a heterodimer with RXR α that transactivates vitamin D response elements (VDRE) present in the regulatory region of the target genes (Danielsson *et al.* 1997, Kato 2000). Although the consensus VDRE is an imperfect direct repeat of (G/A)GGT(G/C)A with a three-nucleotide spacer (DR3), we and others have shown that VDR/RXR α can bind to and transactivate the PXR/CAR responsive elements of CYP2B6, CYP2C9 and CYP3A4 (Thummel *et al.* 2001, Drocourt *et al.* 2002, Makishima *et al.* 2002). Interestingly, previous investigations had identified other VDRE motifs including a DR4 and an inverted palindrome IP9 (Danielsson *et al.* 1997). From a functional point of view, this is expected to generate interferences between the respective signalling pathways controlled by PXR/CAR and VDR. Although the control of CYP2/3 by VDR is not likely to be significant

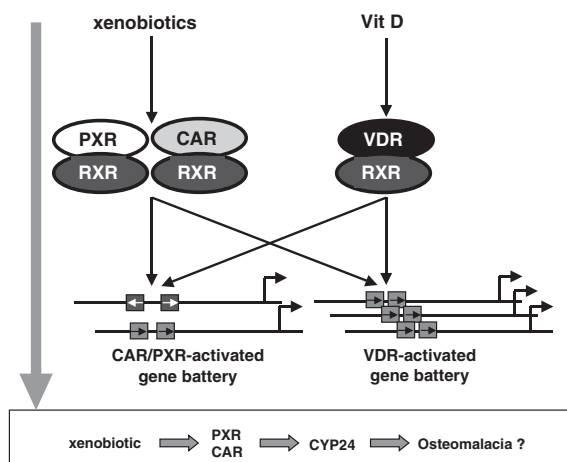


Figure 2. PXR and CAR–VDR cross-talk. It appears that PXR- and CAR-responsive elements present, for example, in CYP2B6 and CYP3A4 gene promoters are recognized and transactivated by VDR. Similarly, the VDR-responsive element present, for example, in CYP24 gene promoter is recognized and transactivated by PXR and CAR. It is possible that this cross-talk is responsible for the development of osteomalacia in patients chronically treated with PXR and CAR activators (phenobarbital, rifampicin, human immunodeficiency virus (HIV) protease inhibitors).

due to the very low blood levels of vitamin D hydroxy-derivatives, the possibility that CAR and PXR control VDR target genes while activated by xenobiotics is likely, as suggested by clinical reports. Indeed, prolonged therapy with rifampicin has been shown to cause vitamin D deficiency. In addition, rifampicin and phenobarbital are among the drugs most frequently reported to be associated with osteomalacia (bone demineralization), a disease linked to a decreased blood level of vitamin D (Brodie *et al.* 1982, Chan 1996). The major route of degradation of vitamin D is the oxidation of the side chain catalysed by vitamin D-24 hydroxylase an enzyme encoded by CYP24, a target gene of VDR (Kato 2000). We therefore predict that xenobiotics can stimulate the expression of this gene (Pascussi *et al.*, unpublished) through PXR/CAR activation, thus producing the observed deficiency in vitamin D which leads to osteomalacia. This is an interesting example of drug-mediated toxicity resulting from nuclear receptor cross-talk. This represents a real concern as such drugs are currently in use, including notably barbiturates, antibiotics, carbamazepine, isophosphamide and human immunodeficiency virus (HIV) protease inhibitors.

Nuclear receptors share partners

Several nuclear receptors including PXR, CAR, VDR, PPAR and LXR require heterodimerization with RXR α to bind DNA and activate gene transcription. Although little is currently known on the relative turnovers of these receptors when activated by their respective ligands, it is conceivable that simultaneous activation of two or more of these may result in a limiting level of RXR α and concomitant interference with one or more of these receptor-mediated pathways. To our knowledge, such a situation has not been described with respect to XMTS gene expression. However, a precedent has been observed with COUP-TFs (Cooney *et al.* 1993). COUP-TF (chicken ovalbumin upstream promoter

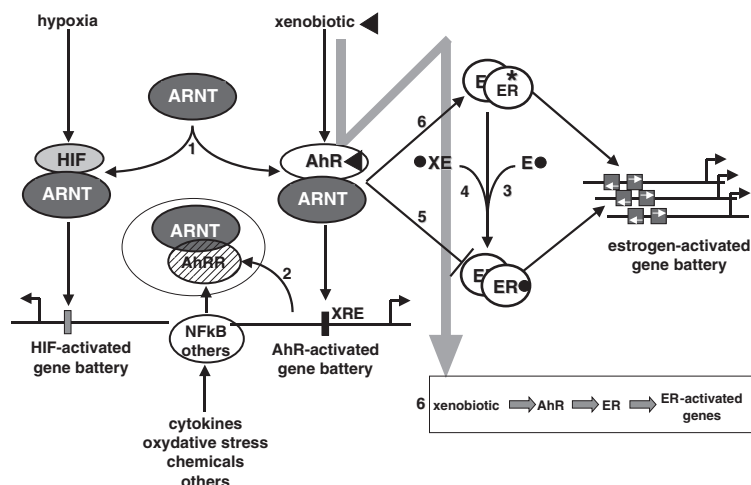


Figure 3. Cross-talk involving AhR, HIF and ER. (1) Cross-talk between AhR and HIF was based on the fact that both receptors share a same partner ARNT. (2) Sequestration of ARNT by AhRR (AhR repressor). Note that AhRR expression is positively regulated by cytokines and other stimuli through NFkB. (3) Normal pathway leading to the activation of genes by ER (oestrogen receptor) under the effect of oestrogens (E). (4) Pathway leading to the activation of genes by ER under the effect of xeno-oestrogens (XE). (5) Repression of ER-mediated oestrogen receptor-target genes by xenobiotics via activation of AhR. (6) Activation of ER-mediated oestrogen receptor-target genes by xenobiotics via activation of AhR; in this case, the heterodimer AhR-ARNT behaves as a coactivator of ER in the absence of oestrogen. As a result, xenobiotics that are ligands of AhR trigger the expression of the battery of genes normally controlled by ER.

transcription factor) designates a family of orphan nuclear receptors that are potent transrepressors of several nuclear receptors including PPAR, HNF-4, ER, VDR, TR, RAR and RXR (Achatz *et al.* 1997, Ktistaki and Talianidis 1997, Giguere 1999). Various mechanisms accounting for COUP-TFs-mediated repression have been identified including: competition for specific nuclear receptor DNA-binding sites, active repression mediated by the N- and C-terminal repression domains of the protein, recruitment of co-repressors and, interestingly, sequestering of RXR (Cooney *et al.* 1993).

An example of cross-talk within this category is the one involving AhR and the hypoxia-inducible factor 1 α (HIF-1 α) (figure 3, arrow 1). Like AhR, HIF-1 α is a member of the basic helix-loop helix PAS family of transcription factors which upon activation dimerizes with ARNT (Semenza 2001). Transcriptional activity of HIF-1 α is controlled by O₂-dependent hydroxylation of key Pro and Asn residues that affect the stability of the protein (via the N- and C-terminal transactivation domain, TAD) and its interaction with coactivators such as CBP/P300 and SRC-1, on the C-terminal transactivation domain (Ema *et al.* 1999). Under normoxia, HIF-1 α is hydroxylated and then is rapidly degraded so that it is unable to recruit coactivators. Under hypoxia, however, hydroxylation of the critical residues in both C- and N-TAD is inhibited and this leads to HIF-1 α protein stabilization, increased accumulation, translocation into the nucleus, formation of heterodimers with ARNT, binding to hypoxia responsive elements (HRE) and recruitment of coactivators. This eventually results in the activation of target genes encoding notably erythropoietin (EPO), the vascular endothelial growth factor and glycolytic enzymes. The fact that both AhR and HIF-1 α form heterodimers with a common partner, ARNT, suggests that cross-talk between both signalling pathways could

occur in such a way that hypoxia would interfere with the expression of AhR-target genes and/or that AhR activators like dioxin would interfere with the expression of genes controlled by HIF-1 α . Poellinger and co-workers (Gradin *et al.* 1996) demonstrated that the affinity of HIF-1 α for ARNT is greater than the affinity of AhR for ARNT, so that once HIF-1 α is activated, it interferes with the dioxin signalling pathway. A similar conclusion was reached by Bradfield and co-workers (Chan *et al.* 1999). In particular, these authors observed a decrease in CYP1A1 mRNA accumulation in parallel with a decrease in the level of the AhR/ARNT-DNA complex in hypoxic dioxin-treated cells with respect to normoxic dioxin-treated cells. However, and consistent with the relative affinities of HIF-1 α and AhR for ARNT, the reverse situation was not as clear, i.e. dioxin did not significantly affect the expression of HIF-1 α -responsive genes. In contrast, Pollenz *et al.* (1999) did not observe any effect due to hypoxia on the distribution and concentration of ARNT, and on the formation of the AhR/ARNT-DNA complex after cell treatment with dioxin. They concluded that the decrease in dioxin-mediated induction of CYP1A1 mRNA under hypoxia is not due to the AhR versus HIF competition but rather results either from limiting amounts of coactivators or from a toxic response of cells to the stress produced by hypoxia. The reasons for these conflicting observations are not clear but several possibilities may be proposed including, the types of cell used by the various investigators (animal versus human), the different treatments employed to produce hypoxic conditions (reduced oxygen levels versus chemically induced hypoxia), the different experimental protocols (notably the timing), and the different relative levels of expression of AhR, HIF, ARNT in the different types of cells. Whether or not functional interference between AhR and HIF occurs in normal cells (notably hepatocytes) is currently unknown and should be evaluated in the future.

In addition to the numerous genes regulated through AhR, the AhR repressor (AhRR) has recently been identified as a target of AhR (Mimura *et al.* 1999, Baba *et al.* 2001). This factor is another member of the PAS family of transcription factors, closely related to AhR. AhRR is located in the nucleus and, in contrast to AhR, binds to ARNT in a ligand-independent manner. The heterodimer AhRR-ARNT binds XRE sequences but has no transactivational activity. Thus, AhRR may exert an inhibitory action on AhR function by two mechanisms (figure 3, arrow 2): (1) sequestration of ARNT through formation of the transcriptionally inactive AhRR-ARNT complex and (2) competition of this complex with the AhR-ARNT heterodimer for DNA target sequences. Thus, the fact that AhRR is a target of AhR suggests the existence of feedback inhibition of the function of AhR. This is reminiscent of previous observations on the superinduction of CYP1A1 in response to AhR ligands in the presence of cycloheximide, a protein synthesis inhibitor, in various cellular systems (Israel *et al.* 1985). Superinduction was interpreted as reflecting the inhibition of the synthesis of a short half-life repressor protein which may well be AhRR. The regulatory region of AhRR contains a NF κ B site that functions as an inducible enhancer in response to the phorbol-ester TPA (Baba *et al.* 2001). It has been shown that NF κ B and AhR-ARNT interact with each other and, in the context of the AhRR promoter (where both responsive elements are correctly juxtaposed), these factors cooperate to enhance the expression of the gene. Interestingly, NF κ B is activated in response to various cytokines such as IL-1, TNF and IFN γ (Scheidereit 1998), which have been shown to inhibit CYP1A expression (Barker *et al.* 1992, Muntane-Relat *et al.*

1995). AhRR could therefore play a role in the negative control of *CYP1A* genes either through cytokines or through other NFkB-activating stimuli.

Functional interactions between nuclear receptors

AhR-ER cross-talk. The pleiotropic action of oestrogens, including the increase in uterine weight, DNA synthesis, progesterone receptor binding, lactoferrin expression, etc., is mediated through the oestrogen receptor (ER) family, which comprises two members, ER α (NR3A1) and ER β (NR3A2) (McDonnell and Norris 2002, Gustafsson 2003). Binding of the hormone is the first step in the classical activation of ER which proceeds through conformational changes leading to the recruitment of appropriate coactivators (notably members of the p160 family SRC-1 and TIF2, or CBP/p300) depending on the nature of the ER complex (including either homodimers of ER α or ER β or heterodimers ER α /ER β), the ligand, and the tissue (figure 3, arrow 3). Many reports have focussed on functional interference between AhR ligands such as dioxin and oestrogen-dependent processes including tumour development, as reviewed (Bock 1994, Safe and Wormke 2003). There are three ways by which xenobiotics might produce interference with oestrogenic-dependent pathways:

- Some non-steroidal man-made chemicals (named xeno-oestrogens) can mimic the action of oestrogens by binding to both ER α and ER β , albeit with a lower affinity as compared with classical ER agonists such as 17 β -oestradiol (E2). Upon binding, xeno-oestrogens affect the ability of ER α and ER β to recruit coactivators and this results in an altered capacity of these receptors to activate target genes (figure 3, arrow 4) (Routledge *et al.* 2000). In general, the affinity of xeno-oestrogens for ER subtypes does not allow the prediction of their final ability to affect coactivator recruitment.
- Negative AhR-ER cross-talk (figure 3, arrow 5). Besides the direct effect of xeno-oestrogens, the modulation by polycyclic aromatic hydrocarbons and halogenated derivatives of oestrogen-mediated pathways has been observed for many years, including both anti-oestrogenic and pro-oestrogenic-related actions (DeVito *et al.* 1992, Jana *et al.* 1999). Since these compounds are classical ligands of the AhR, cross-talk between AhR and ER has been suspected and extensively investigated. AhR ligands including dioxin and derivatives and other AhR activators inhibit several oestrogenic hormone-induced responses in the breast and the female reproductive tract. Extensive investigations have shown that this negative action may result from several mechanisms (Safe and Wormke 2003). First, increased metabolism of 17 β -oestradiol by CYP1 enzymes has been suspected; however, further investigation suggested that this is not likely to account for the cross-talk because the concentration changes in E2 were null or insufficient (Shiverick and Muther 1982). Second, it has been suspected that AhR induces the expression of a negative regulatory transcription factor, a possibility that is not yet fully established and which requires further investigations. Third, an inhibitory xenobiotic-responsive element in the promoter of ER-target genes has been identified in several genes such as cathepsin D, pS2, HSP27 and c-fos with promoter-specific mechanisms of action (Krishnan *et al.* 1995, Gillesby *et al.* 1997, Duan *et al.* 1999, Porter *et al.* 2001). Notably, competitive interactions between AhR and AP-1 for a common binding site in the promoter of pS2 have been demonstrated. Forth, AhR and ER share

common partners including nuclear factor 1, coactivators and co-repressors, and it has been shown that the concentration of these may become limiting upon coactivation of both receptors (Kumar *et al.* 1999, Ricci *et al.* 1999). Finally, a series of investigations suggest that co-exposition of cells to AhR activators and E2 results in increased rate of degradation of ER by the proteasome pathway, so that the oestrogen hormone-induced transactivation is no longer active (Wijayaratne and McDonnell 2001, Wormke *et al.* 2003).

- Positive AhR-ER cross-talk (figure 3, arrow 6). A mechanism that explains the oestrogenic action of polycyclic aromatic hydrocarbons including endometriosis and oestrogen-dependent tumours has recently been elucidated. Ohtake *et al.* (2003) demonstrated that upon activation by agonists such as dioxin or 3-methylcholanthrene, the AhR-ARNT heterodimer associates directly with ER in the absence of ligand. This interaction leads to the generation of a fully competent transcriptional unit which recruits the coactivator CBP/P300 and transactivates ER-target genes, including those involved in uterine gene homeostasis and cell proliferation. Thus, genes that are normally controlled by ER become activated by xenobiotics that are ligands of AhR.

This AhR-ER cross-talk is involved in xenobiotic-related toxic effects. Since the concentration of oestrogen hormones is known to vary with age, tissue, oestrous cycle, and pathophysiological situations, the switch between oestrogenic versus anti-oestrogenic effects of xenobiotics is expected to be dependent on these individual parameters.

Hepatocyte nuclear factor 4 α (HNF4 α ; NR2A1). HNF4 α is a member of the nuclear receptor family and is most highly expressed in the liver and intestine (Sladek *et al.* 1990). First considered as an orphan receptor, it was subsequently shown to bind fatty acyl-CoA thioesters (Hertz *et al.* 1998). This factor acts as a positive transcriptional regulator of many genes in hepatocytes and it is also known to be involved in the control of hepatocyte differentiation (Kuo *et al.* 1992). It was therefore logical to suspect the implication of HNF4 α in the control of the expression of genes involved in detoxication, including notably CYP3As (Huss and Kasper 1998, Ogino *et al.* 1999, Jover *et al.* 2001). Tirona *et al.* (2003) demonstrated that this factor is a positive modulator of the response of the CYP3A4 promoter to PXR and CAR (figure 4). In particular, they identified in the distal part of the CYP3A4 promoter a *cis*-response element (between -7783 and -7771 bp) that confers HNF4 α binding and appears to be at least in part responsible for the modulatory effect of this factor on both basal and inducible expression. The mechanism by which HNF4 α affects PXR and CAR-mediated transactivation of CYP3A4 is as yet unknown. However, since HNF4 α has been postulated as a modulator of chromatin structure (Li *et al.* 2000, Soutoglou and Talianidis 2002), it is possible that this factor acts by relieving compacted chromatin-mediated repression, thus enhancing both basal and inducible expression of CYP3A4.

Short heterodimer partner (SHP, NR0). SHP is a member of the nuclear receptor superfamily that lacks a conventional DNA-binding domain. It is mostly expressed in the liver under the control of FXR (farnesoid X receptor) (Seol *et al.* 1996, 1997). FXR is activated by primary bile acids such as cholic acid

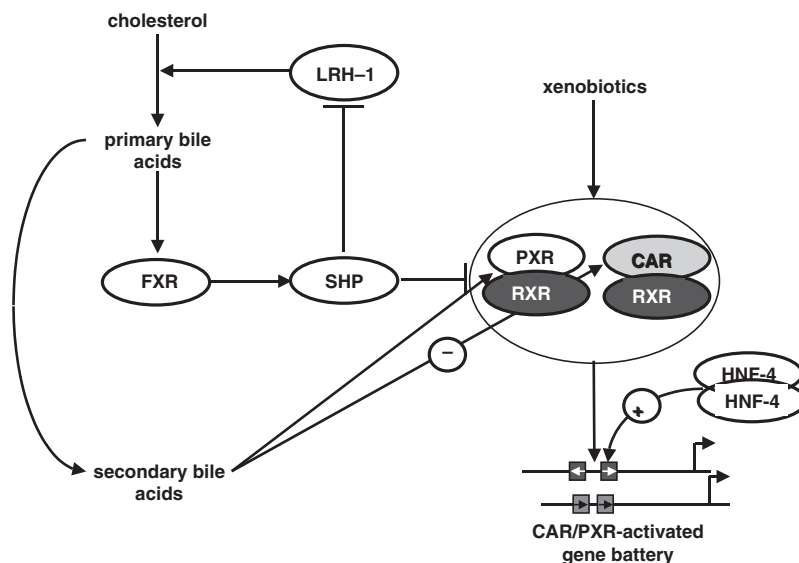


Figure 4. PXR and CAR–SHP cross-talk. Primary bile acids activate FXR, which controls SHP expression. SHP interacts with PXR and CAR (as well as with several other nuclear receptors) and inhibits their transcriptional activity. Secondary bile acids are agonists of PXR and inverse agonists of CAR. As a result, bile acids affect the expression of XMTs by interfering with both PXR and CAR activation and transcriptional activity.

and chenodeoxycholic acid (Parks *et al.* 1999). CYP7A1, the rate-limiting enzyme in bile acid biosynthesis, is known to be subjected to primary bile acid feedback regulation via SHP (Goodwin *et al.* 2000). In fact, SHP represses the expression of CYP7A1 by inhibiting the activity of liver receptor homologue 1 (LRH-1), an orphan nuclear receptor that is known to regulate CYP7A1 expression positively (figure 4). Secondary bile acid derivatives are ligands of PXR and regulate the expression of genes involved in bile acid homeostasis including CYP7A1, Oatp2 and CYP3A (Staudinger *et al.* 2001a, b, Moore *et al.* 2002). SHP has been shown to bind to and inhibit the transcriptional activity of CAR and other nuclear receptors (Seol *et al.* 1996, 1997, Johansson *et al.* 1999). Recently we demonstrated that SHP is also a negative regulator of PXR (Ourlin *et al.* 2003). Using primary hepatocytes, we observed that chenodeoxycholic acid induces SHP mRNA expression and concomitantly decreases the expression of CYP7A1 and reduces rifampicin-mediated induction of CYP3A4, CYP2B6 and CYP2C9. We showed that SHP interacts with PXR in a ligand-dependent manner and inhibits its transcriptional activity. This inhibition is reversed by SRC-1, a transcriptional coactivator of PXR (Kliewer *et al.* 1998). Whether SHP interacts directly with PXR and weakens its binding to DNA as proposed previously for RAR, or whether SHP blocks the AF-2 activation domain of PXR as proposed previously for the oestrogen receptor (Johansson *et al.* 1999) is not known. Previous observations on the modulation of CYP3A expression by bile acids and disruption of the FXR gene in the mouse support these observations (Paolini *et al.* 2000, Schuetz *et al.* 2001, Handschin *et al.* 2002). Thus, SHP interacts with and inhibits the transcriptional activity of both PXR and CAR. This is likely to generate functional interference between bile acid homeostasis and xenobiotic detoxication pathways.

Nuclear receptor expression is controlled by other nuclear receptors. Hepatocyte nuclear factor 4 α

As indicated above, HNF4 α is known to control genes that are preferentially expressed in the liver. Kamiya *et al.* (2003) investigated the expression of PXR during foetal liver development. For this purpose they used an elegant mouse foetal primary culture system in which hepatocytes are prepared either from wild-type animals or from animals expressing the floxed HNF4 α , allowing conditional knock out of this factor after infection of cells with an adenovirus containing the Cre recombinase gene. They observed that the expression of several xenobiotic responsive genes including PXR, CYP3A11, BSEP as well as HNF4 α was increased during the maturation of hepatocytes mediated by treatment of cells with oncostatin M and matrigel. Conversely, conditional knock out of HNF4 α resulted in a total loss of both basal and inducible (by pregnenolone 16 α -carbonitrile) expression of these genes. A *cis*-response element able to bind to and to be transactivated by HNF4 α was identified in the proximal promoter of PXR (between -20 and -50). Thus, HNF4 α appears to control the expression of PXR during foetal liver development. Interestingly, induction of CYP3a11 by pregnenolone 16 α -carbonitrile, as well as PXR expression is maintained in the adult HNF4 α -null mouse. Thus, HNF4 α -dependent regulation of PXR expression is specific to the foetal and perinatal stage but does not occur in the adult. In contrast, while analysing the expression of both PXR and CAR in mice after conditional knock out of HNF4 α , Tirona *et al.* (2003) observed that CAR mRNA was not expressed in the foetus of wild-type animals, but was significantly down regulated in HNF4 α -null mice. Recent results from our laboratory (Vilarem *et al.* unpublished results) also confirm that expression of human CAR in the liver is under the control of HNF4 α . The implication of HNF4 α in the control of the expression of both PXR and CAR is consistent with the fact that xenobiotic metabolizing genes are mainly expressed in the liver.

Glucocorticoid receptor. It has been known for more than 20 years that induction of CYP2B, CYP2C and CYP3A by xenobiotics such as phenobarbital and rifampicin in cultured rodent or human hepatocytes is potentiated by pretreatment of cells with dexamethasone or glucocorticoids (Pascussi *et al.* 2003b, c, *passim*). We demonstrated that this effect is related to the induction of PXR and CAR by glucocorticoids (Pascussi *et al.* 2000a, b, 2001). This was recently confirmed by the identification of a functional glucocorticoid responsive element (GRE) in the human CAR gene promoter (Pascussi *et al.* 2003a). Thus, CAR appears to be a primary glucocorticoid-responsive gene. The presence of a functional GRE in the PXR regulatory region remains to be confirmed. These observations suggest the existence of a cascade of signal transmission GR-CAR/PXR-CYP2/3 (figure 5). The existence of this cascade may have potential consequences for detoxication pathways, since any process that affects the expression and/or transcriptional activity of the glucocorticoid receptor is expected to lead to a reduced expression of PXR/CAR and eventually of CYP and other XMTS regulated by these xenoreceptors.

To verify the existence of this cascade in hepatocytes and cell lines, we tested the effect of colchicine, a compound known to affect the microtubule network (Ben-Chetrit and Levy 1998). Many intracellular signalling pathways are thought to need the microtubule network to become specific and efficient, notably the

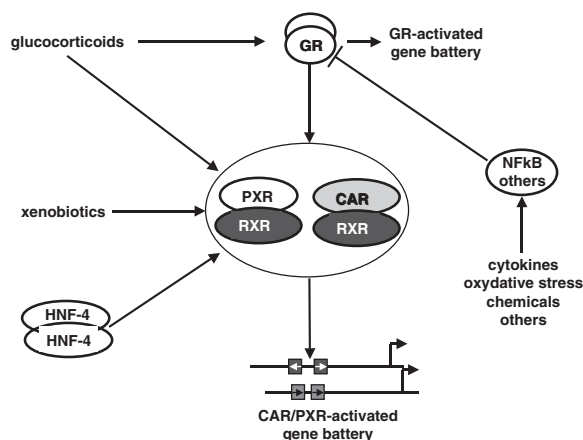


Figure 5. GR-PXR and CAR cross-talk. When activated by glucocorticoid binding, the glucocorticoid receptor (GR) controls the expression of a large battery of genes including PXR and CAR. Cytokines and other stimuli trigger NFkB activation. This factor interacts with and negatively affects GR transcriptional activity. As a result, such stimuli affect the expression of XMTS by interfering with both PXR and CAR expression.

nuclear translocation of the glucocorticoid receptor (Akner *et al.* 1995, Galigniana *et al.* 1998, Galigniana *et al.* 1999, Oren *et al.* 1999). Therefore, disruption of the microtubule network should markedly affect GR translocation to the nucleus and eventually lead to the down-regulation of the battery of genes whose transcriptional activity is GR-dependent, including notably CAR. This is indeed what we observed in colchicine- and nocodazole-treated hepatocytes (Dvorak *et al.* 2003): (1) the decrease in CYP2/3 mRNA expression was accompanied by a concomitant and extensive decrease in TAT and CAR mRNAs and a significant but more modest decrease in PXR mRNA expression; (2) neither GR mRNA expression nor dexamethasone-GR binding were affected by colchicine; (3) the dexamethasone-inducible transcriptional activity of GR in HeLa cells stably transfected with a GRE-driven reporter gene was inhibited by colchicine; and (4) the basal expression of CYP2C9, a GR primary-responsive gene (Gerbal-Chaloin *et al.* 2002), was strongly repressed by colchicine at the mRNA levels.

Many *in vivo* and *in vitro* studies in animals and man have shown that episodes of inflammation and/or infection are frequently associated with a marked decrease in the capacity of the liver to metabolize drugs and xenobiotics (Moochhala 1991), and to regulate bile secretion, as suggested by increased levels of bilirubin and bile accumulation (Hill *et al.* 1997, Trauner *et al.* 1999). Although it is recognized that cytokines and bacterial endotoxins play a critical role in the onset of this decrease (Abdel-Razzak *et al.* 1993, Khatsenko *et al.* 1997, Morgan 1997, Sewer and Morgan 1997, Li-Masters and Morgan 2001, 2002, Beigneux *et al.* 2002), the molecular mechanisms involved have not been fully elucidated. We recently showed that in primary human hepatocytes, cytokines including IL-1 β and IL-6 and lipopolysaccharides (LPS) reduce the expression of both PXR and CAR at the transcriptional level, and concomitantly reduce the expression of CYPs regulated by these receptors (Pascussi *et al.* 2000c). It is well established that activation of transcription factors such as NFkB is mediated by a number of stimuli including cytokines, oxidative stress, and chemicals (Ghosh 1999, Karin 1999). In addition,

this factor has been shown to bind to and inactivate the glucocorticoid receptor (McKay and Cidlowski 1998, Adcock and Caramori 2001). We therefore hypothesized that pro-inflammatory cytokines activate NF κ B thus leading to the inhibition of GR transcriptional activity. This in turn, triggers a reduction of PXR and CAR expression and, by cascade, of CYP2/3A and other XMTS genes, notably those involved in bilirubin homeostasis. Recent results from this laboratory are consistent with this hypothesis (Assénat *et al.* 2004): (1) transactivation of the CAR promoter by GR is down-regulated by LPS or IL-1 β , or after over-expression of the p65 RelA subunit of NF κ B; (2) the suppressive effect of LPS or IL-1 β on CAR mRNA expression in human hepatocytes is reversed by PDTC, a compound known to inhibit NF- κ B action, and by SRI κ B α , the NF- κ B super repressor; and (3) glucocorticoid-induced acetylation of H4 histone at the CAR promoter is markedly decreased by LPS or IL-1 β in primary human hepatocytes and this effect coincides with the accumulation of NF κ B in the nuclei of these cells. The mechanism by which NF κ B inhibits the transcription of CAR is unknown. Both GR and NF- κ B share transcriptional coactivators such as CBP/P300 or SRC-1 (Sheppard *et al.* 1998). It is therefore possible that NF κ B exerts its negative effect either by inhibiting the coactivator-dependent HAT activity and/or by increasing the recruitment of histone deacetylases (HDAC, an activity that in contrast to HAT favours chromatin condensation and leads to reduced gene transcription or silencing) onto the GR transactivation complex in the context of the CAR promoter. Alternatively, since both GR and NF κ B share coactivators, it is conceivable that when one pathway is activated, the other is repressed through competition for the coactivators if their availability becomes limiting. Such a mechanism as been recently shown to account for the TNF α - and LPS-mediated transrepression of AhR-induced Cyp1a1 expression by NF κ B (Ke *et al.* 2001).

Conclusion

In the most classical view of molecular toxicology, the undesirable effects of xenobiotics are primarily ascribed to the chemical reactivity of the parent compounds or to some of their metabolites produced during oxidative metabolism, mediated by CYP and other XMTS. This reactivity leads to covalent binding to proteins and DNA, effects on membrane lipids, etc. (Gregus and Klaassen 1995, Uetrecht 2003). A second aspect of the undesirable effects of xenobiotics results from the fact that these compounds may affect the level and activity of xenobiotic detoxication systems (induction, inhibition). The cross-talk described here between nuclear receptors controlling XMTS expression (such as AhR, PXR and CAR) and other nuclear receptors represent another means by which xenobiotics may exert undesirable effects. It is thus possible to predict functional interferences between the detoxication function and other signalling and metabolic pathways. In addition, these interferences provide new clues for the interpretation of the adverse effects of xenobiotics and for understanding how pathophysiological stimuli affect the organism's ability to detoxify xenobiotics.

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Differences in the metabolism and pharmacokinetics of two structurally similar PPAR agonists in dogs: involvement of taurine conjugation

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1. The metabolism and pharmacokinetics of two structurally similar PPAR agonists, MRL-I, (2*R*)-7-[3-[2-chloro-4-(4-fluorophenoxy)phenoxy]propoxy]-2-ethyl-3,4-dihydro-2*H*-benzopyran-2-carboxylic acid, and MRL-II, (2*R*)-7-[3-[2-chloro-4-(2,2,2-trifluoroethoxy)phenoxy]propoxy]-3,4-dihydro-2-methyl-2*H*-benzopyran-2-carboxylic acid, in dogs were investigated.

2. MRL-I was absorbed rapidly in dogs and exhibited linear pharmacokinetics over the dose range examined, 1–25 mg kg⁻¹. In contrast, the pharmacokinetics of MRL-II were non-linear following both intravenous and oral administration.

3. The acyl glucuronide (AG) conjugate was the only radioactive component detected in bile from dogs dosed with [¹⁴C]MRL-I, whereas bile from dogs dosed with [¹⁴C]MRL-II contained varying amounts of both the AG and taurine conjugates. The percentages of the acyl glucuronide and taurine conjugates of [¹⁴C]MRL-II in dog bile were dose dependent. A higher percentage of radioactivity was associated with the taurine conjugate (about 41%) following intravenous administration at 0.2 mg kg⁻¹ than at 0.9 mg kg⁻¹ (about 14%) or oral administration at 5 mg kg⁻¹ (about 6%). The decrease in the percentage of radioactivity associated with the taurine conjugate at 0.9 mg kg⁻¹ was accompanied by a concomitant increase in the amount of the acyl glucuronide.

4. MRL-I, but not MRL-II, was subject to significant enterohepatic recirculation in dogs. Continuous collection of bile resulted in an 11-fold decrease in the terminal half-life of MRL-I in plasma (1.5 versus 16.6 h), and a 2.4-fold increase in its plasma clearance (4.0 versus 1.7 ml min⁻¹ kg⁻¹) after intravenous administration at 1 mg kg⁻¹.

5. Collectively, the data suggest that the presence and subsequent saturation of the taurine conjugation pathway might have contributed to the non-linear pharmacokinetics of MRL-II in the dog.

Introduction

The peroxisome proliferator-activated receptors (PPARs) α , γ and δ constitute a family of nuclear hormone receptors and are ligand-dependent transcription factors (Desvergne and Wahli 1999, Kersten *et al.* 2000). The PPARs regulate target gene expression by binding to specific peroxisome proliferator response elements as a heterodimer with the retinoid X receptor. PPAR α is highly expressed in liver and plays a role in the β -oxidation of fatty acids and lipid metabolism, whereas PPAR γ is expressed mainly in adipose tissue and stimulates

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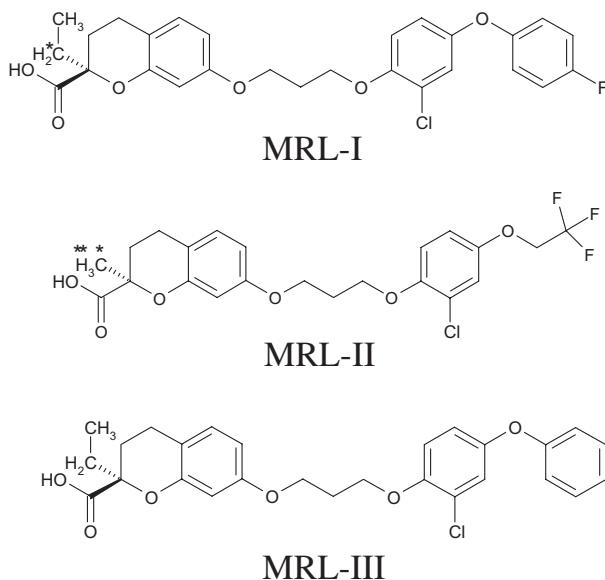


Figure 1. Structures of PPAR agonists. *Position of ^{14}C for [ethyl- ^{14}C]MRL-I and [methyl- ^{14}C]MRL-II or ^{13}C for [$^{13}\text{CD}_3$]MRL-II; **position of deuterium for [$^{13}\text{CD}_3$]MRL-II.

lipolysis of circulating triglycerides, and subsequent uptake of fatty acids into the adipose cell (Desvergne and Wahli 1999, Kersten *et al.* 2000). PPAR δ is expressed ubiquitously and has been shown to be linked to myelinogenesis and glial cell maturation in the central nervous system. It might also play a role in embryo implantation, thus affecting fertility (Berger and Moller 2002).

Synthetic agonists for both PPAR α (fibrates) and PPAR γ (thiazolidinediones: TZDs) are widely used in the treatment of dyslipidemia and diabetes, respectively, and new therapeutic potentials for PPAR ligands are emerging (Wahli *et al.* 1995, Schoonjans *et al.* 1996, Kersten *et al.* 2000, Berger and Moller 2002).

MRL-I, (2*R*)-7-[3-[2-chloro-4-(4-fluorophenoxy)phenoxy]propoxy]-2-ethyl-3,4-dihydro-2*H*-benzopyran-2-carboxylic acid; figure 1, is a PPAR α/γ dual-agonist that is structurally distinct from marketed PPAR agonists. Its plasma glucose-lowering efficacy in db/db mice is similar to that of rosiglitazone, whereas its lipid-lowering efficacy is better than that of fibrates (Koyama *et al.* 2004). MRL-II, (2*R*)-7-[3-[2-chloro-4-(2,2,2-trifluoroethoxy)phenoxy]propoxy]-3,4-dihydro-2-methyl-2*H*-benzopyran-2-carboxylic acid; figure 1, is a structural analogue of MRL-I with PPAR α -selective agonist properties. The cholesterol- and triglyceride-lowering potency of MRL-II was shown to be superior to that of fenofibrate in various *in vivo* models (Koyama *et al.* in preparation).

In the present study, the pharmacokinetics and metabolism of these two structurally similar PPAR agonists were examined in the dog.

Materials and methods

Chemicals

MRL-I, MRL-II, [$^{13}\text{CD}_3$]MRL-II, [ethyl- ^{14}C]MRL-I, [methyl- ^{14}C]MRL-II, MRL-III (figure 1), and the standards of acyl glucuronide and taurine conjugate of MRL-II, were synthesized at Merck Research Laboratories (Rahway, NJ, USA). Radiochemical purities of [ethyl- ^{14}C]MRL-I and [methyl- ^{14}C]MRL-II were $>98\%$. Alamethicin, D-saccharic acid 1,4-lactone and UDPGA were

purchased from Sigma-Aldrich (St Louis, MO, USA). Human UGT supersomes (UGTs 1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 2B7 and 2B15) were obtained from Gentest (Woburn, MA, USA). Acetonitrile and methanol (high-performance liquid chromatography (HPLC) grade) and all other reagents were obtained from Fisher Scientific (Fairlawn, NJ, USA).

Animals

Male beagle dogs, 10–13 kg, were purchased from Marshall Farm (North Rose, NY, USA). They were housed individually and had free access to water and were fed a standard laboratory canine ration (Purina Laboratory Canine Diet 5006, PMI Nutrition International, Brentwood, MO, USA) once daily. Studies were conducted under protocols approved by Merck Research Laboratories Institutional Animal Care and Use Committee.

In vivo pharmacokinetic studies

After an overnight fast, dogs were dosed with MRL-I in 0.5% aqueous methylcellulose by oral gavage at 1, 5 and 25 mg kg⁻¹. Blood (3–4 ml) was drawn from the jugular vein into heparinized containers at regular intervals up to 24 h post-dose. Different groups of dogs ($n = 3/\text{group}$) were dosed orally (1, 5 and 15 mg kg⁻¹) and intravenously (i.v.) (0.5, 1 and 2 mg kg⁻¹) with MRL-II after an overnight fast, with blood collected over 48 h. Doses were prepared using 0.9% saline. The i.v. dosing solutions were sterilized by filtration through a 0.22- μm filter (Millipore, Bedford, MA, USA).

Plasma was obtained by centrifugation at 3000 rpm for 20 min, acidified with 0.5 M formate buffer, pH 3.0 (300 μl buffer ml⁻¹ plasma), and stored at -70°C until analysis by liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS).

Quantification of MRL-I in dog plasma

Concentrations of MRL-I in dog plasma were determined by LC-MS/MS following acetonitrile precipitation. Briefly, 65- μl aliquots of acidified plasma (corresponding to 50 μl non-acidified plasma) were mixed with internal standard (12.5 ng), MRL-III, (2*R*)-7-[3-[2-chloro-4-(4-phenoxy)phenoxy]propoxy]-2-ethyl-3,4-dihydro-2*H*-benzopyran-2-carboxylic acid, followed by the addition of 400 μl acetonitrile. Samples were then spun in a centrifuge at 3000 rpm for 10 min, and supernatants were transferred by a Tomtec (Quadra 96, Model 320, Hamden, CT, USA) and subjected to LC-MS/MS analysis. The LC-MS/MS system consisted of a Perkin Elmer HPLC system and a PE Sciex API 3000 tandem mass spectrometer (Toronto, Ontario, Canada) operated in negative ionization mode using a turbo ionspray. Multiple reaction monitoring (MRM) was used for detection of the analytes. The following ion combinations (precursor $\rightarrow$ product) were used for detection: MRL-I, m/z 499.5 $\rightarrow$ 237 and MRL-III (internal standard), m/z 481.4 $\rightarrow$ 219. The HPLC column was a Beta Basic C18 (50 $\times$ 2 mm, 5 μM , Keystone Scientific, Bellefonte, PA, USA). The column was eluted with 70% A (1 mM ammonium acetate) for 0.4 min, followed by a linear gradient to 90% B (acetonitrile) in 1 min, and a 1.5-min hold at 90% B with a flow rate of 0.2 ml min⁻¹.

Quantification of MRL-II in dog plasma

Concentrations of MRL-II and two of its metabolites, the acyl glucuronide and the taurine conjugate, were determined in dog plasma by LC-MS/MS following acetonitrile precipitation. Briefly, 65- μl aliquots of acidified plasma (corresponding to 50 μl non-acidified plasma) were mixed with internal standard, [¹³CD₃]MRL-II (25 ng) followed by the addition of 500 μl acetonitrile. Samples were then spun in a centrifuge at 3000 rpm for 10 min, and supernatants were transferred by a Tomtec and evaporated to dryness under nitrogen gas at room temperature. Residues were reconstituted in 100 μl of a mixture of acetonitrile and water (30/70, v/v) containing 0.1% trifluoroacetic acid.

The LC-MS/MS system consisted of a Perkin Elmer HPLC system and a PE Sciex API 3000 tandem mass spectrometer equipped with a turbo ionspray operated in the negative ionization mode. MRM was used for detection using the following ion combinations (precursor $\rightarrow$ product): MRL-II, m/z 473.4 $\rightarrow$ 225.1, acyl glucuronide, m/z 649.6 $\rightarrow$ 473.2, taurine conjugate, m/z 579.9 $\rightarrow$ 192.1 and [¹³CD₃]MRL-II (internal standard), m/z 477.3 $\rightarrow$ 142.2. The HPLC column was a 100 $\times$ 2.1 mm ACE 5 phenyl, 5 μm (Mac-Mod, Chadds Ford, PA, USA). The mobile phase consisted of 1 mM ammonium acetate with 0.002% formic acid in water (A) or acetonitrile (B). Separation was achieved at a flow rate of 0.2 ml min⁻¹ with a linear gradient from 30 to 95% B over 4 min, a 1-min hold at 95% B.

Determination of pharmacokinetic parameters

Concentrations of the analytes were computed from the peak area ratios (relative to internal standard) using external calibration curves. The pharmacokinetic parameters were calculated using Watson software (v.6) or Microsoft Excel v.97 SR-1 for non-compartmental models.

In vivo studies in bile duct-cannulated dogs

[^{14}C]MRL-I (100 $\mu\text{Ci}/\text{animal}$) was dosed orally at 2 mg kg^{-1} and [^{14}C]MRL-II (about 350–400 $\mu\text{Ci}/\text{animal}$) was dosed orally at 2 and 5 mg kg^{-1} and i.v. at 0.2 and 0.9 mg kg^{-1} to dogs that had a T-tube type, bile catheter collection system, surgically implanted into their common bile ducts (Access Technologies, Skokie, IL, USA). Bile was diverted into the sampling cannula using a balloon occluder, and collected into a low vacuum collection bulb via a subcutaneously placed collection port. The collection bulbs contained varying amounts of 0.5 M formate buffer, pH 3.0 (as noted below), to stabilize the labile acyl glucuronide metabolite and were weighed pre- and post-collection to estimate the volume of acidified bile collected at each time interval. Bile was collected at the following time intervals: predose, 0–2, 2–4, 4–8, 8–24, 24–48 and 48–72 h. Collection bulbs contained varying volumes of 0.5 M formate buffer, pH 3.0, as follows: 5 ml for the 0–2 and 2–4-h collections, 10 ml for the 4–8-h collection and 15 ml for the 8–24, 24–48 and 48–72-h collections. Bile samples were stored frozen at -70°C until analysis. Urine was collected up to 72 h post-dose into containers containing 0.5 M formate buffer (pH 3.0) at 24-h intervals. In separate studies, the effect of continuous collection of bile on the plasma pharmacokinetics of MRL-I and MRL-II was evaluated after i.v. administration of 1 mg kg^{-1} . Blood (3–4 ml) was collected into heparinized containers by jugular venipuncture at different time points up to 72 h. The blood was immersed immediately in melting ice. Following centrifugation at 3000 rpm for 10 min, 1.0 ml plasma was mixed with 300 μl 0.5 M formate buffer, pH 3.0, and stored at -70°C until analysis by LC-MS/MS. Doses were prepared in 0.9% saline for all studies except for the i.v. dose of [^{14}C]MRL-II, which was prepared in ethanol:PEG400:water (20:40:40, by volume).

Metabolite identification

Bile samples from the dogs dosed with [^{14}C]MRL-I were treated with an equal volume of acetonitrile, spun in a centrifuge at 14 000 rpm for 10 min, and the supernatants were analysed using an HPLC system consisting of a Shimadzu series 200 pump, a Shimadzu series 200 autosampler (Columbia, MD, USA) and a β -Ram scintillation detector (IN/US Systems, Inc., Tampa, FL, USA). The samples were chromatographed on a 3.5- μm Zorbax RX-C18 column (150 $\times$ 4.6 mm, Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of 10 mM ammonium acetate in water (A) and 7.8 mM ammonium acetate in 92/8 acetonitrile/methanol, v/v (B) and the flow rate was 1 ml min^{-1} . The column was eluted with a linear gradient from 25 to 60% B in 30 min, followed by a linear gradient to 90% B in 1 min, and isocratic elution at 90% B for 5 min. Identification of [^{14}C]MRL-I metabolites was achieved by LC-MS/MS using a PE Sciex API 3000 triple quadrupole mass spectrometer. The turbo ionspray source was operated in negative-ion mode at 400°C with an auxiliary nitrogen gas flow of 7.0 l min^{-1} . The HPLC effluent was split 1:4 with 0.2 ml min^{-1} directed into the mass spectrometer, while the remaining 0.8 ml min^{-1} was directed to a fraction collector (Gilson, FC 204, Middleton, WI, USA) where fractions were collected every 30 s over 45 min. The radioactive content of each fraction was determined using a liquid scintillation counter (Beckman LS 6500, Columbia, MD, USA). Alternatively, the samples were analysed by an HPLC equipped with a radioactivity flow-through detector (β -Ram, IN/US) using identical HPLC conditions to those described previously (see above).

Identification of metabolites in bile samples from studies with [^{14}C]MRL-II was achieved by HPLC analysis of acetonitrile extracts and comparison of retention times with chemically synthesized standards. Radiochromatograms were obtained using an on-line Packard Flow Scintillation Analyzer (Downers Grove, IL, USA) at room temperature. Separation of metabolites was achieved using a Perkin Elmer 200 series HPLC system (Shelton, CT, USA) equipped with a 5- μm Betasil Phenyl column (250 $\times$ 4.6 mm, Keystone Scientific). The mobile phase consisted of 1 mM ammonium acetate in water:acetonitrile:acetic acid (95:5:0.1, by volume) (A) and 1 mM ammonium acetate in acetonitrile:water:acetic acid (95:5:0.1, by volume) (B). Separation was achieved under isocratic conditions from 0 to 5 min at 10% B, followed by a 5-min linear gradient to 45% B, an isocratic condition for 10 min at 45% B, then a 10-min linear gradient to 55% B, a linear gradient to 95% B from 30 to 34 min, followed by isocratic conditions at 95% B for 7 min to wash the column. The flow rate was 1 ml min^{-1} .

In vitro glucuronidation in human UGT supersomes

[^{14}C]MRL-I and [^{14}C]MRL-II were incubated with human UGT supersomes in a reaction mixture containing 62 mM phosphate buffer, pH 7.5, 0.025 mg ml^{-1} alamethicin, 1 mM MgCl_2 , 5 mM D-saccharic acid 1,4-lactone and 5 mM UDPGA at a substrate concentration of 10 μM . The final volume of the incubation was 250 μl . The microsomal incubations were carried out at protein concentrations of 0.5 mg ml^{-1} , following pre-incubation with alamethicin at 4°C for 15 min, followed by incubation for 3 min at 37°C with D-saccharic acid 1,4-lactone, MgCl_2 in phosphate buffer (pH 7.5), and either [^{14}C]MRL-I or [^{14}C]MRL-II dissolved in methanol and at a final concentration of 10 μM . The reactions were started by the addition of UDPGA and incubation continued for 30 min. The incubations were terminated by adding 62.5 μl ice-cold acetonitrile. Samples were kept on ice for 30 min and then spun in a centrifuge at 14 000 rpm for 10 min. The supernatants were analysed using a Perkin

Elmer 200 series HPLC system equipped with a column and the eluent was analysed using an on-line Packard Flow Scintillation Analyzer (see below).

The samples from the incubations of [^{14}C]MRL-I were injected onto a Zorbax SB-Phenyl column (250×4.6 mm; Agilent Technologies) and eluted with 0.1% acetic acid in 10 mM ammonium acetate (A) and 0.1% acetic acid in a mixture of acetonitrile and 100 mM ammonium acetate in methanol (92.8:7.2, v/v) (B). Elution was achieved with 30% B for 10 min, followed by a linear gradient to 80% B in 40 min, and then to 90% B in 9 min. The flow rate was 1 ml min^{-1} .

For the analysis of samples from the incubation with [^{14}C]MRL-II, a Beta Basic C18 (10×4.6 mm, $5 \mu\text{m}$, Keystone Scientific) was used. The mobile phase consisted of 0.1% acetic acid in water: acetonitrile (95:5, v/v) (A) and 0.1% acetic acid in acetonitrile:water (95:5, v/v) (B). Elution was achieved under isocratic conditions from 0 to 7 min at 10% B, followed by a linear gradient to 100% B in 16 min and an isocratic conditions for 4 min at 100% B. The flow rate was 1.5 ml min^{-1} .

Results and discussion

MRL-I and MRL-II (figure 1) are structurally similar compounds with different affinities towards PPAR receptors; MRL-I is a dual PPAR α/γ agonist and MRL-II is selective for the α receptor. Here, we report that, in addition to their distinct pharmacological activities, MRL-I and MRL-II exhibited different pharmacokinetics and metabolism in dogs.

MRL-I and MRL-II were absorbed rapidly in dogs following oral administration, with T_{max} values of about 0.5–2 h. MRL-I exhibited linear pharmacokinetics within the dose range examined, from 1 to 25 mg kg^{-1} (table 1). In contrast, the pharmacokinetics of MRL-II were non-linear following both i.v. and oral administration (tables 1 and 2). The non-linearity was apparent most when comparing the plasma clearances (CL_p) following 0.5 and 2 mg kg^{-1} i.v. administration, resulting in means of about 6.5 and $1.7 \text{ ml min}^{-1} \text{ kg}^{-1}$, respectively.

[^{14}C]MRL-I was eliminated exclusively by way of its acyl glucuronide followed by its excretion into the bile (data not shown). In contrast, [^{14}C]MRL-II was eliminated by both acyl glucuronidation and taurine conjugation (figure 2) with subsequent excretion into the bile.

Table 1. Pharmacokinetic parameters for MRL-I and MRL-II following oral administration to male beagle dogs.^a

Compound	Dose (mg kg^{-1})	AUC _{0–24h} ($\mu\text{M}\cdot\text{h}$)	Dose-normalized AUC ($\mu\text{M}\cdot\text{h}\cdot\text{kg mg}^{-1}$)	C_{max} (μM)	T_{max} (h)
MRL-I	1	5.25 ± 0.54	5.25 ± 0.54	1.91 ± 0.04	0.5 ± 0.0
	5	27.1 ± 2.30	5.42 ± 0.46	12.0 ± 1.10	0.6 ± 0.1
	25	93.5 ± 12.7	3.74 ± 0.51	30.7 ± 5.63	0.9 ± 0.4
MRL-II	1	10.2 ± 4.0	10.2 ± 4.0	3.8 ± 0.8	1.2 ± 0.8
	5	107 ± 14	21.4 ± 2.8	35.6 ± 4.8	0.4 ± 0.1
	15	652 ± 279	43.5 ± 18.6	102 ± 14	2.0 ± 1.0

^aValues are mean $\pm$ standard deviation ($n=3$ male dogs).

Table 2. Pharmacokinetic parameters of MRL-II following intravenous administration to male beagle dogs.^a

Dose (mg kg^{-1})	AUC _{0–∞} ($\mu\text{M}\cdot\text{h}$)	CL_p ($\text{ml min}^{-1} \text{ kg}^{-1}$)	Vd_{ss} (l kg^{-1})	$T_{1/2}$ (h)
0.5	2.5 ± 0.6	6.5 ± 1.8	1.5 ± 0.4	3.0 ± 1.3
1	15.8 ± 7.1	2.6 ± 1.3	0.6 ± 0.2	3.0 ± 1.1
2	39.2 ± 5.0	1.7 ± 0.03	0.6 ± 0.1	6.7 ± 1.4

^aValues are mean $\pm$ standard deviation ($n=3$ male dogs).

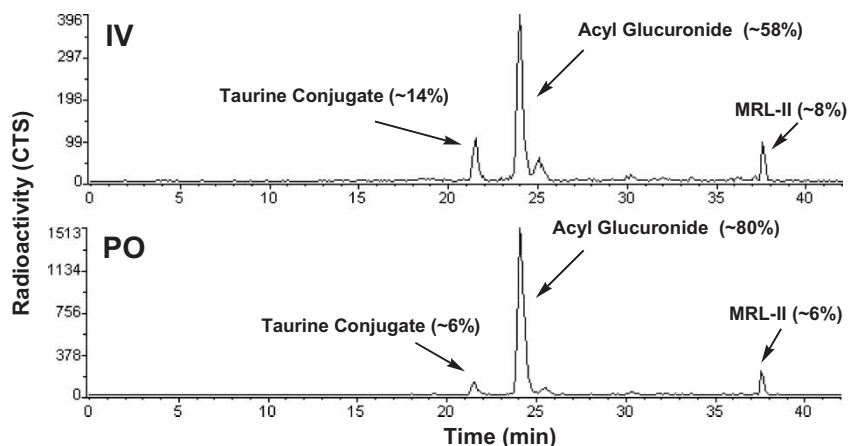


Figure 2. Metabolite profiles of dog bile following intravenous and oral administration of $[^{14}\text{C}]$ MRL-II at 1 and 5 mg kg^{-1} , respectively. Data are the percentage of radioactivity injected onto an high-performance liquid chromatography column.

Conjugation with an amino acid or with glucuronic acid is an important elimination pathway for carboxylic acid-containing xenobiotics. The most commonly encountered amino acid conjugates are those of glycine, taurine and glutamine, with the exact nature of the amino acid being dependent on the species and the structure of the xenobiotic carboxylic acid (Caldwell *et al.* 1979, Emudianughe *et al.* 1983, 1987, Hutt and Caldwell 1990). The amino acid conjugation of xenobiotic carboxylic acids occurs via a two-step process. The initial step is the activation of the carboxyl group to a reactive coenzyme A thioester by an acyl-CoA synthetase or ATP-dependent acid: CoA ligase followed by acyl transfer to an amino acid residue by an acyltransferase (Killenberg and Webster 1980, Caldwell 1982). The present results from the *in vivo* metabolism studies indicated that there were differences in the specificities of these two enzymes for MRL-I and MRL-II, which may be caused by differences in the nature of the substituents on the alpha carbon adjacent to the carboxylic acid group (ethyl versus methyl) as well as other functional groups within the two molecules, i.e. 4-fluorophenyl versus ethyltrifluoromethyl moieties.

In spite of their structural similarities, only MRL-II produced detectable levels of the taurine conjugate. However, both MRL-II and MRL-I produced the acyl glucuronide metabolites. Dose has been shown to be a determinant of the principal metabolic options for xenobiotic carboxylic acids, namely conjugation with an amino acid versus glucuronic acid. Amino acid conjugation appears to predominate at low doses, while at higher doses, glucuronic acid conjugation becomes more important, suggesting that amino acid conjugation is a high-affinity, low-capacity system, whereas glucuronic acid conjugation is a high-capacity system with a broader substrate selectivity (Hutt and Caldwell 1990). The effect of dose on the pattern of metabolism of xenobiotic carboxylic acids has been reported previously. For example, Dixon *et al.* (1977) showed that at low doses, 1-naphthylacetic acid underwent both glycine and glucuronic acid conjugation. However, the proportion of glycine conjugation decreased with a concomitant increase of glucuronidation as the dose was increased. The implications of capacity-limited amino acid conjugation on the pharmacokinetics of salicylic

Table 3. Contribution of radioactive components in pooled bile following administration of [^{14}C]MRL-II.^a

Dose	Radioactivity (%)		
	Parent	Acyl glucuronide	Taurine conjugate
0.2 mg kg ⁻¹ , i.v. ^b	9	26	41
0.9 mg kg ⁻¹ , i.v. ^b	8	58	14
5 mg kg ⁻¹ , oral ^c	6	80	6

^aSamples from individual animal were pooled by the fraction of the volume at different time points (0–72 h) and pooled again by the same volume across animals.

^b*n* = 2 male dogs.

^c*n* = 3 male dogs.

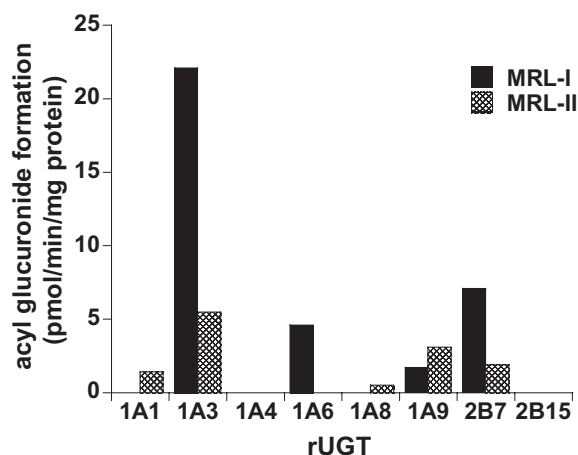


Figure 3. Acyl glucuronidation of MRL-I and MRL-II in human UGT supersomes. Data are derived from a single determination.

acid have also been described. At therapeutic doses, glycine conjugation of salicylic acid is saturable (Levy *et al.* 1965a, b), whereas glucuronidation is saturable only at higher doses, resulting in dose-dependent pharmacokinetics in humans (Levy *et al.* 1972, Levy and Giacomini 1978).

Therefore, saturation of taurine conjugation was considered contributory to the non-linear kinetics of MRL-II in the dog. The percentages of the two metabolites, the acyl glucuronide and the taurine conjugates, in pooled dog bile (0–72 h) changed with increasing dose (table 3). A higher percentage of radioactivity was associated with the taurine conjugate (about 41%) following 0.2 mg kg⁻¹ i.v. administration compared with about 14% at 0.9 mg kg⁻¹ i.v. and about 6% following a 5 mg kg⁻¹ oral dose. Also, the percentage of the acyl glucuronide in dog bile increased with increasing dose (table 3). The results are consistent with saturation of the taurine conjugation pathway resulting in a change in the pattern of metabolism to more acyl glucuronide formation.

The *in vitro* acyl glucuronidation of MRL-I and MRL-II was studied in baculovirus-expressed human UGT supersomes (figure 3). MRL-I was a good substrate for UGT1A3 and to a lesser extent for UGTs1A6, 1A9 and 2B7, whereas UGTs1A3, 1A9 and 2B7 exhibited similar activity towards MRL-II, with

Table 4. Pharmacokinetic parameters of MRL-I following intravenous administration to bile duct-cannulated male dogs at 1 mg kg^{-1a}.

	AUC ₀₋ (μM·h)	CL _p (ml min ⁻¹ kg ⁻¹)	Vd _{ss} (l kg ⁻¹)	T _{1/2} (h)
Bile collected	8.6 ± 2.2	4.0 ± 1.0	0.3 ± 0.1	1.5 ± 0.2
Bile circulated	23.9 ± 15.6	1.7 ± 0.8	1.5 ± 0.7	16.6 ± 3.5

^aValues are mean ± standard deviation (n = 3 male dogs).

Table 5. Pharmacokinetic parameters of MRL-II following intravenous administration to bile duct-cannulated male dogs at 1 mg kg^{-1a}.

	AUC ₀₋ (μM·h)	CL _p (ml min ⁻¹ kg ⁻¹)	Vd _{ss} (l kg ⁻¹)	T _{1/2} (h)
Bile collected	6.1, 9.6	5.7, 3.7	1.1, 1.4	1.1, 1.6
Bile circulated	9.9, 8.7	3.6, 4.0	1.4, 0.8	2.2, 2.7

^an = 2 male dogs.

UGTs1A1 and 1A8 showing a much lower extent of glucuronidation. These results indicated different specificities of UGT enzymes for MRL-I and MRL-II. The overall rate of *in vitro* glucuronidation of MRL-I was higher than that of MRL-II, which was consistent with the *in vivo* metabolite profiles of MRL-I.

There are many reports addressing the pharmacokinetic and clinical implications of enterohepatic circulation of drugs that undergo extensive glucuronidation (Ouellet and Pollack 1995, Ezzet *et al.* 2001, Patrick *et al.* 2002, Roberts *et al.* 2002). Since glucuronidation was the major elimination pathway for MRL-I and MRL-II, it was examined whether these compounds were subject to enterohepatic recirculation in the dog. Continuous collection of bile from bile duct-cannulated dogs over 72 h resulted in an 11-fold decrease in the mean terminal half-life of MRL-I in plasma (1.5 versus 16.6 h), and a 2.4-fold increase in its mean plasma clearance (4.0 versus 1.7 ml min⁻¹ kg⁻¹) after i.v. administration at 1 mg kg⁻¹ compared with similar studies in the same dogs where the bile was allowed to circulate normally. These data indicated that MRL-I was subjected to extensive enterohepatic recirculation in this species (table 4). However, the pharmacokinetic parameters of MRL-II in bile duct-cannulated dogs were not affected significantly by the continuous collection of bile (table 5). These results suggested that there may be differences in the rate of hydrolysis of the acyl glucuronide conjugates, although, *in vitro*, the acyl glucuronide of MRL-I was more stable than that of MRL-II. The half-lives of rearrangement/hydrolysis of these two acyl glucuronides were 150 and 26 min in human plasma and 700 and 100 min in dog bile at 37°C, respectively (data not shown). The stability of these acyl glucuronides in dog intestinal contents was not determined.

In conclusion, differences in the metabolism and pharmacokinetics of two structurally similar PPAR agonists in dogs have been demonstrated. The compound that formed a taurine conjugate in addition to an acyl glucuronide, namely MRL-II, exhibited non-linear pharmacokinetics. In contrast, MRL-I did not form a taurine conjugate, although it did form an acyl glucuronide as a major metabolite and was subject to enterohepatic recirculation. Further, the pharmacokinetics of this compound were dose proportional.

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***In vivo* metabolism and kinetics of ethylene glycol monobutyl ether and its metabolites, 2-butoxyacetaldehyde and 2-butoxyacetic acid, as measured in blood, liver and forestomach of mice**

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1. Ethylene glycol monobutyl ether (EGBE) causes forestomach hyperplasia and neoplasia in mice when administered chronically by inhalation.

2. The study was initiated to test the physiologically based pharmacokinetic (PBPK) model prediction that 2-butoxyacetaldehyde (BAL), a transient, labile intermediate in the oxidation of EGBE to butoxyacetic acid (BAA), is unlikely to achieve concentrations sufficient to cause DNA damage in target tissues.

3. Male and female B6C3F1 mice were administered a high oral dose of EGBE (600 mg kg⁻¹), and tissues were collected at 5, 15, 45 and 90 min following the dose. The tissues were processed for determination of EGBE, BAL and BAA by gas chromatography-mass spectrometry.

4. BAL was detected at low concentrations in all tissues sampled and at all time points following EGBE administration (about 0.3–33 µM). BAL concentrations were highest in the initial samples (5 min) in all tissues and declined from that point.

5. BAL concentrations in liver and forestomach tissues corresponded to the peak concentrations predicted by an already published PBPK model, and are higher than BAL concentrations that could be achieved by inhalation exposure to EGBE.

6. Mouse inhalation exposure to EGBE is therefore unlikely to generate BAL concentrations in tissues sufficient to initiate a carcinogenic response.

Introduction

Ethylene glycol monobutyl ether (EGBE) causes forestomach hyperplasia and neoplasia in female mice and liver haemangiosarcomas in male mice when administered chronically by inhalation (US NTP 2000). It is proposed that the hepatic tumours are a result of chronic haemolysis producing iron deposition and resultant oxidative stress in this organ (Boatman *et al.* 2004). Anaemia as a result of haemolysis is a primary toxic effect observed in rodent studies with EGBE (US NTP 2000). Oxidation of EGBE to 2-butoxyacetic acid (BAA) is the cause of the observed haemolysis, with red blood cells from rodents more susceptible than blood from humans or certain other species (Ghanayem and Sullivan 1993). Considerable evidence also exists to support the contention that the forestomach tumours in female mice likely originated from a non-linear, chronic cytotoxicity/regenerative hyperplasia mode of action related to EGBE or BAA that is unlikely to occur in humans (Boatman *et al.* 2004). However, a potential role for a direct

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genotoxic effect of the aldehyde intermediate, 2-butoxyacetaldehyde (BAL), on DNA has not been completely ruled out (US EPA 2003). BAL is a transient, labile intermediate that is unlikely to achieve concentrations sufficient to cause DNA damage in target tissues based on physiologically based pharmacokinetic (PBPK) simulations (Tyler *et al.* 2003).

Therefore, the current study was initiated to test the PBPK predictions and to determine the extent of the *in vivo* formation of BAL and BAA from EGBE in blood, liver and forestomach tissues. Oral administrations, near the maximum tolerated non-lethal dose for EGBE, were employed to simulate a worst-case inhalation dose that would have resulted in the deposition of a large amount of the relatively non-volatile EGBE in the forestomach following its condensation in the nasal mucosa and ingestion during grooming.

Materials and methods

Animals

Male and female B6C3F1 (hybrid) mice (B6C3F1/CrlBR) were purchased from Charles River Laboratories (Raleigh, NC, USA) and were 7–10 weeks of age at dosing. The mice were fed certified rodent diet (Rodent Diet #5002 Pellet, PMI Feeds, Inc., St Louis, MO, USA) and allowed access to domestic tap water *ad libitum*.

Test chemicals

EGBE was obtained from Sigma-Aldrich (St Louis, MO, USA). Analyses of the EGBE test chemical employing pentafluorophenyl hydrazine (PFPH, Sigma-Aldrich) derivatization and gas chromatography with mass spectrometric (GC-MS) detection indicated the presence of a trace amount of BAL as a contaminant in the EGBE test compound at approximately 0.05%. The EGBE test chemical was purified by fractional distillation of the EGBE over 2,4-dinitrophenylhydrazine (2,4-DNPH, Sigma-Aldrich) under acidic conditions (Hoshika and Muto 1978), which reduced the BAL content to approximately 0.005% of the EGBE test chemical. BAL was synthesized in house by the method of Leopold (1985). GC-MS analysis of the BAL indicated a purity of 93.7%, with EGBE identified as the major impurity. BAA was obtained from TCI America (Portland, OR, USA). The purity of BAA determined by GC-MS analysis was 97.7%, with the major impurity identified as butyl glycolate.

In vivo EGBE administration and sample processing

Mice were fasted for 6 h before dose administration. EGBE was dissolved in distilled water at approximately 60 mg ml^{-1} and administered to groups of four male and female mice by oral gavage at 600 mg kg^{-1} EGBE, which is approximately one-half of the mouse oral LD_{50} . Additional animals were dosed with distilled water to provide tissue blanks, which were harvested at 5-min post-dosing. At 5, 15, 45 and 90 min following the dose, animals were anaesthetized by carbon dioxide inhalation, and approximately 100-mg aliquots of blood, liver and forestomach tissues were collected by weight. A 100- μl aliquot of internal standard solution in distilled water containing 514.42 μM ethylene glycol monohexyl ether (EGHE, Fluka, Buchs SG, Switzerland), 51.35 μM hexanal (Sigma-Aldrich), and 525.31 μM propoxyacetic acid (PAA, prepared in house) was added by weight to each tissue. The tissue plus internal standard solution was homogenized for approximately 10 s (Polytron with PT 7 generator at one-half full speed; Brinkman Instruments, Inc., Westbury, NY, USA), and 0.4 ml 100 mM PFPH in 0.1 M hydrochloric acid were added. This mixture was homogenized thoroughly with the Polytron, which required approximately 15 s for the blood preparations, 30 s for the liver and 1 min or more for the forestomach. The preparations were allowed to stand for 15 min at room temperature to allow complete derivatization of carbonyl groups by PFPH and then extracted with 0.8 ml ethyl acetate. The phases were separated by centrifugation, and approximately 0.4 ml organic phase was methylated by the addition of 25 μl ethereal diazomethane.

GC-MS analysis method

The extracted tissue samples were analysed by gas chromatography-mass spectrometry with selected ion monitoring (GC-MS/SIM, Model 6890-5973N, Agilent Technologies, Inc., Palo Alto, CA, USA) using either negative chemical ionization (NCI mode) for BAL and hexanal as internal standard, or electron-impact ionization (EI mode) for EGBE and BAA, and EGHE and PAA as internal standards. The GC-MS response was calibrated in both NCI and EI modes by spiking mouse whole blood with known amounts of the EGBE, BAL and BAA analytes contained in the internal standard

solution, and processing these standards as described above for the tissues. This method assumes similar extraction efficiencies would be obtained from the liver and forestomach tissues as seen in the mouse blood standards. A DB-1701, 30 m $\times$ 0.25 mm $\times$ 0.25 μ m film capillary column (Agilent Technologies) with a 3–5 m $\times$ 0.22–0.25 mm i.d. deactivated silica inlet retention gap was used to perform separations. A 1 μ l splitless, ramped-flow injection with a 225°C inlet and helium carrier gas was used. The oven temperature program was 40°C initial temperature for 1 min, ramped at 10°C min⁻¹ to 220°C then held for 1 min. NCI mode detection (2 ml min⁻¹ methane gas) for BAL-PFPH monitored the 276.2 m/z ion, and for hexanal-PFPH, the 260.2 m/z ion. EI mode detection monitored the 87.2 m/z ion for EGBE, the 85.2 m/z ion for EGHE, and the 74.1 m/z ion for methyl-BAA and methyl-PAA.

Results

At 5 min following oral administration of 600 mg kg⁻¹ EGBE, high concentrations of the parent compound were detected in the forestomach (including the forestomach contents) of both female (123 $\pm$ 25 mM) and male (129 $\pm$ 19 mM) mice, while the blood samples and liver tissues contained roughly 50-fold less EGBE (figures 1 and 2). The EGBE concentrations declined rapidly in subsequent blood and liver samples, but were still measurable at 90 min post-dosing. EGBE concentrations in the forestomach declined only gradually beyond 5 min post-dosing and were still an average of 62% (female mice) and 31% (male mice) of the 5-min peak concentrations at 90 min post-dosing.

Low concentrations of BAL were detected in all organs at maximum levels at 5 min post-dosing, and were roughly 10-fold higher in the forestomach compared with the blood and liver. The BAL concentrations were observed to decline rapidly in subsequent samples, but at concentrations that were two to four orders of magnitude lower than the parent compound. In contrast, the BAA concentrations in all organs were elevated in the 5-min sample, and continued to increase before levelling off at the 45- and 90-min sample times. The concentrations of BAA in the forestomachs of male and female mice were significantly lower at all time points compared with BAA concentrations in the blood and liver ($p < 0.0001$, two-tailed Student's t -test).

There appears to be no difference between male and female mice in organ concentrations of EGBE or BAA at comparable time points following dosing. However, the BAL tissue concentrations are frequently higher in female mice compared with male mice. Significantly higher BAL concentrations were identified at 45 and 90 min in the liver, and at 90 min in the forestomachs of female mice ($p < 0.05$, two-tailed Student's t -test) compared with male mice.

The contents of the forestomach at the time of excision were highly variable following the 6-h fast, ranging from only fluid content in some mice to relatively full of partially digested feed in others. Isolation of the forestomach from the main stomach by bisection with scissors resulted in expulsion and variable loss of the stomach contents, dependent in part on the amount of content present. In addition, the tough forestomach tissue required hand mincing with scissors before homogenization, introducing further variability in losses of the contents. In spite of the variability in content and tissue processing, variability in analyte concentrations in the forestomach is comparable to that in the other tissues.

The forestomachs from two mice dosed with 600 mg kg⁻¹ EGBE were processed at 5 min (male) or 90 min (female) post-dosing by removing the contents and rinsing the tissue in saline before analysis. EGBE and BAL concentrations in these rinsed forestomachs were substantially below the concentrations detected in non-rinsed forestomachs (approximately a 60% reduction at 5 min, and an 85%

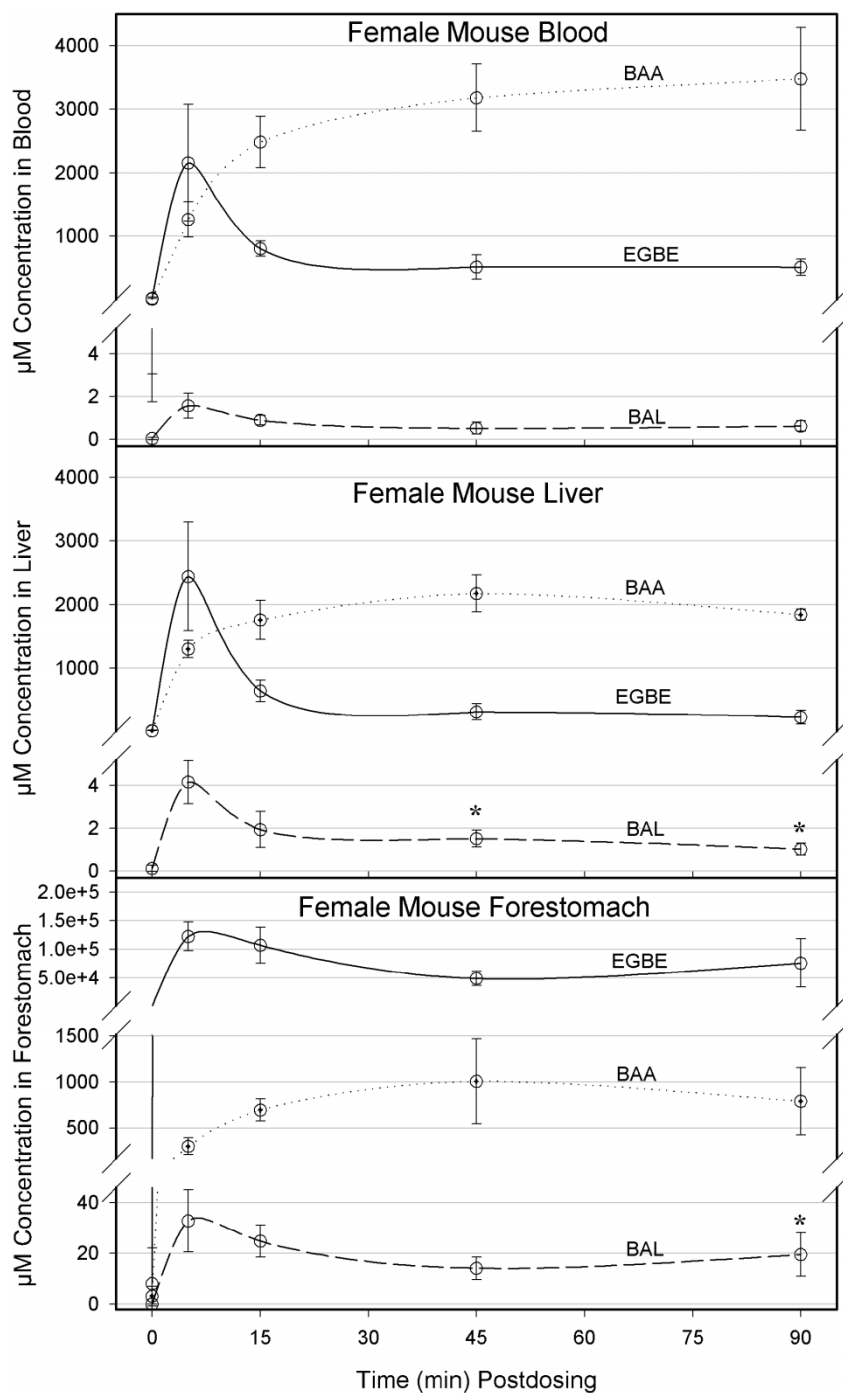


Figure 1. Ethylene glycol monobutyl ether (EGBE), 2-butoxyacetaldehyde (BAL) and butoxyacetic acid (BAA) concentrations measured in tissues of female mice at selected intervals following *in vivo* oral doses of 600 mg kg^{-1} EGBE or distilled water blank (0 min data point) as described in the Materials and methods. The y-axes contain differing scales for the analytes. Data are presented as the mean $\pm$ SD for $n=4$. *Mean BAL concentrations were significantly higher at these time points in female mice compared with males ($p < 0.05$, two-tailed Student's *t*-test).

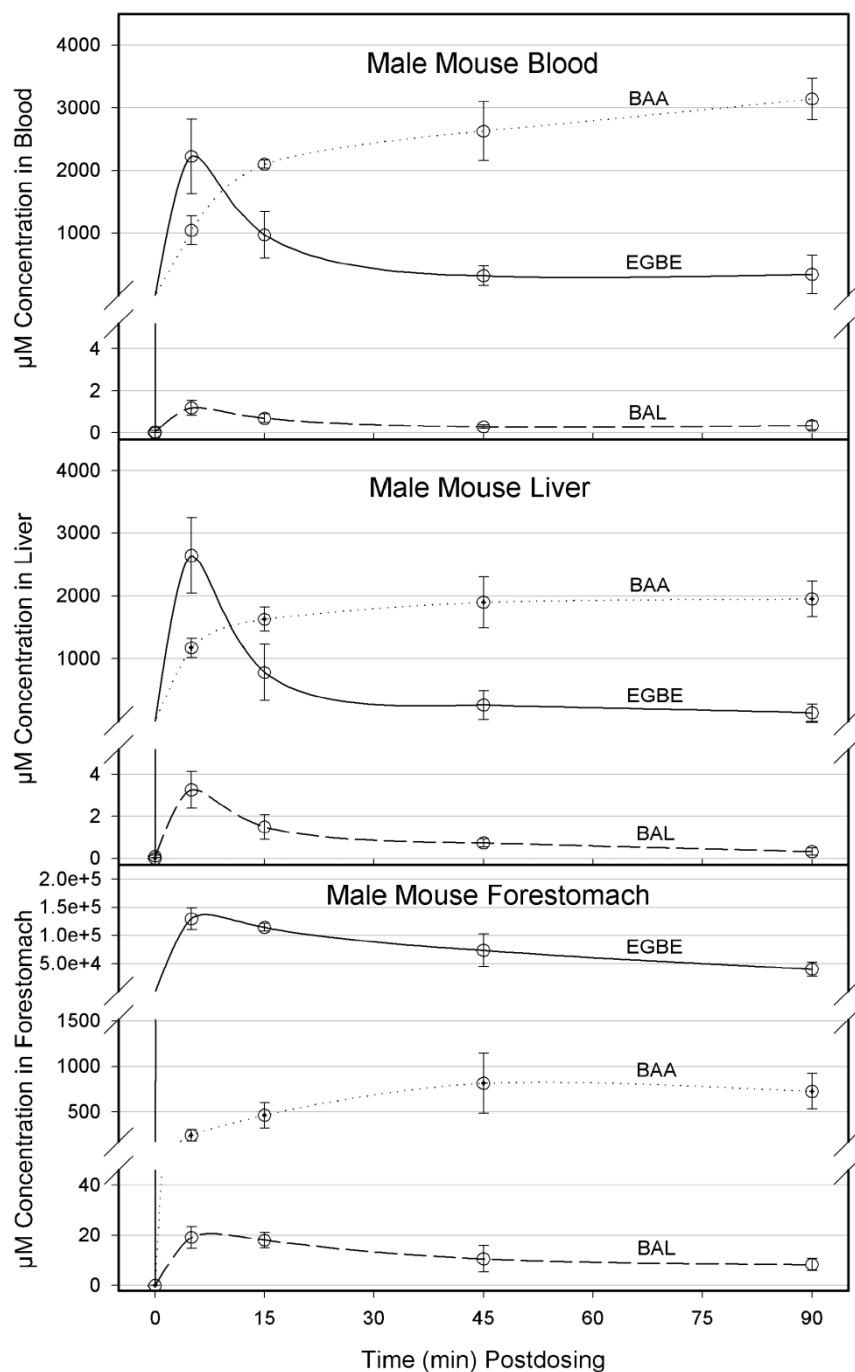


Figure 2. Ethylene glycol monobutyl ether (EGBE), 2-butoxyacetaldehyde (BAL) and butoxyacetic acid (BAA) concentrations measured in tissues of male mice at selected intervals following *in vivo* oral doses of 600 mg kg⁻¹ EGBE or distilled water blank (0 min data point) as described in the Materials and methods. The y-axes contain differing scales for the analytes. Data are presented as the mean $\pm$ SD for $n = 4$.

reduction at 90 min), but the BAA concentrations appeared to be unaffected by the rinsing procedure, and were actually 150% (5 min) and 200% (90 min) of the mean unwashed forestomach concentration.

Discussion

The primary analytical objective of this study was to determine the occurrence BAL in blood, liver and forestomach tissue after an oral dose of EGBE in mice. The 600 mg kg⁻¹ EGBE administered in these studies is near the maximum tolerated non-lethal dose, and was intended to maximize the possibility of detecting the BAL metabolic intermediate. This objective was accomplished in that BAL was detectable at low concentrations in all tissues assayed between 5 and 90 min following the 600 mg kg⁻¹ EGBE oral dose. As shown in figures 1 and 2, BAL concentrations were highest in the forestomach (including the forestomach contents), roughly 10-fold higher than in blood or liver. BAL concentrations were highest in the 5-min samples and were 19.1 ± 4.3 µM in male mice and 32.8 ± 12.3 µM in female mice. These peak concentrations were consistent with simulated values obtained from the PBPK model of Corley *et al.* (2004) that predicted peak BAL gastrointestinal (GI) concentrations of 18 and 33 µM in male and female mice, respectively, following an oral gavage of 600 mg kg⁻¹ EGBE. The model was modified using the Green *et al.* (2002) rate constants determined in forestomach and glandular stomach tissues of female B6C3F1 mice and F344 rats with the following assumptions: (1) the *in vivo* rate constants from the model correspond to the first, rate-limiting step in the metabolism of EGBE to BAL; (2) the ratio of the rate of *in vitro* oxidation of BAL to BAA/EGBE to BAL was used to estimate the *in vivo* $V_{\max}$ for oxidation of BAL to BAA (the parallelogram approach); (3) the K_m for conversion of BAL to BAA reported by Green *et al.* (2002) was used; (4) forestomach rate constants were applied to the entire GI tract and to the liver; and (5) BAL does not leave tissues where it is formed while EGBE and BAA circulate in the body.

The Corley *et al.* (2004) PBPK model simulations of peak BAL concentrations in the liver were similar to those in GI tissues (i.e. 17 and 29 µM in male and female mice, respectively) because the metabolic rate constants determined by Green *et al.* (2002) were also used for the liver. The current data indicate that the metabolic clearance of BAL in the liver of mice may in fact be faster than in the forestomach tissues.

Organ concentrations of BAL that might be anticipated from a worst-case inhalation exposure to EGBE appear to have been grossly exceeded by the 600 mg kg⁻¹ EGBE oral dose. Since the BAL concentrations expected in GI tissues of mice following a 600 mg kg⁻¹ oral dose of EGBE could be accurately predicted with the above model, the model was also used to predict peak BAL concentrations in GI tissues following a 6-h inhalation exposure at the theoretical maximum vapour concentration of 1160 ppm EGBE. The predicted concentration for BAL was 0.8 µM, which is approximately 40-fold less than the peak BAL concentrations measured in the forestomachs of female mice in the current study. These data indicate that the GI exposure level to BAL resulting from the 600 mg kg⁻¹ EGBE oral dose could not be achieved in a 6-h inhalation study.

Aldehydes occur as natural constituents in a wide variety of foods, and are also used as food additives (Feron *et al.* 1991). Low molecular weight aldehydes, such

as formaldehyde and acetaldehyde, are of weak genotoxic potential, generally manifesting these effects at cytotoxic dose levels. For those aldehydes tested in animals, there is evidence for carcinogenicity of formaldehyde and acetaldehyde, producing nasal tumours, but only equivocal evidence for carcinogenic activity in humans. It has been suggested that cytotoxicity plays a crucial role in the carcinogenic response in animals to formaldehyde and acetaldehyde. Similarly, the *in vitro* evidence for the genotoxicity of BAL is equivocal. No DNA damage was observed in a recent *in vitro* comet-screening assay that exposed a mouse endothelial cell line to up to 1.0 mM BAL for up to 24 h (Klaunig and Kamendulis 2003). However, mixed results have been reported on the genotoxicity of BAL by other investigators (Elliot and Ashby 1997). The lowest genotoxic concentration of BAL reported is 40 μ M. The current oral studies, using a worst-case high-oral dose, produced peak tissue concentrations of BAL that are less than the 40 μ M minimum genotoxic concentration in *in vitro* assays. It appears unlikely that mouse exposures to EGBE by inhalation could generate BAL concentrations in tissues sufficient to produce either cytotoxicity or carcinogenicity.

The fact that the EGBE dose was delivered to the stomach, and contained a small amount of BAL as an impurity, may have contributed to the higher initial BAL concentrations in the forestomach relative to the blood and liver. The mean BAL concentration measured in dose solutions was 0.005% of the amount of EGBE. As a comparison, the mean forestomach EGBE content in female mice sampled at 5 min was 123 ± 25 mM, of which 6.1 μ M would be the BAL contamination in this amount of EGBE. The mean BAL concentration assayed in the forestomachs 5 min after dosing the female mice was 32.8 ± 12.3 μ M, therefore, the BAL contamination in the dose may account for up to 19% of the initial BAL content of these forestomachs. Using the same calculation at the 5-min time point for male mice, approximately 34% of the initial forestomach BAL content in male mice may be attributed to BAL contamination in the dose.

Green *et al.* (2002) have shown that both alcohol and aldehyde dehydrogenases are heavily concentrated in the stratified squamous epithelium of the forestomach in mice. Considering the high concentrations of EGBE present in the forestomach at all time points, the persistence of BAL in the forestomach probably was due to continuous production of BAL, and not to its slow clearance. BAL concentrations in tissues were highest in the initial samples (5 min) and declined at a greater rate in blood and liver than in the forestomach. Similar elimination patterns were observed in mice by Poet *et al.* (2003) for both EGBE and BAA, where these chemicals were eliminated more slowly from forestomach tissues than from blood or other tissues following both oral and intraperitoneal dosing. It was proposed by these latter authors that the relatively higher BAA concentrations in forestomach tissues were a consequence of retention or secretion of EGBE into the stomach followed by metabolism to BAA.

In the current studies, the high concentrations of EGBE and, to a lesser extent, BAL in the forestomach tissues presumably were due to the extended dwell time of the administered dose in this organ. To investigate this, forestomachs from two mice were rinsed in saline to remove the contents before analysis. The EGBE and BAL concentrations in these rinsed forestomachs were reduced by > 60% (male, 5 min) and > 80% (female, 90 min) of the mean of non-rinsed mouse forestomachs of the same sex mice at the same time point. In sharp contrast, the BAA content of

the rinsed forestomachs was 150 and 200% of the mean non-rinsed forestomachs at the comparable data points. The BAA concentrations appear higher in the washed forestomachs because the BAA is apparently contained primarily in the forestomach tissues, and the forestomach contents, which apparently contain little BAA, in effect diluted the tissue BAA concentration when the two components were combined. Systemic metabolism and distribution, as evidenced by higher concentrations of BAA in blood and liver over the entire course of the study, could account for the concentrations of BAA in the forestomach tissues. There appears to be no accumulation of BAA in the forestomach during the 90-min period of these experiments.

The EGBE concentration in the aqueous dose solution was 500 mM and was deposited directly into the forestomach via the oral route. EGBE concentrations of greater than 100 mM were measured initially in both male and female mouse forestomachs, and declined to the 50 mM range by 90 min post-dosing. BAA concentrations in the forestomach ranged from 0.25 mM initially to near 1 mM by 45 min post dosing. In contrast, the concentrations of BAL measured in the forestomach were 10- (early time points) to 100-fold (later time points) less than the BAA concentrations. Liver, the major metabolic organ, initially contained millimolar quantities of EGBE, which was oxidized via the aldehyde intermediate to millimolar concentrations of BAA. The BAL intermediate was detectable in the liver, but at concentrations that were greater than 300-fold lower than BAA in the initial samples and greater than 1000-fold lower than BAA in the later samples. Blood functions mainly as a transport organ for these analytes with little dehydrogenase capability and thus reflects systemic concentrations of the analytes. BAA was present at millimolar concentrations in the initial blood samples and increased threefold in concentration by the later samples due to continuing rapid oxidation of the large reservoir of EGBE. In contrast, the initial blood BAL concentrations were approximately 1000-fold lower than the acid concentrations, and declined to concentrations that were greater than 5000-fold lower than BAA in the later blood samples. In terms of contact with EGBE metabolites, tissues analysed in this study were exposed to one to three orders of magnitude more of the acid metabolite compared to the aldehyde.

The analytical method developed for this study focused on the sensitive detection of BAL (as the PFPH derivative) in mouse tissues. Underivatized BAL was found to be unstable in aqueous solution, and its MS detection was not particularly sensitive. Aqueous-phase derivatization with PFPH provided a method of trapping the aldehyde immediately after tissue sampling, and produced a molecule amenable to high sensitivity detection. The GC-MS/NCI/SIM analysis of the PFPH derivatives resolved two peaks for both BAL and the hexanal internal standard (figure 3, top frame). The two peaks have nearly identical mass spectra and are presumed to be the syn- and anti-isomers of the hydrazone derivative that are resolved on the high-resolution capillary column. The final analysis method employed for BAL used GC-MS with negative chemical ionization and SIM detection to provide increased sensitivity. This method provided a limit of quantitation of approximately 0.25 μ M for BAL in the mouse tissues with negligible background. During the studies, all samples were analysed for BAL on the day of preparation to avoid concerns about degradation of the PFPH derivatives over time. Periodic reanalysis of BAL standards indicated an analytical accuracy of 80.4% of the gravimetric values with 16.9% deviation. The low BAL

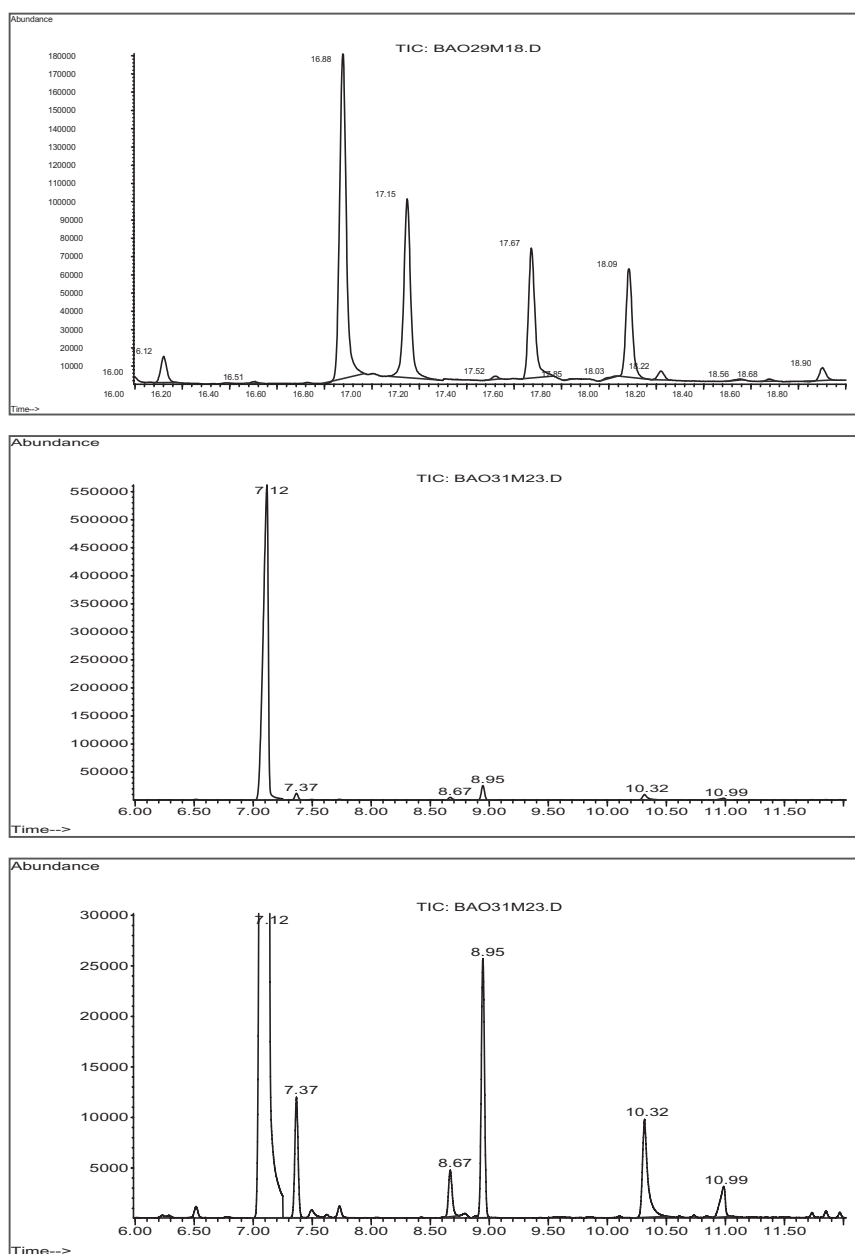


Figure 3. Typical gas chromatography with mass spectrometric/selected ion monitoring (SIM) chromatograms of EGBE metabolite analyses in a female mouse forestomach collected 15 min following a 600 mg kg^{-1} oral dose of ethylene glycol monobutyl ether (EGBE). The top frame is a portion of a negative chemical ionization-SIM chromatogram showing the syn- and anti-isomers of pentafluorophenyl hydrazine (PFPH)-2-butoxyacetaldehyde at 17.67 and 18.09 min (222.1 amu, 17.67 min peak used for quantitation), and the syn- and anti-isomers of PFPH-hexanal (224.1 amu) internal standard at 16.88 (used for quantitation) and 17.15 min. The middle frame is a portion of an electron impact ionization-SIM (87.2 amu) chromatogram of the same sample showing the large EGBE parent compound peak at 7.12 min. The bottom frame is the same chromatogram as the middle frame with the y-axis expanded to show EGHE (10.32 min, 85.2 amu), the internal standard for EGBE, and methyl-butoxyacetic acid (8.95 min, 74.1 amu), and its internal standard, methyl-propoxyacetic acid (7.37 min, 74.1 amu).

concentrations may be due to repeated analysis of the standards for several days following their preparation.

A second analytical method provided GC-MS/EI/SIM detection of EGBE with EGHE as its internal standard, and detection of BAA with PAA as its internal standard (figure 3, middle and bottom frames, both acids as the methyl ester). Low-level detection was not required for these analytes since they were present in relatively high concentration in all tissues following the EGBE doses. The BAA analyses proved to be reliable in all samples, with low-level contamination occurring in occasional blanks as the only analytical problem. Periodic reanalysis of BAA standards indicated an analytical accuracy of 98.3% of the gravimetric values with 0.06% deviation. The GC-MS/EI/SIM analysis for EGBE was more problematic. Initial analyses for EGBE and its internal standard, EGHE, resulted in sharp, symmetrical peaks with accurate integration. However, presumably as a result of the activation of the analytical column by the high hydrazine content of the samples, the alcohol analyte peak shapes progressively deteriorated during the studies. Periodic reanalysis of EGBE standards indicated an analytical accuracy of 99.1% of the gravimetric values, but with 26.2% deviation reflecting the poor precision of this analysis. In addition, the EGBE concentrations in the forestomach were well above the maximum 10 mM calibration level. Quantitative results for EGBE in forestomach reported here should be regarded as approximations.

Conclusions

The peak concentrations of BAL detected in mouse forestomach following a 600 mg kg⁻¹ oral dose of EGBE were similar to those simulated by an existing PBPK model, and appear to be well above concentrations that could be obtained in a 6-h inhalation exposure. All the tissues analysed contained concentrations of the parent compound and BAA that were multiple orders of magnitude higher than the aldehyde intermediate. It appears that mouse exposure to EGBE by inhalation is unlikely to generate BAL concentrations in tissues sufficient to initiate a carcinogenic response, assuming that BAL is genotoxic.

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Prediction of differences in *in vivo* oral clearance of *N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100) between extensive and poor metabolizers from *in vitro* metabolic data in human liver microsomes lacking CYP2D6 activity and recombinant CYPs

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1. It has previously been reported that *N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) was predominantly metabolized by cytochrome P450 (CYP) 2D6 in human liver microsomes (HLM). In the present study, the contribution of CYP forms involved in the formation of the major metabolites of NE-100 in human liver lacking CYP2D6 activity (PM-HLM) has been predicted by use of *in vitro* kinetic data on recombinant CYPs microsomes (rCYPs).

2. In PM-HLM, NE-100 is predicted to be metabolized to *N*-despropyl-NE-100 (NE-098), *p*-hydroxy-NE-100 (NE-152) and *m*-hydroxyl-NE-100 (NE-163), but not to *O*-demethy-NE-100 (NE-125), which is a major metabolite in pooled human liver microsomes (EM-HLM). The relative activity factor approach assumed that NE-098 formation is predominantly catalysed by CYP3A4 and CYP2C9 and the NE-152+163mix (a mixture of two hydroxylated metabolites, NE-152 and NE-163) formation is only catalysed by CYP3A4.

3. The predicted contribution rates of CYP3A4 and CYP2C9 for NE-098 formation were 58.1 and 34.6%, respectively, in PM-HLM. These predicted results were strongly supported by kinetic and inhibition studies using PM-HLM. The intrinsic clearance of NE-100 predicted from rCYPs (the predicted $CL_{int-HLM-total}$) corresponded to those observed from EM- and PM-HLM (the observed $CL_{int-HLM}$).

4. The *in vivo* oral clearance (CL_{oral}) of NE-100 in extensive metabolizers and poor metabolizers of CYP2D6 was predicted to be 50 times higher in extensive metabolizers than poor metabolizers using *in vitro-in vivo* scaling method based on the dispersion model. These data suggest that polymorphism of CYP2D6 might greatly affect NE-100 metabolism *in vivo*.

Introduction

N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100), a selective sigma 1 ligand, is under development as a therapeutic agent for the treatment of schizophrenia (Okuyama *et al.* 1993). Using

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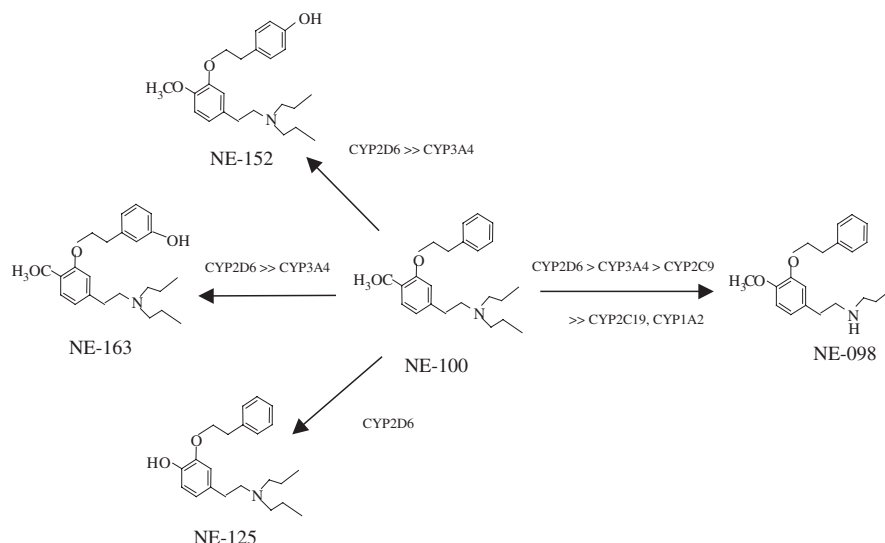


Figure 1. Primary metabolic pathways of NE-100 in human liver microsomes and cytochrome P450 forms involved in the formation of each metabolite. NE-152 + 163mix represents a mixture of the two hydroxylated metabolites (NE-152 and NE-163).

pooled human liver microsomes (HLM) and human intestinal microsomes (HIM), it was previously reported that the metabolism of NE-100 in liver is likely to be important for pharmacokinetics of NE-100 rather than that in the small intestine (Yamamoto *et al.* 2003a, b). As shown in figure 1, NE-100 is metabolized to *N*-despropyl-NE-100 (NE-098), *p*-hydroxy-NE-100 (NE-152), *m*-hydroxyl-NE-100 (NE-163) and *O*-demethy-NE-100 (NE-125) in HLM. Inhibition and kinetic studies have shown that the formation of NE-125 and NE-152 + 163mix are mediated by cytochrome P450 (CYP) 2D6, but the formation of NE-098 is catalysed by several CYP forms such as CYP3A4, CYP2D6, CYP2C9, CYP2C19 and CYP1A2. Among these CYP forms, CYP2D6 was estimated to be important for the overall metabolism of NE-100 by use of the relative activity factor (RAF) approach (Yamamoto *et al.* 2003a, b).

Considering a clinical trial of NE-100, the large contribution of CYP2D6 to metabolism suggests that the pharmacokinetics of NE-100 might be substantially affected by polymorphic expression of CYP2D6 and by inhibition of CYP2D6 activity with other co-administered drugs. On the other hand, the poor metabolizer (PM) status of CYP2D6 exists in about 5–10% in Caucasians (Broly *et al.* 1991) and less than 1% in Asians (Horai *et al.* 1989, Guengerich 1995). For drugs such as haloperidol, metoprolol, venlafaxine and atomoxetine, which are mainly metabolized by CYP2D6, their pharmacokinetics after oral administration were substantially different between EM and PM (Lennard *et al.* 1986, Llerena *et al.* 1992, Lessard *et al.* 1999, Sauer *et al.* 2003). Inhibition of CYP2D6-mediated metabolism also causes drug–drug interactions (Murray 1992). Large decreases of *in vivo* oral clearance (CL_{oral}) of metoprolol or venlafaxine have been observed when propafene or quinidine was given simultaneously, respectively (Wagner *et al.* 1987, Lessard *et al.* 1999). Although interindividual differences in plasma drug concentrations are based on many factors such as body weight, age, hepatic blood flow and expression level of the enzyme, genetic polymorphism of enzyme and

drug–drug interactions strongly affect the difference. Therefore, for drugs showing a narrow therapeutic window, identification of the major metabolizing enzymes and prediction of differences in plasma concentrations between EM and PM at the drug development stage is of importance to prevent serious clinical side-effects based on these factors. Therefore, before initiating clinical trials, an understanding of the contribution of CYP2D6 polymorphism to drug pharmacokinetics is important. As shown in previous *in vitro* studies using EM-HLM and rCYP, NE-100 is predominantly metabolized by CYP2D6 (Yamamoto *et al.* 2003b). *In vitro* studies using HLMs lacking CYP2D6 activity (PM-HLM) may provide useful information in NE-100 metabolism and the prediction of differences in the plasma concentration between CYP2D6 EM and PM. However, a stable supply of PM-HLM is very difficult to obtain. Therefore, an alternative method is required for identification of the metabolism and disposition of the major metabolites of NE-100 in PM.

In the current study, CYP forms involved in NE-100 metabolism in PM-HLM were also predicted using *in vitro* metabolic data from rCYPs and the RAF approach, then the prediction was compared with the observations on PM-HLM. In addition, *in vivo* CL_{oral} of NE-100 in the PM subject was predicted from *in vitro* metabolic data using the *in vivo*–*in vitro* scaling method based on the dispersion model (Rane *et al.* 1977, Roberts and Rowland 1986a, b, Wilkinson 1987).

Materials and methods

Chemicals and reagents

NE-100 hydrochloride and its metabolites, NE-125 (*N,N*-dipropyl-2-[4-hydroxy-3-(2-phenylethoxy)phenyl]ethylamine), NE-098 (*N,N*-propyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine), NE-152 (*N,N*-dipropyl-2-[4-methoxy-3-(2-(4-hydroxyphenyl)ethoxy)phenyl]ethylamine) and NE-163 (*N,N*-dipropyl-2-[4-methoxy-3-(2-(3-hydroxyphenyl)ethoxy)phenyl]ethylamine), were synthesized in Taisho Pharmaceutical Co. Ltd (Saitama, Japan). [^{14}C] NE-100, labelled at the phenoxy ring (97.5%, radiochemical purity, 8.53 MBq mg $^{-1}$), was synthesized at Amersham Biosciences UK, Ltd (Little Chalfont, UK). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and β -NADP $^{+}$ were from Wako Pure Chemical Industries (Osaka, Japan). Furafulylline, sulfaphenazole, *S*-mephentoin, quinidine and ketoconazole were purchased from Ultrafine Chemicals Co. (Manchester, UK). Troleandomycin and proadifen hydrochloride (SKF-525A) were purchased from Sigma-Aldrich (St Louis, MO, USA) and Funakoshi Co. Ltd (Tokyo, Japan), respectively. All other chemicals and solvents used were of the highest quality commercially available.

Enzymes

Pooled human liver microsomes (EM-HLM; H161-lot 3), human liver microsomes lacking CYP2D6 activity (PM-HLM; H030-lot 1) and microsomes of a human B lymphoblast cells expressing recombinant human CYP enzymes (i.e. CYP1A2, 2C9-Arg, 2C19, 2D6*1-Val and 3A4) were purchased from BD Gentest Corporation (Woburn, MA, USA).

Human blood and plasma collection

Human blood was collected into Venoject II tubes containing sodium heparin (Terumo, Tokyo, Japan) from 10 in-house Japanese healthy volunteers (age 29–43 years, sex male, weight 50–70 kg). Plasma was obtained by centrifuging the blood at 3000g for 5 min at 4°C, then stored at –40°C.

Relative contribution rate of CYP forms to the formation activity of each metabolite in human liver microsomes lacking CYP2D6 activity (PM-HLM)

Contribution rate under linear conditions for metabolite formation. The contribution rates of each CYP form to the formation of each metabolite in PM-HLM were estimated based on the kinetic data obtained from rCYPs reported in Yamamoto *et al.* (2003b, table 1) using the RAF approach (Crespi 1995). The intrinsic clearances ($CL_{\text{int.HLM-CYP-X}}$) for the formation of each metabolite in PM-HLM

Table 1. Kinetic parameters of formation activity of NE-100 metabolites in recombinant CYPs and the predicted $CL_{\text{int.HLM-CYP}}$ of PM-HLM for each CYP form.

Metabolites	CYP forms	Recombinant CYPs			HLM lacking CYP2D6 activity (PM-HLM)
		^1K_m (μM)	$^1V_{\text{max}}$ (pmol min^{-1} mg^{-1} protein)	$^1CL_{\text{int.cDNA-CYP-X}}$ (ml min^{-1} mg^{-1} protein)	Predicted $CL_{\text{int.HLM-CYP-X}}$ (ml min^{-1} mg^{-1} protein of HLM equivalent)
NE-098	2D6	0.1	38.6	0.405	0.000
	3A4	15.3	156.5	0.010	0.056
	2C9	93.9	942.3	0.010	0.034
	2C19	29.2	36.3	0.001	0.002
	1A2	15.1	12.5	0.001	0.005
	$^2\text{Total}$				0.097
NE-152 + 163mix	2D6	0.07	77.2	1.192	0.000
	3A4	9.8	19.1	0.002	0.011
	2C9	—	—	—	—
	2C19	—	—	—	—
	1A2	—	—	—	—
	$^2\text{Total}$				0.011
NE-125	2D6	0.06	118.4	2.121	0.000
	3A4	—	—	—	—
	2C9	23.6	10.9	0.001	0.002
	2C19	144.9	9.9	0.001 >	0.001 >
	1A2	—	—	—	—
	$^2\text{Total}$				0.002

Kinetic studies on the formation of NE-098, NE-152 + 163mix and NE-125 in the recombinant human P450s (CYP2C9-Arg, 2D6-Val, and 3A4) were done using substrate concentration from 0.05 to 20.0 μM . For CYP1A2 and CYP2C19, the kinetic parameters were estimated using results obtained from a preliminary study on microsomal protein concentration. K_m and V_{max} values in recombinant CYPs were determined using Eadie-Hofstee plots. $CL_{\text{int.cDNA-CYP-X}}$ was calculated from ratio of V_{max} to K_m . Predicted $CL_{\text{int.HLM-CYP-X}}$ was calculated from kinetic parameters on recombinant CYPs using RAF approaches, as described in the Materials and methods.

¹These kinetic parameters obtained from recombinant CYPs microsomal samples have already been reported (Yamamoto *et al.* 2003b).

²Total represents the total intrinsic clearance (predicted $CL_{\text{int.HLM-total}}$) for the formation of each metabolite in PM-HLM and the intrinsic clearance was expressed as the sum of the $CL_{\text{int.HLM-CYP-X}}$ for each CYP form except that for CYP2D6 as follows: for each metabolite formations, the predicted $CL_{\text{int.HLM-total}} = CL_{\text{int.HLM-3A4}} + CL_{\text{int.HLM-2C9}} + CL_{\text{int.HLM-1A2}} + CL_{\text{int.HLM-2C19}}$.

—, Not detected. In this study, RAF for each CYP form were calculated based on the ratio of activity for a relatively specific reaction in HLM lacking CYP2D6 activity (PM-HLM, H030-lot 1) divided by the activity for that same reaction in recombinant CYPs. The specific activity for each CYP form in donor sample (H030-lot 1) used for calculating the RAF has been described in the data sheet provided by the manufacturer. Since PM-HLM lacks CYP2D6 activity, the RAF for CYP2D6 was considered to be zero.

were calculated as follows:

$$CL_{\text{int.HLM-CYP-X}} = CL_{\text{int.cDNA-CYP-X}} \times \text{RAF-CYP-X}, \quad (1)$$

where $CL_{\text{int.cDNA-CYP-X}}$ and RAF-CYP-X are the intrinsic clearance of the formation for each metabolite obtained with recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 respectively, and the RAFs for each CYP form. According to the data sheet supplied by the manufacturer, the RAF of CYP2D6 was considered to be zero, since bufuralol 1'-hydroxylation activity, the specific activity of CYP2D6, was not detected in PM-HLM (H030-lot 1). The total intrinsic clearance ($CL_{\text{int.HLM-total}}$) for formation of each metabolite in PM-HLM was expressed as the sum of the $CL_{\text{int.HLM-CYP-X}}$ for each CYP form (table 1). The contribution rates of each CYP form to the $CL_{\text{int.HLM-total}}$ for the formation of each metabolite were estimated as the per cent of $CL_{\text{int.HLM-CYP-X}}$ to $CL_{\text{int.HLM-total}}$.

Relative rate of contribution of CYP forms as a function of substrate concentration. The relative contribution rates of each CYP form to the formation of each metabolite depending on substrate concentration in PM-HLM were predicted using a kinetic parameter (K_m , V_{max}) with recombinant CYP forms and the RAFs. The formation activities of individual metabolites ($V_{HLM-CYP}$) for each CYP form in PM-HLM at various substrate concentrations were calculated as follows:

$$V_{HLM-CYP-X} = (V_{max,cDNA-CYP-X} \times \text{RAF}_{CYP-X} \times S) / (K_{m,cDNA-CYP-X} + S), \quad (2)$$

where S , $K_{m,cDNA-CYP-X}$, $V_{max,cDNA-CYP-X}$ and RAF_{CYP-X} are substrate concentration, affinity constants, the maximum formation activity of each metabolite and RAFs for each CYP, respectively. The total formation activity ($V_{HLM-total}$) of each metabolite at various substrate concentrations in PM-HLM was expressed as the sum of the $V_{HLM-CYP-X}$ for each recombinant CYP form. The contribution rates of each CYP form to the formation activity of individual metabolites in PM-HLM at various substrate concentrations were estimated as the per cent of $V_{HLM-CYP-X}$ to $V_{HLM-total}$.

Kinetic study of NE-100 metabolism by human liver microsomes lacking CYP2D6 activity (PM-HLM)

Kinetic studies on NE-100 disappearance and the formation of NE-100 metabolites were undertaken using PM-HLM. Incubation medium contained 0.20 mg ml⁻¹ PM-HLM, 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.4 mM β -NADP⁺, 8 mM glucose 6-phosphate, 8 mM MgCl₂, 0.5 IU ml⁻¹ glucose 6-phosphate dehydrogenase) and various concentrations of NE-100, in a final volume of 500 μ l. After pre-incubation at 37°C for 5 min, the reaction was initiated by adding NE-100 at concentrations ranging from 0.05 to 20.0 μ M for 1–30 min and was terminated by adding 1 ml methanol. Linearity in the disappearance velocity of NE-100 and the formation activity for each metabolite, with respect to the concentration of microsomes and the incubation time, was assessed by least-squares linear regression using Microsoft Excel.

Kinetic study of NE-100 metabolism by pooled human liver microsomes (EM-HLM)

The kinetic study for NE-100 disappearance was undertaken using EM-HLM. The metabolism of NE-100 in EM-HLM (0.20 mg ml⁻¹ microsomal protein) was initiated by adding NE-100 at concentrations ranging from 0.05 to 20.0 μ M for 0.2–30 min. Other reaction conditions and procedures were the same as described above.

Kinetic study of NE-100 metabolism by recombinant human CYP forms

Kinetic studies for NE-100 disappearance were undertaken using rCYPs (CYP1A2, 2C9-Arg, 2C19, 2D6-Val and 3A4). The reaction contained recombinant CYP2D6, CYP2C9 or CYP3A4 microsomal proteins of 0.20, 0.46 or 0.43 mg ml⁻¹, respectively, was initiated by adding NE-100 at concentrations ranging from 0.05 to 20.0 μ M. Incubation times were for 0.2–30 min for CYP2D6 and for 0.2–30 min in CYP2C9 and CYP3A4, respectively. Other reaction conditions were the same as described for HLM.

Chemical inhibition study

The effects of selective inhibitors of CYP forms on the formation of NE-100 metabolites in PM-HLM were undertaken using 0.1 and 10.0 μ M NE-100. The concentration of inhibitors used and experimental conditions (except for the incubation time) were as described by Yamamoto *et al.* (2003a). Incubation times were 10 min (for 0.1 μ M NE-100) and 15 min (for 10.0 μ M NE-100). Final methanol concentration in the incubation medium was 0.5%. Control experiments were undertaken under the same conditions but without an inhibitor.

Plasma protein binding of NE-100

To 1.96 ml human plasma, 0.04 ml distilled water solution containing NE-100 were added to give final concentrations of 1, 10 and 100 μ M. After incubation for 30 min at 37°C, 0.05 ml plasma sample were taken from each plasma sample to measure the total plasma concentration and the residual 0.5 ml was transferred to an ultrafiltration tube (Centrifree YM-30, Millipore Corp., Bedford, MA, USA) in triplicate. The tubes were centrifuged for 15 min (1000g at 25°C) and 100 μ l filtrate were removed to measure the unbound plasma concentration. Plasma and filtrated samples were subjected to liquid scintillation counting with 10 ml liquid scintillator. Potassium phosphate buffer instead of plasma was similarly treated for the correction of adsorption.

Microsomal protein binding of NE-100 in incubation medium

Microsomal protein binding in the incubation medium, including each microsomal sample (EM-HLM, PM-HLM, rCYP2D6, rCYP3A4 and rCYP2C9) was determined using the same method described above. The incubation medium contained 0.20 mg ml⁻¹ (for EM-HLM, PM-HLM and

rCYP2D6), 0.46 mg ml⁻¹ (for rCYP3A4) or 0.43 mg ml⁻¹ (for rCYP2C9), 50 mM potassium phosphate buffer (pH 7.4) and various concentrations of NE-100 (0.1, 1, 10 and 100 µM as the final concentration).

Blood-plasma concentration ratio (R_B) of NE-100 in humans

To 0.98 ml heparinized human whole blood, 0.02 ml of a distilled water solution containing NE-100 were added to give final concentrations of 0.1, 1 and 10 µM. After incubation for 15 min at 37°C, 0.05 ml blood sample was taken to measure the blood concentration. The plasma samples were obtained by centrifuging 0.5 ml of the remaining blood for 5 min (1000g at 25°C) in triplicate. Each blood sample was solubilized with a mixture of Soluene-350 (Packard, Meriden, CT, USA) and isopropanol in 1 ml, decolourized by adding 30% H₂O₂, and the blood and plasma samples were subjected to liquid scintillation counting with 10 ml liquid scintillator.

Assay of unchanged NE-100 and its metabolites

The reaction mixtures containing methanol were shaken and centrifuged at 3000 rpm for 10 min, then the supernatant was evaporated to dryness and redissolved in methanol. To separate the unchanged NE-100 and its metabolite in the sample, two-dimensional thin layer chromatography (2D-TLC) analysis was undertaken using TLC plates and solvent systems (Yamamoto *et al.* 2003b). Radioluminography, BAS2000 system (Fuji-Film, Tokyo, Japan), was used to quantify these metabolites. As NE-152 and NE-163 could not be quantitatively separated by the TLC method in this experiment, these metabolites were measured as a mixture of NE-152 and NE-163 and expressed as NE-152 + 163mix.

Data analysis

Assuming one- or two-enzyme models as described by Nakajima *et al.* (1999), kinetic data on enzyme, K_m and V_{max} (K_{m1} and V_{max1} for high affinity component, K_{m2} and V_{max2} for low affinity component) for NE-100 disappearance in EM-HLM were estimated using a residual analysis on Eadie-Hofstee plots based on visual inspection. The kinetic data for NE-100 disappearance and formation of each metabolite in PM-HLM were estimated by the least-squares linear regression using Microsoft Excel on Eadie-Hofstee plots. Intrinsic clearance (CL_{int}) was calculated as ratio of the V_{max} to the K_m . Any statistical significance of differences between the control and inhibitor treatments was determined using Dunnett's test (SAS system for windows, v.6.1; SAS Institute, Inc., Cary, NC, USA). $p < 0.05$ was considered as being statistically significant.

Prediction of in vivo CL_{oral} of NE-100 in CYP2D6 extensive and poor metabolizers from in vitro metabolic data

To predict differences of *in vivo* oral clearance (CL_{oral}) of NE-100 between extensive (EM) and poor metabolizers (PM) of CYP2D6, various *in vitro* studies were undertaken using human blood, plasma, EM-HLM, PM-HLM, and rCYP. The *in vivo* hepatic clearance (CL_H) of NE-100 was calculated from the intrinsic clearance for the disappearance of NE-100 in EM and PM using the *in vivo-in vitro* scaling based on the dispersion model (Roberts and Rowland 1986a, Sugiyama *et al.* 1988), because there are some successful predictions of *in vivo* hepatic clearance by use of the model regarding high-clearance drugs (Houston 1994, Iwatsubo *et al.* 1996, 1997a, Houston and Caelile 1997). The relationship between CL_H , hepatic blood flow (Q_H) and hepatic availability (F_H) is expressed as follows:

$$CL_H = Q_H(1 - F_H) \quad (3)$$

$$F_H = 4a / \{(1 + a)^2 \exp((a - 1)/2 \times D_N) - (1 - a)^2 \exp(-(1 + a)/2 \times D_N)\} \quad (4)$$

$$a = (1 + 4R_N \times D_N)^{1/2} \quad (5)$$

$$R_N = f_B \times CL_{int,free} / Q_H \quad (6)$$

$$CL_{int,free} = \sum (V_{max-n} / (K_{m-n} \times f_{Ms})), \quad n = 1 \text{ or } 2$$

$$\text{or } \sum ((V_{max,cDNA-CYP-X} \times \text{RAF}_{-CYP-X}) / (K_{m,cDNA-CYP-X} \times f_{Ms,CYP-X})) \quad (7)$$

$$f_B = f_P / R_B \quad (8)$$

$$R_B = C_{blood} / C_{plasma}, \quad (9)$$

where R_B is ratio of blood concentration (C_{blood}) to plasma concentration (C_{plasma}) of NE-100; f_P , f_B and f_{Ms} are unbound fraction of NE-100 in plasma, blood and incubation medium, respectively; $CL_{int,free}$ (observed) is the hepatic intrinsic clearance of NE-100 disappearance in EM- and PM-HLM, taking into consideration the f_{Ms} in each incubation medium; and $CL_{int,free}$ (predicted) is the hepatic intrinsic

clearance corrected in EM- and PM-HLM from kinetic parameter in rCYPs using RAF approach, taking into consideration the f_{M_s} in each incubation medium. In addition, the $CL_{int,free}$ values are expressed as per g liver using the microsomal protein contents in 1 g liver (52.5 mg protein g⁻¹ liver) (Iwatsubo *et al.* 1997a). R_N and D_N are the efficiency and dispersion numbers, respectively. Q_H and D_N values used for calculation of CL_H were 0.95 ml min⁻¹ g⁻¹ liver (Bischoff *et al.* 1971, Dedrick *et al.* 1973) and 0.17 (experience value) (Roberts and Rowland 1986b, Iwatsubo *et al.* 1996, 1997a). Oral clearance (CL_{oral}) was calculated using the following equation (Iwatsubo *et al.* 1997b):

$$CL_{oral} = (CL_H + CL_R + CL_L + F_H \times CL_{GM}) / (F_H \times F_a \times F_g), \quad (10)$$

where CL_R , CL_L and CL_{GM} are the clearances in renal, lung and intestinal membrane, respectively; and F_a and F_g are the fraction absorbed from the gastrointestinal tract to the portal circulation blood, and the intestinal availability. For calculations of CL_{oral} for EM and PM, it was assumed that gut metabolism was the same in EM and PM and that CL_L and CL_{GM} were negligible. Furthermore, the CL_R for humans was regarded as 0 ml min⁻¹ because the unchanged drug was not detected in the urine after intravenous and oral administration of NE-100 to rats (data not shown). The $F_a \times F_g$ used was 0.733, which is the elimination ratio (oral/intravenous) of total radioactivity in urine after oral and intravenous administration of [¹⁴C] NE-100 to rats (data not shown).

Results

Prediction of relative contribution rate of CYP forms in human liver microsomes lacking CYP2D6 activity

As shown in table 1 and figure 2(A), predicted $CL_{int,HLM-total}$ values of PM-HLM for each metabolite from kinetic data on rCYPs were 0.097 (for NE-098 formation), 0.011 ml min⁻¹ mg⁻¹ protein (for NE-152 + 163mix formation) and 0.002 ml min⁻¹ mg⁻¹ protein (for NE-125 formation). As described in the Materials and methods, the contributions of CYP3A4, CYP2C9, CYP2C19 and CYP1A2 to NE-098 formation were estimated as 58.1, 34.6, 2.4 and 4.9%, respectively. On the other hand, it was determined that NE-152 + 163mix is formed only by CYP3A4. The contribution of CYP forms to NE-125 formation was estimated to be 92.8% for CYP2C9 and 7.2% for CYP2C19.

Relative contribution rate of each CYP form as a function of substrate concentration

Figure 2(B–E) shows the predicted change in contribution of CYP forms for the overall metabolism of NE-100 and three metabolite formations as a function of the concentration of substrate, respectively. The formation of NE-098 at low concentration of NE-100 was predominantly catalysed by CYP3A4, but CYP2C9 contributed to the formation more than CYP3A4 at substrate concentrations over 15 µM. The formation of NE-152 + 163mix is catalysed by only CYP3A4 and NE-125, which is the major metabolite in EM-HLM, and was mainly formed by CYP2C9. The contribution rates of CYP2C9 and CYP2C19 to NE-125 formation at 10 µM were estimated to be 90 and 10%, but the $CL_{int,HLM-total}$ of PM-HLM for NE-125 formation was negligible compared with that for other metabolite formation. Even when the substrate concentration was increased, the contribution rates of CYP forms to the formation of these metabolites in PM-HLM do not extensively change. As shown in figure 3(A), the contribution rate of NE-098 formation to the overall metabolism of NE-100 in PM-HLM was estimated to be over 80% using the RAF approach.

Kinetic study of NE-100 metabolism in human liver microsomes lacking CYP2D6 activity

Possible metabolic pathways of NE-100 in PM-HLM are *N*-depropylation (forming NE-098) and hydroxylation of the phenyl ring (forming NE-152 and

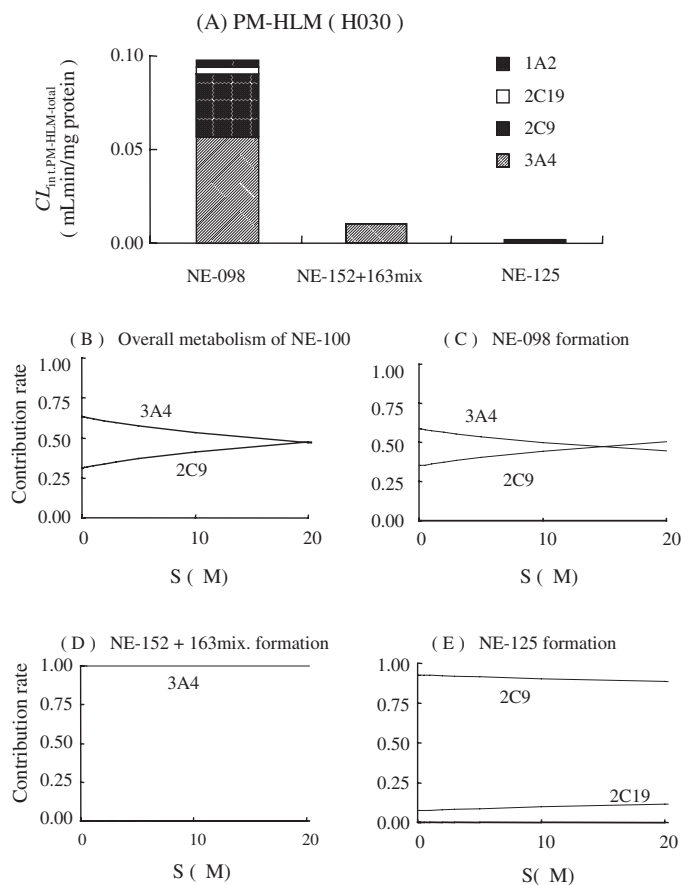


Figure 2. Hepatic intrinsic clearance and relative contribution for each metabolite formation in human liver microsomes lacking cytochrome P450 (CYP) 2D6 activity. Clearance was calculated by extrapolating *in vitro* kinetic data with recombinant CYP microsomes (rCYPs) to human liver activity using a relative activity factor (RAF) approach. Predicted $CL_{int,HLM-total}$ was calculated from kinetic parameters on recombinant CYPs using the RAF approach as described in the Materials and methods (figure 2A). The kinetic data used were reported in Yamamoto *et al.* (2003b). The RAFs for each CYP form were calculated as the ratio of the specific activities described in the data sheet provided by the manufacturer in HLM and rCYPs. Substrate-dependent changes in the contribution rates of each CYP form for the overall metabolism of NE-100, NE-098, NE-152 + 163mix and NE-125 formation are shown in figure 2(B–E), respectively.

NE163). As NE-152 and NE-163 could not be quantitatively separated by the TLC method in this experiment, these metabolites were measured as a mixture and expressed as NE-152 + 163mix. Eadie–Hofstee plots for NE-098 and NE-152 + 163mix formations in the PM-HLM showed a monophasic profile (data not shown). The K_m values of NE-098 and NE-152 + 163mix formation were similar, but the V_{max} of NE-098 formation was 21 times higher than that of NE-152 + 163mix formation (table 2). The $CL_{int,HLM}$ is also 14.1 times different between these metabolites. The kinetic study with varying NE-100 concentrations confirmed that a major metabolic pathway in PM-HLM is NE-098 formation and the contribution rate of the formation to the entire metabolism of NE-100 is over 80% (figure 3B).

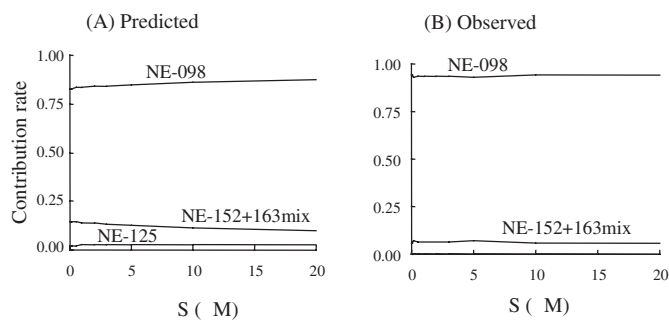


Figure 3. Comparison between the predicted (A) and observed (B) contribution rates of the formation of each NE-100 metabolite in PM-HLM. The contribution rates of the formation for each metabolite were calculated from the ratio of formation activity for each metabolite to the sum of activities of NE-125, NE-152 + 163mix and NE-098 at each substrate concentration. (A) Formation activities of metabolites were estimated using the relative activity factor approach from *in vitro* kinetic parameters of recombinant CYPs. (B) Formation activities of these metabolites were observed in human liver microsomes lacking CYP2D6 activity.

Table 2. Kinetics parameters for the formation of NE-100 metabolites in human liver microsomes lacking CYP2D6 activity.

	K_m (μM)	$V_{\max}$ ($\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$)	$\text{CL}_{\text{int. PM-HLM}}$ ($\text{ml min}^{-1} \text{mg}^{-1} \text{protein}$)
NE-098	27.1	1878.6	0.069
NE-152 + 163mix	18.2	89.3	0.005

K_m and $V_{\max}$ values were calculated using Eadie-Hofstee plots and $\text{CL}_{\text{int.HLM}}$ was calculated based on the ratio of $V_{\max}$ to K_m as described in the Materials and methods.

PM-HLM represents human liver microsomes lacking CYP2D6 activity.

Inhibition study in human liver microsomes lacking CYP2D6 activity

As shown in figure 4, at two substrate concentrations (0.1 and 10 μM), the formation of NE-098 and NE-152 + 163mix was inhibited by about >80% by 100 μM SKF-525A (non-selective inhibitor). The formation of NE-152 + 163mix was significantly inhibited by ketoconazole and troleandomycin (selective CYP3A4 inhibitors), but the formation of NE-098 was inhibited by multiple CYP inhibitors at both concentrations of the substrate. Ketoconazole inhibited the formation activity of NE-098 by 62.2 and 55.0% at 0.1 and 10 μM NE-100, respectively. Sulfaphenazole, a selective CYP2C9 inhibitor, inhibited the formation activity of NE-098 by 44.1 and 37.7% at low and high concentrations of the substrate but *S*-mephenytoin, a substrate of CYP2C19, inhibited only slightly the formation activity of NE-098. These results suggest that CYP forms involved in the formation of these metabolites in PM-HLM are mainly CYP3A4 and the contribution rates of CYP forms to these metabolite formations do not change at the ranges of substrate concentrations used in this inhibition study.

Comparison between the predicted $\text{CL}_{\text{int-HLM-total}}$ and the observed $\text{CL}_{\text{int-HLM}}$ in EM-HLM and PM-HLM

Figure 5 shows Eadie-Hofstee plots for NE-100 disappearance in EM and PM-HLM, and table 3 shows the kinetic parameters obtained from EM-HLM, PM-HLM and rCYPs. The plot for EM-HLM gave biphasic curves but the plot

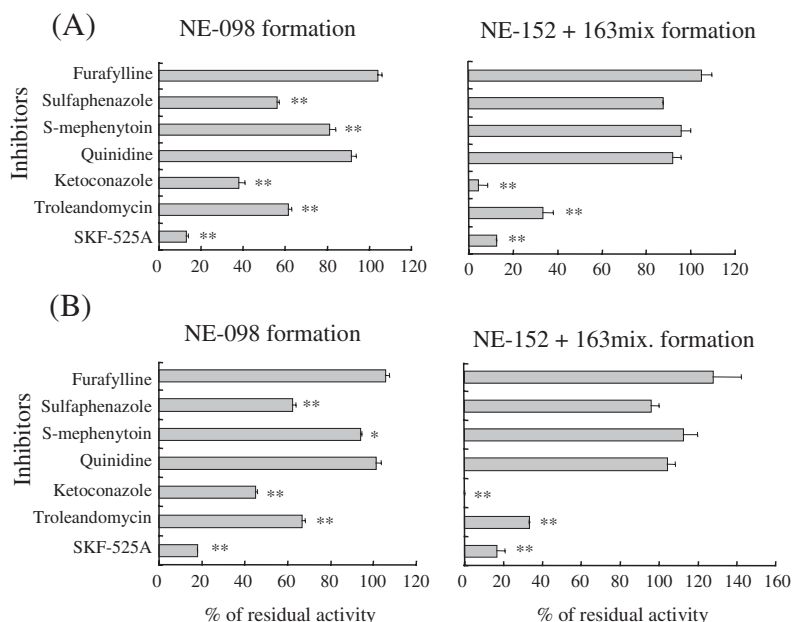


Figure 4. Effect of specific inhibitors of cytochrome P450 forms on formation activities of NE-100 metabolites by human liver microsomes lacking CYP2D6 activity (H030). Substrate concentrations used were 0.1 μM (A) and 10 μM (B). Each value is the mean of triplicate determinations. Concentrations of inhibitor used are as follows: furafylline (10 μM), sulfaphenazole (10 μM), S-mephenytoin (50 μM), quinidine (1 μM), ketoconazole (0.5 μM), troleandomycin (10 μM) and SKF-525A (100 μM). ** $p < 0.01$, * $p < 0.05$.

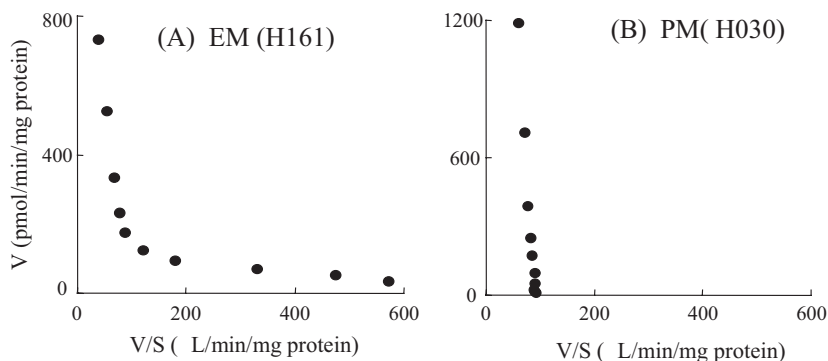


Figure 5. Eadie-Hofstee plots for NE-100 disappearance in pooled human liver microsomes (A) and human liver microsomes lacking CYP2D6 activity (B). The ranges of substrate concentration used were from 0.05 to 20 μM . V ; NE-100 disappearance velocity; S , NE-100 concentration. EM, PM, extensive and poor metabolizer of CYP2D6, respectively. Data are the mean of duplicate determinations.

for PM-HLM was monophasic, thus indicating that both high ($K_{m1} = 0.15 \mu\text{M}$) and low affinity enzymes ($K_{m2} = 7.2 \mu\text{M}$) are involved in NE-100 metabolism in EM-HLM but that only the low affinity enzyme ($K_m = 37.9 \mu\text{M}$) is responsible for the metabolism in PM-HLM. The intrinsic clearance (the observed $CL_{\text{int-HLM}}$) observed in EM- and PM-HLM was 0.872 and 0.089 $\text{ml min}^{-1} \text{mg}^{-1}$ protein, respectively. The K_m in recombinant CYP2D6 was the lowest among the rCYPs

Table 3. Kinetic parameters for the disappearance activity of NE-100 in recombinant CYPs, HLM and HLM lacking CYP2D6 activity.

CYP forms	Recombinant CYPs			Predicted $CL_{int,HLM-CYP-X}$ ($ml\ min^{-1}\ mg^{-1}\ protein$ of HLM equivalent)	
	K_m	V_{max}	$CL_{int,cDNA-CYP-X}$	EM-HLM	PM-HLM
	(μM)	($pmol\ min^{-1}\ mg^{-1}\ protein$)	($ml\ min^{-1}\ mg^{-1}\ protein$)	(H161)	(H030)
2D6	0.1	289.3	5.671	0.913	0.000
3A4	12.8	216.7	0.017	0.076	0.094
2C9	79.9	1096.8	0.014	0.015	0.046
2C19	14.2	17.6	0.001	0.002	0.001
1A2	19.9	21.1	0.001	0.002	0.006
EM				1.008 ¹	
PM					0.148 ²
HLM	K_{m1}	K_{m2}	V_{max1}	V_{max2}	Observed $CL_{int,HLM}$
	(μM)		($pmol\ min^{-1}\ mg^{-1}\ protein$)		($ml\ min^{-1}\ mg^{-1}\ protein$)
EM (H161)	0.15	7.2	116.4	754.6	0.872
PM (H030)		37.9		3356.0	0.089

Kinetic studies on NE-100 disappearance in recombinant human P450s (CYP2C9-Arg, 2D6-Val, and 3A4) were done using substrate concentrations from 0.05 to 20.0 μM . For CYP1A2 and CYP2C19, the kinetic parameters were estimated based on the results obtained from a preliminary study on microsomal protein concentration. K_m and V_{max} values in recombinant CYPs determined using Eadie-Hofstee plots. $CL_{int,cDNA-CYP-X}$ was calculated from ratio of V_{max} to K_m on recombinant CYPs. Predicted $CL_{int,HLM-CYP-X}$ was calculated based on kinetic parameters on recombinant CYPs using RAF approaches, as described in the Materials and methods.

¹The predicted $CL_{int,HLM-total}$ for EM-HLM (pooled human liver microsomes) was calculated as sum of $CL_{int,HLM-cDNA-CYP-X}$ obtained from five recombinant CYPs.

²The predicted $CL_{int,HLM-total}$ for PM-HLM (human liver microsomes lacking CYP2D6 activity) was calculated as sum of $CL_{int,HLM-cDNA-CYP-X}$ obtained from four recombinant CYPs except CYP2D6. In this study, RAFs for each CYP form were calculated based on the ratio of the activity for a relatively specific reaction in EM-HLM (H161) or PM-HLM (H030) divided by the activity for the same reaction in recombinant CYP. The specific activity for each CYP form used for calculating the RAFs were described in the data sheet provided by the manufacturer.

Assuming one- or two-enzyme models as shown by Nakajima *et al.* (1999) kinetic data on enzyme, K_m and V_{max} values (K_{m1} and V_{max1} for high-affinity component, K_{m2} and V_{max2} for low-affinity component) for the disappearance of NE-100 in EM-HLM were estimated using residual analysis on Eadie-Hofstee plots based on visual inspection. For NE-100 metabolism in EM-HLM, the K_{m1} and V_{max1} represent apparent affinity constant and apparent maximum activity of NE-100 disappearance for high-affinity components, and K_{m2} and V_{max2} represent apparent affinity constant and apparent maximum activity for low-affinity components. $CL_{int,HLM}$ of NE-100 disappearance in EM-HLM were calculated as the sum of the ratio of V_{max} to K_m in the high- and low affinity components based on two enzyme models. For NE-100 metabolism in PM-HLM, K_m and V_{max} represent apparent affinity component and apparent maximum activity of NE-100 disappearance. The $CL_{int,HLM}$ in EM-HLM was calculated as the ratio of V_{max} to K_m .

tested and was close to the K_{m1} in EM-HLM. On the other hand, the K_m values in other rCYPs were close to the K_{m2} in EM-HLM and the K_m in PM-HLM. The intrinsic clearance (the predicted $CL_{int,HLM-total}$) for EM- and PM-HLM estimated from the kinetic parameters using the RAF approach was 1.008 and 0.148 $ml\ min^{-1}\ mg^{-1}\ protein$, respectively. When the intrinsic clearance for NE-100 disappearance, NE-098, NE-152 + 163mix and NE-125 formation obtained from both HLMs were plotted in the same figure, there was a good correlation between the predicted $CL_{int,HLM-total}$ and the observed $CL_{int,HLM}$ (figure 6).

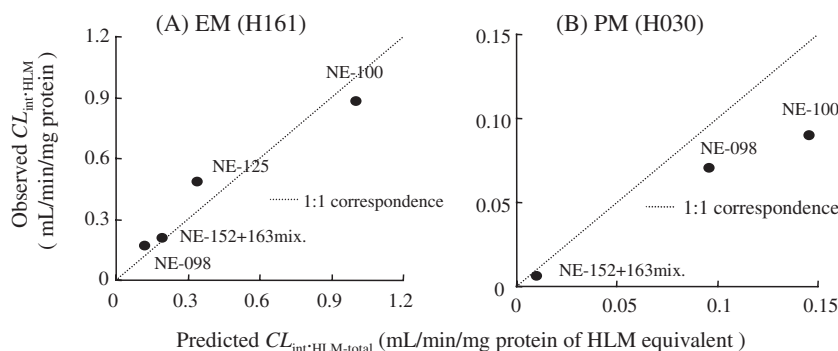


Figure 6. Correlation between the predicted $CL_{int,HLM-total}$ of NE-100 disappearance and formation of each metabolite from recombinant cytochrome P450 (CYP) and the observed $CL_{int,HLM}$ of NE-100 disappearance and formations of each metabolite from pooled human liver microsomes (A) or from human liver microsomes lacking CYP2D6 activity (B) $CL_{int,HLM-total}$ expresses the sum of $CL_{int,HLM-CYP}$ for NE-100 disappearance or formations of each metabolite, which was calculated by extrapolating *in vitro* kinetic data with each recombinant CYP form to human liver activity using a relative activity factor approach as described in the Materials and methods. $CL_{int,HLM}$ expresses the CL_{int} observed in human liver microsomes (EM-HLM) or human liver microsomes lacking CYP2D6 activity (PM-HLM). For NE-125, NE-152 + 163mix and NE-098 formations, the observed $CL_{int,HLM}$ and predicted $CL_{int,HLM-total}$ for EM-HLM were reported by Yamamoto *et al.* (2003b). Since no NE-125 was formed in the PM-HLM, the data are not shown here. Dotted lines represent means of equal value between the predicted $CL_{int,HLM-total}$ and the observed $CL_{int,HLM}$.

Prediction of *in vivo* CL_{oral} of NE-100 in extensive and poor metabolizers of CYP2D6 from *in vitro* data of metabolism

The various *in vitro* parameters for predicting the *in vivo* CL_{oral} of EM and of PM from *in vitro* data are summarized in table 4. After the hepatic intrinsic clearance ($CL_{int-free}$) was calculated by taking into consideration the unbound fractions of NE-100 in the incubation medium, the CL_{oral} of NE-100 for EM and PM was predicted using the dispersion model. The predicted values of the CL_{oral} for EM and PM using the metabolic data from rCYP and the RAF approach were 229.2 and 4.7 $l\ min^{-1}$, respectively. The difference in the CL_{oral} between EM and PM was predicted to be 48.4-fold. On the other hand, the predicted CL_{oral} for EM and PM using the metabolic data in pooled HLM and HLM lacking CYP2D6 activity were 201.7 and 2.3 $l\ min^{-1}$. The difference in the CL_{oral} between EM and PM was predicted to be 88.2-fold.

Discussion

Comparing the metabolism of NE-100 between human liver and intestine, it was concluded that hepatic metabolism mainly contributes to the first-pass metabolism of NE-100 more so than does the small intestinal metabolism. NE-100 is metabolized to NE-098, NE-152, NE-163 and NE-125 in EM-HLM. Judging from inhibition, kinetic and recombinant CYP studies, NE-125 (the major metabolite in EM-HLM) and NE-152 + 163mix were predominantly formed by CYP2D6 and that NE-098 was formed by multiple CYP forms including CYP2D6 in EM-HLM. These data strongly suggest that CYP2D6 is a dominant form for NE-100 metabolism in humans at a substrate concentration corresponding to plasma levels in patients (Yamamoto *et al.* 2003a, b).

Table 4. *In vitro* parameters of NE-100 used for prediction of *in vivo* CL_{oral} based on the disappearance of NE-100 between extensive and poor metabolizers of CYP2D6.

Parameters		Human liver microsomes		Recombinant CYPs				
		EM-HLM	PM-HLM	EM-HLM		PM-HLM		
Observed								
$CL_{int. free}$	(ml min ⁻¹ g ⁻¹ liver)	65.2	6.8	–		–		
Predicted								
$CL_{int. free}$	(ml min ⁻¹ g ⁻¹ liver)	–	–	67.8		11.6		
f_{Ms}		0.70	0.68	$f_{Ms,2D6}$, (0.80,	$f_{Ms,3A4}$, 0.65,	$f_{Ms,2C9}$ 0.68,	$f_{Ms,2C19}$ 1.00,	$f_{Ms,1A2}$ 1.00)
f_p								0.076
R_B	$= C_{blood}/C_{plasma}$							0.698
CL_R	(ml min ⁻¹ body)							0.000
$F_a \cdot F_g$								0.733
Q_H	(ml min ⁻¹ g ⁻¹ liver)							0.95
Liver weight	(g liver kg ⁻¹ body)							24.3
Microsomes contents	(mg protein g ⁻¹ liver)							52.5
D_N								0.17
Body weight	(kg)							70.0

EM-HLM and PM-HLM are human liver microsomes from extensive metabolizers and a poor metabolizer of CYP2D6, respectively. Observed $CL_{int.free}$ represents the intrinsic clearance of the disappearance of NE-100 observed in EM-HLM and PM-HLM, taking into consideration the unbound fraction of NE-100 in incubation medium (f_{Ms}). Predicted $CL_{int.free}$ represents the intrinsic clearance of the disappearance of NE-100 calculated from kinetic parameters on recombinant CYPs using RAF approach EM-HLM and PM-HLM, taking into consideration the unbound fraction of NE-100 in each incubation medium ($f_{Ms,2D6}$, $f_{Ms,3A4}$, $f_{Ms,2C19}$, $f_{Ms,2C9}$, $f_{Ms,1A2}$). $f_{Ms,2C9}$, $f_{Ms,1A2}$, $f_{Ms,2C9}$ and $f_{Ms,1A2}$ were assumed as 1.000. An $F_a \cdot F_g$ was used (0.724) which is the elimination ratio (po/iv) of total radioactivity in urine after oral administration of NE-100 to rats. CL_R (*in vivo* renal clearance) of NE-100 was as 0 ml min⁻¹, because unchanged drugs were not detected in rat urine after oral administration. To predict difference in CL_{oral} based on deficiency of CYP2D6 activity between EM and PM, we assumed that CL_L and CL_{GM} were negligible in this study. f_p , unbound fraction in human plasma; R_B , human blood–plasma concentration ratio; Q_H , hepatic blood flow; D_N , dispersion number.

Table 5. Prediction of differences in the predicted *in vivo* CL_{oral} between extensive and poor metabolizers of CYP2D6.

Microsomes	Predicted CL_{oral} ($l \cdot min^{-1}$)		CL_{oral} ratio (EM/PM)
	EM	PM	
rCYP	229.2	4.7	48.4
HLM	201.7	2.3	88.2

Oral clearance (CL_{oral}) of NE-100 for EM and PM was predicted from various *in vitro* parameters (such as shown in table 4) using the dispersion model. HLM: these data were predicted using kinetic data obtained on human liver microsomes. rCYP: these data were predicted using kinetic data obtained on five recombinant CYPs. CL_{oral} ratio represents ratio of CL_{oral} in EM to that in PM.

In the present study, the major metabolite and contribution rate of CYP forms involved in the formation of NE-100 metabolites wherein NE-100 was administered to a patient lacking CYP2D6 activity (PM) were predicted using the kinetic parameters obtained from rCYPs, except for CYP2D6 and the RAF approach. The predicted $CL_{int-HLM-total}$ for NE-098 formation in PM-HLM was the highest among the metabolites of NE-100 and was nine times greater than that for NE-152 + 163mix. The predicted $CL_{int-HLM-total}$ for NE-125, a major metabolite in EM-HLM, was negligible. This result shows that the major metabolite is NE-098 in PM-HLM, although the major metabolite is NE-125 in EM-HLM. At NE-100 concentrations corresponding to *in vivo* conditions ($K_m \gg S$), it was predicted that CYP3A4 and CYP2C9 are the major CYP forms catalysing the NE-098 formation and CYP3A4 is the only CYP form catalysing NE-152 + 163mix formation. As shown in figure 2(B), CYP forms involved in the overall metabolism of NE-100 are predicted to be CYP3A4 and CYP2C9. Even when substrate concentrations were increased, the contribution rates of CYP forms to the formation of these metabolites in PM-HLM did not significantly change (figure 2). Kinetic and inhibition studies were undertaken to confirm the predicted findings using HLM obtained from CYP2D6 PM. A kinetic study using PM-HLM showed that NE-100 was metabolized to NE-098 and NE-152 + 163mix but not to NE-125. An inhibition study using PM-HLM supported the predicted finding that CYP forms involved in NE-098 and NE-152 + 163mix formations were mainly CYP3A4 and CYP2C9, and only CYP3A4, respectively. The effect of specific inhibitors on formation of these metabolites was not dependent on the substrate concentration. This result suggests that the contribution of CYP forms involved in NE-100 metabolism in PM-HLM can be estimated using kinetic data obtained from rCYPs without using PM-HLM. For NE-100 disappearance, the predicted $CL_{int-HLM-total}$ from the kinetic data using rCYPs corresponded well to the observed $CL_{int-HLM}$ from HLM (figure 6). This result strongly suggests that the RAF approach is useful not only for the prediction of contribution rates of each CYP form, but also for that of CL_{int} in EM- and PM-HLM. This study based on the RAF approach showed that CYP2D6 and CYP3A4 play predominant roles in NE-100 metabolism in CYP2D6 EM and PM, respectively, and that the major metabolite in NE-100 differs between CYP2D6 EM and PM.

Due to the large contribution of CYP2D6 to the metabolism of many drugs, the polymorphism of CYP2D6 often produces extensive differences in plasma

concentrations after oral administration. Thus, the prediction of differences between EM and PM is required before the start of clinical trials. To predict CL_{oral} , hepatic (CL_{H}) and renal (CL_{R}) clearances are most important parameters, because many clinically used drugs are mainly eliminated through hepatic metabolism and urinary excretion. It has been reported that the prediction of CL_{H} in humans based on laboratory animal data is more difficult than that of CL_{R} because of substantial species differences (Dedrick *et al.* 1973, Boxenbaum 1980, 1982, Sawada *et al.* 1984, Lin 1995). Based on physiological models, *in vivo-in vitro* scaling methods have been to predict the *in vivo* CL_{H} for the number of drugs given orally (Iwatsubo *et al.* 1996, 1997a, b, Lave *et al.* 1997, Higashikawa *et al.* 1999). However, precise determination of *in vitro* data, such as K_{m} , V_{max} and CL_{int} , is necessary for this method. Most K_{m} values reported in the literature are based on the total substrate concentration added to the incubation because the purpose of the study is to identify the major enzymes involved in the *in vitro* metabolic pathways of drug. Furthermore, a more precise kinetic parameter based on f_{Ms} was required to predict *in vivo* CL_{H} from *in vitro* intrinsic clearance. Obach (1996) reported that felodipine and propranolol bind to microsomal protein in the incubation medium, and that the predicted CL_{H} well approximated to the observed CL_{H} by using kinetic parameters based on f_{Ms} .

Among physiological models, the dispersion model has been reported to be useful for the prediction of *in vivo* pharmacokinetic data such as E_{R} (hepatic extraction ratio), CL_{H} and AUC_{oral} for many oral drugs (Iwatsubo *et al.* 1996, 1997b). Since the CL_{int} of NE-100 is large and CYP2D6 is substantially involved in its metabolism, the present study predicted the differences in *in vivo* oral clearance (CL_{oral}) of NE-100 between EM and PM from *in vitro* $CL_{\text{int,free}}$ (taking into account the f_{Ms}), using the dispersion model. The predicted CL_{oral} for EM and PM using *in vitro* metabolic data from rCYPs and the RAF approach were 229.2 and 4.7 lmin^{-1} , respectively. The ratio between these CL_{oral} (CL_{oral} ratio) was approximately 50. The predicted CL_{oral} for EM and PM based on the *in vitro* metabolic data actually obtained from EM- and PM-HLM was 201.7 and 2.3 lmin^{-1} , respectively, and the ratio between these CL_{oral} (CL_{oral} ratio) was approximately 90. These predicted results suggest that large differences in plasma concentrations might be observed between EM and PM after the oral administration of NE-100 to humans and that CYP2D6 polymorphism might substantially influence on the *in vivo* NE-100 metabolism.

In conclusion, the major metabolites of NE-100 and the CYP forms producing them in PM-HLM were predicted using the RAF approach and these results corresponded well to the result observed from the PM-HLM study. Considering the difficulty of PM-HLM supply, the rCYPs and the RAF approach is a useful tool for the prediction of CL_{int} and the contribution of CYP forms involved in drug metabolism in PM-HLM as shown in this study. In addition, using the predicted CL_{int} (taking into account the f_{Ms}) for EM- and PM-HLM and the *in vitro-in vivo* scaling method gives the exactly predicted variation of CL_{oral} between CYP2D6 EM and PM. By changing the magnitude of the CL_{int} for a major CYP involved in drug metabolism, therefore, the application of the *in vitro* metabolic data from rCYPs, the RAF approach and the dispersion model might be possible to predict not only difference in the *in vivo* CL_{oral} caused by P450 polymorphism but also that based on inhibition of P450 by other co-administered drugs.

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Selectivity of 7-alkoxycoumarins as probe substrates for rat hepatic cytochrome P450 forms is influenced by the substitution pattern on the coumarin nucleus

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1. The O-dealkylation of 7-alkoxycoumarins is widely used as an assay to characterize cytochrome P450 (CYP) activity. These substrates can also undergo oxidative attack at additional sites on the coumarin nucleus, which may influence their apparent selectivity for particular CYP forms.

2. Accordingly, the effect of blockade of these additional sites was investigated on the selectivity towards rat hepatic CYP forms, with emphasis on the CYP1A and 2B forms.

3. Blockade of the 3-/4- and 6-positions resulted in substrates for which the CYP1A/2 selectivity of the unsubstituted 7-alkoxycoumarins was altered to a CYP2B selectivity; this was achieved with little overall change in the molecular dimensions of the substrate. Limited analysis of other inducible CYP forms indicated at most only small effects of structure modification on activity.

4. The findings suggest that the sensitivity of probe substrates for CYP forms may be limited by the occurrence of competing side reactions of the substrate, and that better probes may be derived by blocking the sites of these side reactions.

Introduction

The cytochromes P450 (CYP) encompass a family of forms with distinct but overlapping substrate specificities, which play a major role in the metabolism of a wide variety of xenobiotics, a feature being the ready inducibility of some of the forms by other xenobiotics (Lin and Lu 2001). The possibilities of xenobiotic–xenobiotic interactions, in the form of inhibition or induction of specific CYP forms, are issues of concern, particularly in the development of new therapeutic agents (Yuan *et al.* 2002). Induction of CYPs may also be important in animal preclinical studies of novel xenobiotics, as several non-genotoxic CYP inducers have been shown to produce liver tumours in rodents (Renwick *et al.* 2001). For these reasons, there is a continuing search for more form-selective substrates as probes to delineate these actions of xenobiotics (Yuan *et al.* 2002, Lu *et al.* 2003).

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The *O*-dealkylation of 7-alkoxycoumarins has long been a popular probe reaction for CYP-dependent activity in liver microsomes, the alkoxy coumarin being converted to a highly fluorescent 7-hydroxycoumarin metabolite. The relative ease of running the assay and the high sensitivity afforded by the fluorescence measurement have made these reactions suitable for a high-throughput approach to *in vitro* drug metabolism (Crespi and Stresser 2000, Nakamura *et al.* 2001).

7-Ethoxycoumarin (7EC) *O*-deethylation is the prototype of this class of reaction (Ullrich and Weber 1972). A feature of this reaction is its high inducibility by inducers of the CYP1A family; in contrast, there is only modest inducibility by inducers of the CYP2B or other CYP family (Ullrich *et al.* 1973, Kamataki *et al.* 1980). Although use of 7-methoxycoumarin (7MC) as substrate reverses this relative selectivity (CYP2B > CYP1A), the levels of induction achieved are small and they limit use of 7MC as a sensitive selective CYP2B substrate (Kamataki *et al.* 1980, Matsubara *et al.* 1983, Reen *et al.* 1991, Fry *et al.* 1992).

A number of previous studies have indicated that 7EC and other 7-alkoxycoumarins may be metabolized by routes other than *O*-dealkylation (Indahl and Scheline 1971, Matsubara *et al.* 1983, Walsh and Miwa 1984, Jung *et al.* 1985, DeLuca *et al.* 1988, Reen *et al.* 1991, Ball *et al.* 1996, Okuyuma *et al.* 1997). Of these studies, three have identified the routes of metabolism. Hydroxylation of 7EC at 3- and 6-positions were the principal routes identified (in liver microsome studies), which themselves were differentially regulated by CYP1A and CYP2B inducers (Walsh and Miwa 1984, Jung *et al.* 1985). These findings are consistent with the metabolic fate of 7MC in rats (Indahl and Scheline 1971). As far as the present authors are aware, there is no evidence that 7-alkoxycoumarins undergo opening of the pyrone ring as occurs with coumarin itself (Indahl and Scheline 1971).

In light of these observations we have postulated that the inducibility of the *O*-dealkylation of 7-alkoxycoumarins may be influenced by the relative inducibility of these alternative routes, and this was tested by determining the *O*-dealkylation of a range of substituted 7-alkoxycoumarins in rat liver microsomes. In the course of these studies, two new 7-alkoxycoumarin substrates were identified that are highly and selectively induced by CYP2B inducers.

Substitution at the 6-position was achieved by use of ethyl or chloro groups, whilst substitution at the 3-/4-position was achieved by use of methyl, trifluoromethyl or acetyl groups, either as a di-substitution or as a single substitution at the 4-position. The rationale for the latter was based on the observation that the hepatotoxicity of coumarin, which is believed to be mediated by a CYP-dependent 3,4-oxidation pathway, could be blocked by methyl substitution at either the 3- or 4-position (Fernyhough *et al.* 1994). The structure of coumarin and the numbering system used for the compounds studied are given in figure 1, whilst the structures of the 7-substituted coumarins that have been studied are presented in table 1.

Materials and methods

Animals

Male Wistar rats (7–8 weeks old) were obtained from the University of Nottingham Biomedical Services Unit. Animals were housed at a constant room temperature of $21 \pm 1^\circ\text{C}$, on a 12 h light–dark cycle, and were allowed free access to standard laboratory diet and water at all times. Care of the animals

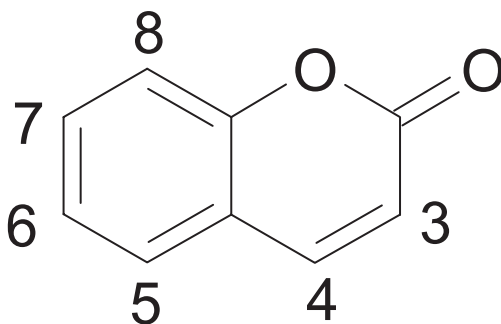
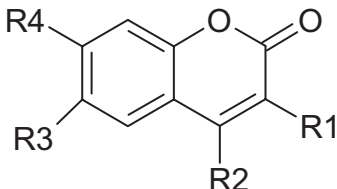


Figure 1. Structure of coumarin, with the numbering system used herein.

Table 1. Coumarins used as substrates.

					
Substrate	R1	R2	R3	R4	Melting point (°C)
1 [7EC]	H	H	H	OC ₂ H ₅	88.7–90.2 (lit. 88–89) ¹
2	H	CH ₃	H	OC ₂ H ₅	116.4–116.8 (lit. 115) ²
3	H	CH ₃	C ₂ H ₅	OC ₂ H ₅	152.7–153.7
4	CH ₃	CH ₃	C ₂ H ₅	OC ₂ H ₅	134.2–134.5
5	H	CH ₃	H	OCH ₃	purchased
6	H	CH ₃	C ₂ H ₅	OCH ₃	163.0–163.5 (lit. 162–163) ³
7	H	CH ₃	C ₂ H ₅	OC ₅ H ₁₁	76.5–77.0
8	COCH ₃	H	H	OC ₂ H ₅	174.1–175.1
9	COCH ₃	H	H	OCH ₃	170.1–171.2 (lit. 168–169) ⁴
10	H	CF ₃	Cl	OCH ₃	178.6–179.3
11	H	CF ₃	Cl	OC ₂ H ₅	156.8–157.5
12	H	CH ₃	O-CH ₂ -O		purchased

¹Aitio (1978); ²Abdou *et al.* (1988); ³Usgaonkar *et al.* (1953); ⁴Rangaswami *et al.* (1941).

conformed to UK Home Office guidelines. The protocols for treatment of animals with the cytochrome P450 inducers phenobarbitone (PB), β -naphthoflavone (BNF), pregnenolone 16 α -carbonitrile (PCN), isosafrole (ISAF) and isoniazid (IZO) have been described previously (Fry *et al.* 1992).

Human liver microsomes

Pooled human liver microsomes were obtained from XenoTech llc (lot no. 0010079; distributed by Totam Biologicals, Northampton, UK). The data sheet for this product indicated that the microsomes were obtained from a pool of 16 donors of age range 17–67 years of age (10 male, 14 Caucasian, two African-American).

Supply and synthesis of coumarins

Melting points (uncorrected) were determined on a Stuart MP3 digital apparatus, with a ramping rate of 1°C min⁻¹, commencing 10°C below the approximate melting point obtained from the literature or preliminary experiment. Thin-layer chromatography (TLC) was performed on aluminium-backed silica gel with fluorescence indicator (Whatman, Maidstone, UK), with a solvent system of hexane (67–70°C)/ethyl acetate (3/1, v/v).

The following hydroxycoumarins were purchased: 7-hydroxy-4-methylcoumarin (Sigma Chemical Co., Poole, UK); 7-hydroxycoumarin and 6,7-dihydroxy-4-methylcoumarin (Aldrich, Poole, UK); and

6-chloro-7-hydroxy-4-trifluoromethylcoumarin (Maybridge, Tintagel, UK). 3-Acetyl-7-hydroxycoumarin was synthesized by condensation of ethyl acetoacetate with 2,4-dihydroxybenzaldehyde as described by Rajanibala *et al.* (1955). 6-Ethyl-7-hydroxy-4-methylcoumarin and 6-ethyl-7-hydroxy-3,4-dimethylcoumarin were prepared by condensation of the appropriate resorcinol with ethyl acetoacetate (for the 4-methyl derivative) or ethyl α -methylacetoacetate (for the 3,4-dimethyl derivative) in the presence of concentrated sulphuric acid, essentially as described by Mann and Saunders (1960) for the synthesis of 7-hydroxy-4-methylcoumarin. The synthesized compounds produced single spots on TLC, and sharp melting points which agreed with literature values:

- 3-Acetyl-7-hydroxycoumarin: 239.5–240.9°C; lit. 240°C (Shah and Shah 1954).
- 6-Ethyl-7-hydroxy-4-methylcoumarin: 216.4–216.8°C; lit. 215.5–216.5°C (Tsujihara *et al.* 1999).
- 6-Ethyl-7-hydroxy-3,4-dimethylcoumarin: 250.0–251.0°C; lit. 248°C (Thakor and Shah 1947).

7-Methoxy-4-methylcoumarin was purchased (Sigma), as was 6,7-methylenedioxy-4-methylcoumarin (4-methylayapin; Tokyo Kasei, Japan—supplied by Fluorochem Ltd, Glossop, UK); the 7-methoxy-4-methylcoumarin was purified by recrystallization from methanol before use. The 3-acetyl-7-alkoxycoumarins were synthesized by Knoevenagel condensation of ethyl acetoacetate with the appropriate 3-alkoxysalicylaldehyde (Knoevenagel and Schröter 1898). The remaining 7-alkoxycoumarins were synthesized by alkylation of the corresponding 7-hydroxycoumarins by a modification of the method described by Dean and Parton (1969) for the alkylation of 6,7-dihydroxycoumarin. Briefly, the 7-hydroxycoumarin (0.03 mole) and K_2CO_3 (0.05 mole) were stirred together in DMSO (20 ml) at room temperature during the dropwise addition of iodoalkane (0.04 mole) over 15 min. The whole was left stirring at room temperature for 24 h, after which the reaction was stopped with 150 ml water. The whole was extracted ($\times 2$) with chloroform (50 ml), and the pooled extracts were evaporated to dryness after removal of water with anhydrous $MgSO_4$. The product obtained was recrystallized from 50% aq. methanol or ethanol. All products gave a single spot on TLC and sharp melting points, which agreed with literature values for those compounds reported previously (table 1).

Preparation of liver microsomes and hepatocytes

Microsomal fractions were prepared from the pooled livers of four to six rats as described (Fry *et al.* 1992) and stored at $-80^\circ C$ until use. Rat hepatocytes were isolated as described (Hammond and Fry 1997) and seeded onto six-well collagen-coated plates in modified Williams' medium E (containing 1 mg ml^{-1} bovine serum albumin, 2 mM glutamine, 30 nM dexamethasone, 10 nM insulin, 5 mM nicotinamide, 5 ng ml^{-1} sodium selenite, 5 $\mu\text{g ml}^{-1}$ transferrin, 0.2 $\mu\text{g ml}^{-1}$ copper sulphate, 0.75 $\mu\text{g ml}^{-1}$ zinc sulphate and 50 $\mu\text{g ml}^{-1}$ gentamicin). The medium was changed after 2 h, and thereafter at 24 h intervals. At 24 and 48 h, phenobarbitone (final concentration range 50–500 μM) was added to the medium, and at 72 h, enzyme activity was determined in the intact cells. Cell protein was dissolved in 0.5 M NaOH and determined using the Bradford assay (Hammond and Fry 1997).

Determination of alkoxy coumarin O-dealkylase activity

Dealkylase activities were determined in rat liver microsomes based on the method of Paterson *et al.* (1984). The incubation mixture contained 10 μM alkoxy coumarin, 5 mM magnesium sulphate, 0.5 mM $NADP^+$, 5 mM isocitrate and 1 Unit ml^{-1} isocitrate dehydrogenase in 0.1 M Tris-HCl, pH 7.4, containing 1 mg ml^{-1} bovine serum albumin (BSA). Aliquots of incubation mix, 0.9 ml, were incubated at $37^\circ C$ in a shaking water bath for 5 min and then the reaction was started by adding 0.1 ml microsomes (0.4–2.0 mg protein ml^{-1} depending on source of microsomes, i.e. inducer treatment), diluted in the Tris buffer containing BSA. Samples were incubated for 5–15 min, depending on inducer treatment, and then the reaction was stopped with 0.2 ml 15% w/v trichloroacetic acid, and the protein pelleted by centrifugation. In a 96-well plate, 250 μl 0.4 M NaOH-glycine buffer, pH 10.4, was added to 50 μl protein-free supernatant, and the fluorescence measured on a Cytofluor 2350 (Applied Biosystems, Warrington, UK), at an excitation wavelength of 390 nm, and an emission wavelength of 450 nm, this being the optimal wavelength pair for 7-hydroxycoumarin. Fluorescence was measured relative to the appropriate 7-hydroxycoumarin standards included in the assay. An alkoxy coumarin concentration of 10 μM was used to measure selectively the high-affinity component of 7-alkoxy coumarin O-dealkylase activity, as has been described for 7MC and 7EC as substrates (Boobis *et al.* 1981, Paterson *et al.* 1984); this substrate concentration was used for all substrates to enable direct comparisons to be made.

O-dealkylase activity towards coumarin 6 was determined in intact hepatocytes by the method described for 7-methoxycoumarin as substrate (Hammond and Fry 1997). In brief, hepatocyte monolayers were incubated with substrate (20 μM) for 2 h, after which the medium was treated with β -glucuronidase + sulphatase to release the 7-hydroxycoumarin from its conjugates, the 7-hydroxycoumarin being measured as described above.

Determination of pentoxyresorufin O-dealkylase activity

Determination of resorufin production from pentoxyresorufin was carried out analogously to that described for alkoxy coumarin O-dealkylase activity. Briefly, the incubation mix was similar to that

described above, with the modification that the substrate was 5 μ M 7-pentoxoresorufin, and 10 μ M dicoumarol was also added. Aliquots of incubation mix, 0.9 ml, were incubated at 37°C in a shaking water bath for 5 min, and then the reaction was started by adding 0.1 ml microsomes (1–3.5 mg protein ml⁻¹ depending on source of microsomes, i.e. inducer treatment), diluted in the Tris buffer containing BSA. Samples were incubated for 10–30 min, depending on inducer treatment, and then the reaction was stopped with 0.5 ml 15% w/v zinc sulphate. Saturated barium hydroxide (0.5 ml) was then added, and the tubes put on ice for 5 min. Protein was removed by centrifugation and the fluorescence measured in the protein-free supernatant on a Cytofluor 2350 (excitation wavelength of 530 nm, emission wavelength of 590 nm). Fluorescence was measured relative to a resorufin standard included in the assay.

Inhibition experiments

The effect of the CYP2B inhibitor deprenyl was investigated using a double incubation protocol. Incubation mix containing 10 times the final microsome concentration, inhibitor but no substrate, was pre-incubated at 37°C, and then 0.1 ml removed and added to fresh incubation mix containing substrate. Deprenyl was obtained from Sigma.

Lipid solubility and molecular geometry calculations

Lipid solubility (expressed as log *P*) was calculated using the Interactive Analysis LogP Predictor (<http://www.logp.com>).

Molecular models for all compounds studied were generated using the Insight[®] II program from Molecular Simulations, Inc. (San Diego, CA, USA) and energy minimized using MOPAC with the AM1 Hamiltonian program. The calculation of area/depth² ratios was performed as follows: the molecules were individually aligned along their principal axes (using a locally written program), so the major axis was parallel to the *x*-axis and the minor axis parallel to the *z*-axis. The atoms with largest and smallest *x*, *y* and *z* coordinates were identified, and the dimensions of the necessary bounding box thus determined, with allowance for the Van der Waals radius of the limiting atoms.

The area/depth² ratios for three calibration interactors and 7EC (coumarin 1) calculated by the technique described herein were similar to those calculated using the molecular dimensions defined by Lewis *et al.* (1986)—DDT (present work, 2.0; calculated, 0.8); aflatoxin (3.2; 3.2); 7EC (3.0; 3.7) and benzo[*a*]pyrene (10.6; 11.9)—this attesting to the validity of the method of calculation used in this study.

Calculations and statistical analysis

Reaction curves were fitted and kinetic constants calculated using the GraphPad Prism program. Statistical significance was tested using unpaired *t*-tests or ANOVA/Dunnnett's test as appropriate, also run on Prism. *p* ≤ 0.05 was determined to be significant.

Results

O-dealkylase activities with coumarins 1–9

In the first set of experiments, the *O*-dealkylase activity with coumarins 1–9 as substrates was determined in liver microsomes from untreated rats (termed 'control') and from rats treated to induce specific CYP forms. These treatments (and the form induced) were: β -naphthoflavone (BNF: CYP1A1/2); isosafrole (ISAF: CYP1A2); phenobarbitone (PB: CYP2B1 and CYP3A1); isoniazid (ISO: CYP2E1); and pregnenolone-16 α -carbonitrile (PCN: CYP3A1). The results obtained are presented in figures 2 and 3, whilst the magnitude of induction achieved with PB and BNF for each substrate is presented in table 2.

There was an approximately eightfold variation in the constitutive enzyme activity (figure 2), the highest activity being observed with 3-acetyl-7-ethoxycoumarin (coumarin 8). The key features for a high constitutive activity were a 7-*O*-ethyl moiety and a substituent at the 3-position, as exemplified in coumarins 4 and 8.

The *O*-dealkylation of 7EC (coumarin 1) was significantly elevated following treatments with various inducers in the order BNF > PB > ISAF = ISO. These findings are in keeping with results from other studies (Fry *et al.* 1992). The

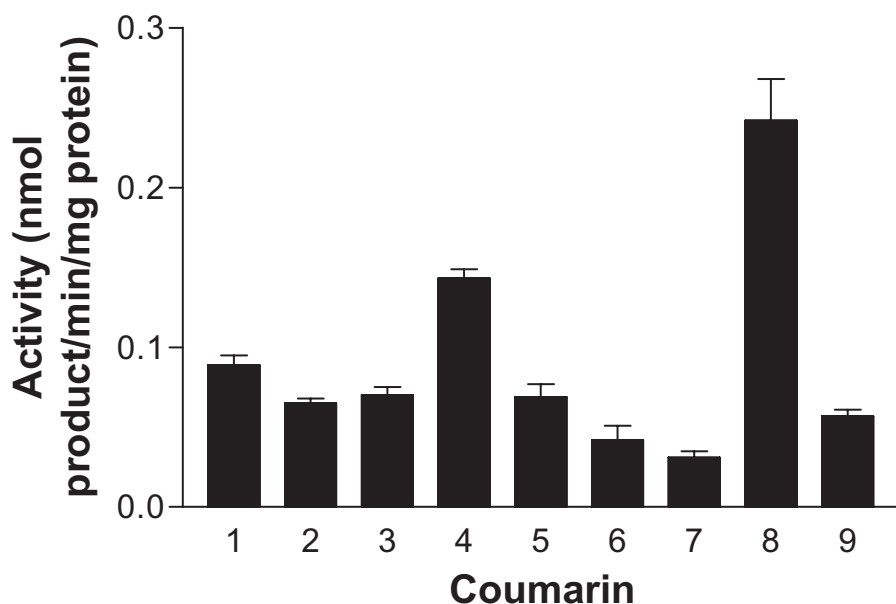


Figure 2. Constitutive 7-*O*-dealkylase activity in rat liver microsomes measured with coumarins 1–9. Enzyme activity was measured as described in the text, and is reported as the mean $\pm$ SEM of seven measurements performed on microsomes isolated from a pooled batch of six rat livers.

observation that the increase observed with BNF (which induces CYP1A1 and CYP1A2) is much greater than that observed with ISAF (induces CYP1A2) indicates that the bulk of the BNF-inducible activity is mediated by CYP1A1. By a similar reasoning (comparing treatment with PB and PCN), all the PB-inducible activity is mediated by CYP2B1.

The addition of a 4-methyl moiety to 7EC (i.e. coumarin 2) was without effect on PB-inducible activity but blunted the response to BNF and abolished the response to both ISAF and ISO.

The addition of a 6-ethyl group to coumarin 2 (i.e. coumarin 3) changed the selectivity with regard to BNF and PB inducibility in favour of PB. Essentially identical results were obtained with a further methyl substitution at the 3-position (coumarin 4).

A change in favour of PB inducibility was also achieved when the 7-ethoxy group of coumarin 2 was replaced by a 7-methoxy group (coumarin 5), although the inducibility by PB was relatively modest (sevenfold induction). This observation has been made by previous workers (Kamatani *et al.* 1980, Matsubara *et al.* 1983, Reen *et al.* 1991, Fry *et al.* 1992), when either the high affinity form or the sum of the high- and low-affinity forms were measured (Fry *et al.* 1992).

A combination of the features described above—a 4-methyl substitution, a 6-ethyl substitution and a 7-methoxy group—produced a substrate (coumarin 6) that was highly selective for the PB-inducible forms of P450. Activity was not induced by BNF treatment whilst PCN treatment produced a fourfold increase in activity which was not statistically significant under the conduct of this trial. By comparison, activity was induced 58-fold by PB treatment.

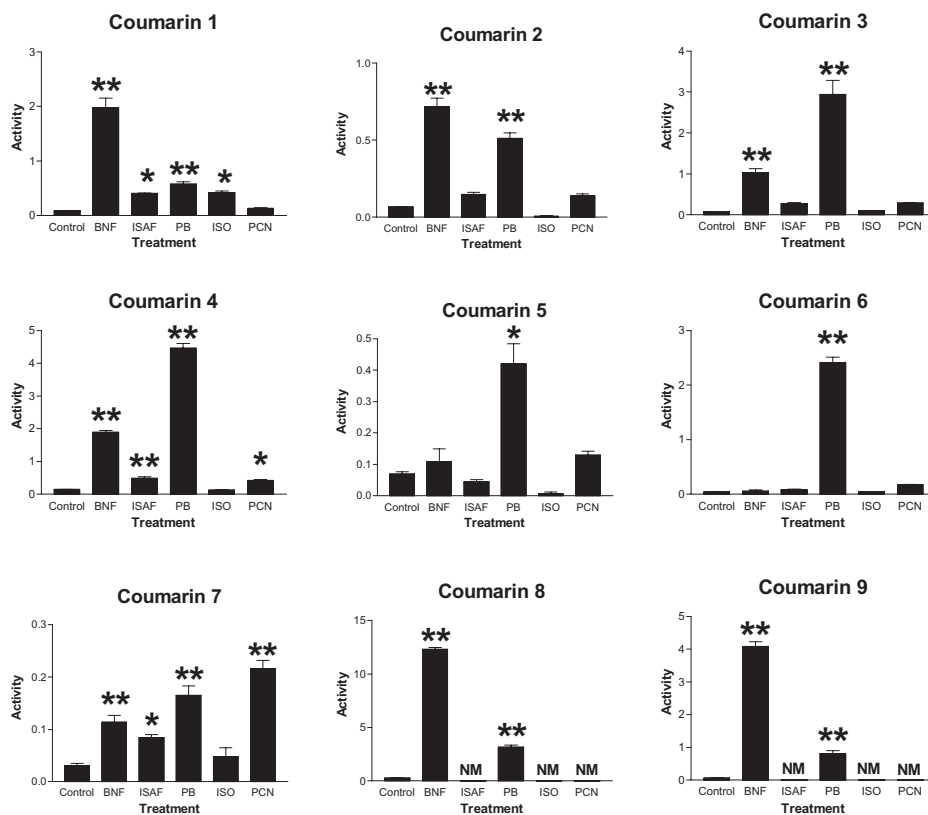


Figure 3. 7-O-dealkylase activity in liver microsomes isolated from untreated rats (control) or rats treated with BNF, ISAF, PB, ISO or PCN, measured with coumarins 1–9. Enzyme activity was measured as described in the text, and is reported as the mean $\pm$ SEM of four to seven measurements performed on microsomes isolated from a pooled batch of four to six rat livers, in units of nmol product min⁻¹ mg⁻¹ protein. Where indicated, values are significantly different from control at **p* = 0.05 and ***p* < 0.01 (ANOVA/Dunnett's test).

Replacement of a 7-pentoxy group for the 7-methoxy group in coumarin 6 (to produce coumarin 7) abolished this PB selectivity, producing low levels of induction, and, indeed, a bigger increase in activity following PCN treatment (approximately sevenfold).

3-Acetyl substitution of 7EC produced a substrate (coumarin 8) whose metabolism was highly induced by BNF (more so than with 7EC as substrate), and induced to a much lesser extent by PB. For this type of substrate, replacement of the 7-ethoxy group with a 7-methoxy group (coumarin 9) had no effect on this selectivity of induction, although the magnitude of inducibility by BNF was enhanced.

Further analysis of the responses of coumarins 1–9 to BNF and PB

Having determined a range of selectivity towards BNF- and PB-inducibility, attempts were then made to relate this to molecular geometry and lipid solubility of the coumarin substrates. The results are presented in table 2, which also includes molecular geometry data for three additional compounds to act as calibrators to the original study by Lewis *et al.* (1986).

Table 2. Selectivity of 7-alkoxycoumarins as substrates for BNF- and PB-inducible forms of cytochrome P450 in relation to molecular size and lipid solubility.

Coumarin	Fold induction by:		Area/depth ² ratio	Log <i>P</i>
	BNF	PB		
1	22	6	3.0	2.08
2	11	8	4.6	2.63
3	15	42	4.5	3.67
4	13	31	4.3	4.22
5	< 2	6	6.8	2.12
6	1	58	4.5	3.18
7	4	5	n.d.	5.14
8	51	13	4.3	1.36
9	72	14	4.0	0.87
		DDT	2.0	
		Aflatoxin	3.2	
		Benzo[α]pyrene	10.6	

Fold induction is defined as the ratio of the BNF or PB value to that of the control.

Molecular size and lipid solubility were calculated as described in the Materials and methods.

n.d., Not determined.

With regard to lipophilicity, low lipophilicity seemed to favour CYP1A selectivity (as judged by inducibility by BNF), whereas CYP2B selectivity (as judged by inducibility by PB) was favoured by high lipophilicity (figure 4), although there appeared to be a narrow lipophilicity 'window' for this latter relationship.

For the molecular geometry analysis, DDT was chosen as an example of a selective CYP2B1 interactor, whilst aflatoxin and benzo[α]pyrene were chosen as examples of selective CYP1A1 interactors (for references, see Lewis *et al.* 1986), and area/depth² ratios were used as a measure of 'molecular planarity' as reported by Lewis (2000). With this information, the data calculated for the coumarin substrates would suggest that they all lie towards the CYP1A1 end of the molecular geometry spectrum, whilst the enzyme activity data indicates that two of the substrates for the *O*-dealkylation (coumarins 5 and 6) are highly selective for CYP2B1. The area/depth² ratio for coumarin 7 could not be calculated because of difficulties associated with the conformationally flexible alkyl side chain at C7.

Further studies with coumarin 6

Having established 6-ethyl-7-methoxy-4-methylcoumarin (coumarin 6) to be a selective substrate for the PB-inducible CYP forms, the kinetics of the *O*-dealkylation with this substrate were established. The concentration dependence of this reaction in control rat liver microsomes and liver microsomes from PB-treated rat is presented in figure 5. The data obtained were fitted to a number of models and found to conform to a single enzyme model ($R^2 \geq 0.998$), for which the kinetic constants indicated in table 3 were obtained. Eadie-Hofstee analysis of these data produced further evidence of a single enzyme model (analysis not shown). A maximal velocity of 0.31 nmol product min⁻¹ mg⁻¹ protein was calculated for the control rat liver microsomes, which was increased 10-fold following treatment with PB. The K_m following PB treatment (5 μ M) was approximately one-sixth that

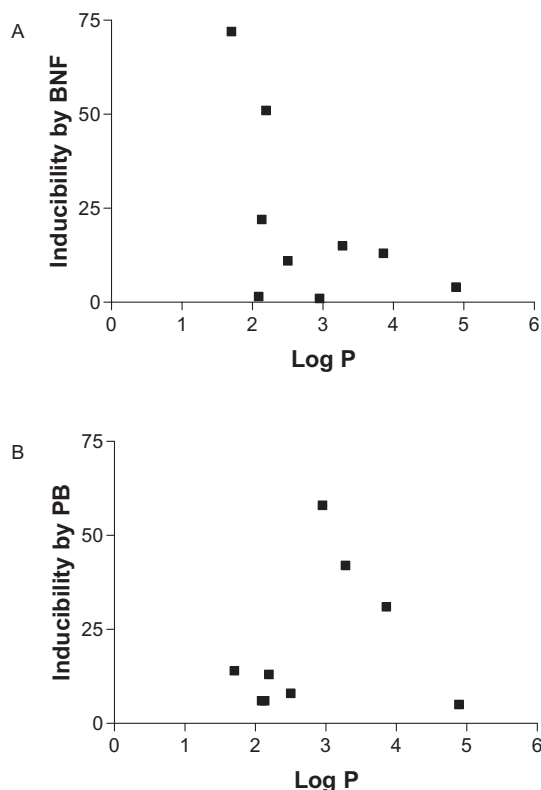


Figure 4. Relation of lipophilicity (measured as log *P*) to inducibility of *O*-dealkylation of coumarins 1-9 by treatment with BNF (A) or PB (B). The data used to derive these graphs are presented in table 2.

obtained in control rat liver microsomes (30 μ M). Low and variable activities at the lower end of the substrate concentration range, and a suggestion of inhibition/inactivation at the higher end, made kinetic determinations in liver microsomes from BNF-treated rats unreliable (data not shown).

Deprenyl, a selective CYP2B1 inhibitor (Sharma *et al.* 1996), significantly inhibited *O*-dealkylation of coumarin 6 in liver microsomes from PB-treated rats, but was without effect on the activity measured in liver microsomes from untreated rats or rats treated with BNF (figure 6). Deprenyl at the same concentration (1 μ M) inhibited pentoxyresorufin *O*-dealkylase (PROD) activity in liver microsomes from PB-treated rats to a similar extent (50%; data not shown). PROD activity is accepted as being a marker for CYP2B1 activity (when measured in liver microsomes from PB-treated rats), and these data provide further evidence for the involvement of CYP2B1 in the *O*-dealkylase activity towards coumarin 6 in liver microsomes from PB-treated rats.

O-dealkylation of coumarin 6 can also be used to detect PB induction in rat cultured hepatocytes, as illustrated by the data presented in figure 7. Note that this analysis could be performed on the metabolite released into the culture medium, and did not require the preparation of cell homogenates as the enzyme source. Again, deprenyl was an effective inhibitor of this induced activity (data not shown). These data indicate that maximal induction was obtained with 100 μ M

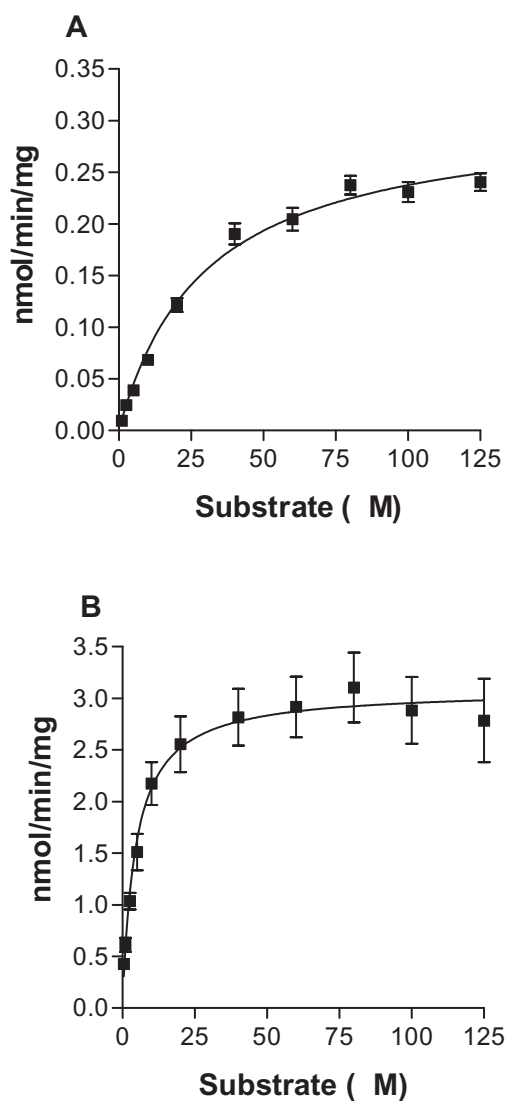


Figure 5. Concentration dependence of *O*-dealkylation of 6-ethyl-7-methoxy-4-methylcoumarin in liver microsomes from untreated rats (A) and liver microsomes from rats treated with PB (B). Enzyme activity was measured as described in the text, and is reported as the mean $\pm$ SEM of six to eight measurements performed on microsomes isolated from a pooled batch of six rat livers, in units of $\text{nmol product min}^{-1} \text{mg}^{-1}$ protein. The line was drawn by non-linear regression, assuming monophasic kinetics, on the Prism program.

Table 3. Kinetic constants for *O*-dealkylation of 6-ethyl-7-methoxy-4-methylcoumarin in liver microsomes from untreated rats (control) and rats treated with PB.

Treatment	K_m (μM)	$V_{\max}$ ($\text{nmol product min}^{-1} \text{mg}^{-1}$)
None (control)	29.7 ± 3.6	0.31 ± 0.01
PB	5.0 ± 0.4	3.21 ± 0.06

Data were calculated by non-linear regression using the means of six to eight individual reaction curves.

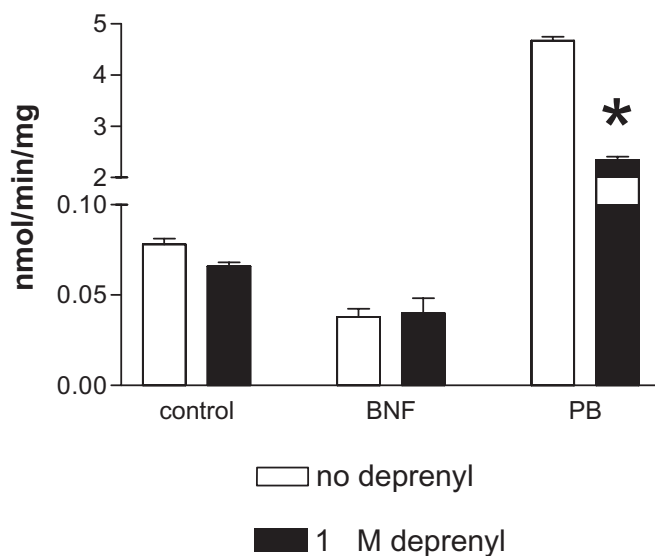


Figure 6. Effect of deprenyl on the *O*-dealkylation of coumarin 6 in liver microsomes from untreated rats and rats treated with BNF or PB. Enzyme activity was measured in the absence and presence of deprenyl (1 μ M) as described in the text, and is reported as the mean $\pm$ SEM of five to eight measurements performed on microsomes isolated from a pooled batch of six rat livers, in units of nmol product $\text{min}^{-1} \text{mg}^{-1}$ protein. Where indicated, values are significantly different from control at $*p < 0.01$ (paired *t*-test).

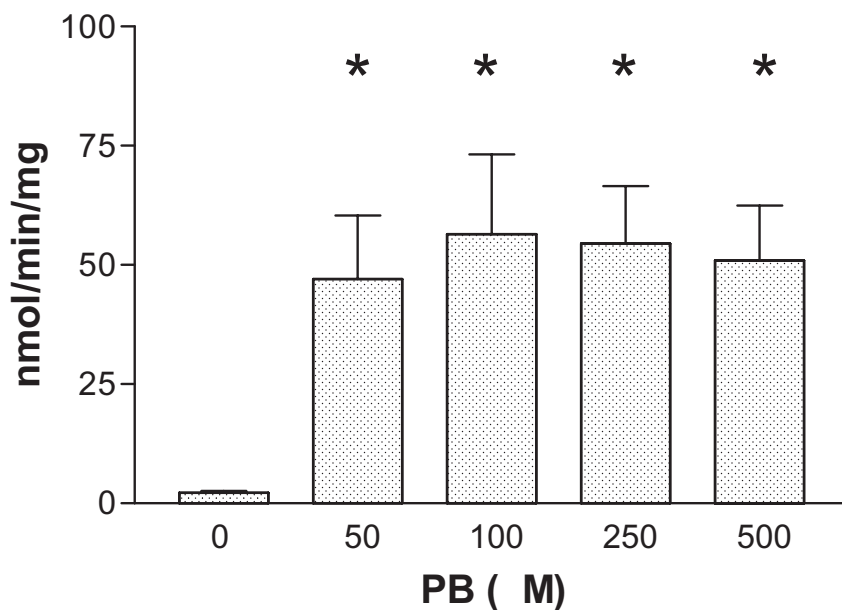


Figure 7. Effect of treatment with PB on the *O*-dealkylation of coumarin 6 in rat hepatocyte cultures. Rat hepatocytes were cultured in the absence or presence of PB for 48 h, after which time the cells were incubated with coumarin 6 (20 μ M) for 2 h. The medium was then analysed for product as described in the text. Data are the mean $\pm$ SEM of four to five separate cultures. Where indicated, values are significantly different from control at $*p < 0.01$ (ANOVA/Dunnett's test).

PB, the induced activity being approximately 30-fold greater than that measured in untreated cells.

Finally, *O*-dealkylation activity towards coumarin 6 can also be detected in human liver microsomes, albeit at lower levels than that measured in rat liver microsomes (table 4).

O-dealkylase activities measured with coumarins 10–12

The previous studies had indicated that blocking the alternative sites of metabolism of 7-ethoxycoumarin, together with replacement of the 7-ethoxy group with a 7-methoxy group, markedly altered the selectivity of the substrate towards CYP forms, leading to a substrate that was highly selective for CYP2B1. The generality of these observations was tested by use of the novel 7-alkoxy-coumarin substrates, coumarins 10 and 11, in which the 3-/4-position was blocked by a 4-(trifluoro)methyl group, while the 6-position was blocked by a chloro-group. The data for the enzyme activity measured in liver microsomes from untreated rats and rats treated with BNF or PB are presented in table 5.

As with coumarins 1–9, the 7-ethoxy analogue (coumarin 11) possessed a higher basal activity than did the 7-methoxy analogue (coumarin 10). There was a tendency for an elevated activity towards coumarin 11 in liver microsomes from BNF-treated rats, but this did not reach statistical significance, whilst PB treatment produced a marked (22-fold) enhancement of activity. BNF treatment was without effect on *O*-dealkylation of coumarin 10, whilst PB treatment, again, caused a marked (40-fold) enhancement of this activity.

In the final study, 6,7-methylenedioxy-4-methylcoumarin (coumarin 12) was tested as a possible form-selective substrate. The rationale for this rested on the observation that numerous other methylenedioxy compounds undergo CYP-dependent demethylenation to the corresponding *o*-dihydroxy-compound (Fukuto *et al.* 1991), and this could be considered as being analogous to a 7-*O*-dealkylation

Table 4. *O*-dealkylase activities in rat and human liver microsomes.

Substrate	<i>O</i> -dealkylase activity (nmol product min ⁻¹ mg ⁻¹)	
	Rat	Human
7-Ethoxycoumarin	0.089 ± 0.006	0.138 ± 0.009
6-Ethyl-7-methoxy-4-methyl-coumarin	0.042 ± 0.009	0.026 ± 0.006

Data are the mean ± SEM of five to seven measurements made on microsomes prepared from a pool of six rat livers, or on pooled human liver microsomes.

Table 5. *O*-dealkylase activity in liver microsomes from untreated rats and from rats treated with BNF or PB, assessed using coumarins 10–12 as substrates.

Coumarin	<i>O</i> -dealkylase activity (nmol product min ⁻¹ mg ⁻¹)		
	Control	BNF	PB
10	0.063 ± 0.012	0.058 ± 0.015	2.590* ± 0.171
11	0.126 ± 0.028	0.261 ± 0.056	2.720* ± 0.404
12	0.153 ± 0.010	0.418* ± 0.009	0.608* ± 0.016

Data are the mean ± SEM of three to six separate determinations.

*Significantly different from control at *p* < 0.01.

with a hindered 6-position. The results of this study are presented in table 5, and indicate that *O*-demethylenation was significantly, but modestly, increased following treatment with BNF or PB, and showed little selectivity as a marker reaction.

Discussion

The results indicate that ring substitution of 7-alkoxycoumarins impacts upon various properties of these compounds as substrates for *O*-dealkylase activity of the cytochrome P450 system in rat liver microsomes. In the course of these studies, two coumarins (6, 10) have been identified as highly selective substrates for the CYP2B forms. Moreover, coumarin 6 possesses additional attributes in that it appears to be a useful substrate for CYP activity in species other than rat (easily measurable activity in human liver microsomes), it can be used as a marker substrate for CYP2B inducibility in rat hepatocyte cultures, and it exhibits monophasic kinetics. Taken together, these observations suggest that coumarin 6 may be a useful CYP probe substrate in a variety of experimental situations.

A limitation to the use of 7EC (and 7MC) as a CYP substrate is that it exhibits biphasic kinetics in both rat and human liver microsomes (Boobis *et al.* 1981, Paterson *et al.* 1984), which often complicates interpretation of changes in such enzyme activity. However, coumarin 6 exhibited only monophasic kinetics. Other workers have established that other ring-substituted 7-alkoxycoumarins also exhibit monophasic kinetics (Deluca *et al.* 1988, White 1988, Giunazi *et al.* 1996), and it appears that substitution at the 3-/4-position is key to this. It would seem likely that such substitution blocks binding of 7EC to at least one form involved in 7EC *O*-dealkylase activity.

The mechanism underlying this selectivity towards either the CYP1A or CYP2B form has been explored in the context of lipophilicity, molecular geometry and interference with alternative sites of metabolism.

The lipophilicity data suggested a reciprocal relationship between CYP1A and CYP2B form selectivity, the less lipophilic substrates being favoured by CYP1A and the more lipophilic being favoured by CYP2B. However, other factors must play a contributory role in determining CYP form selectivity, as judged by the marked difference in BNF inducibility of *O*-dealkylation of coumarins 1 and 5, in spite of very similar log *P* values, and by the poor inducibility of *O*-dealkylation of the most lipophilic coumarin (7) studied.

Lewis *et al.* have proposed that CYP1A1 and CYP2B1 substrates (and inhibitors and inducers; collectively termed 'interactors') may be discriminated on the basis of physico-chemical properties: CYP1A1 substrates tend to be large, planar molecules (Lewis *et al.* 1986), whereas CYP2B1 substrates tend to be lipophilic and have more non-planar molecular geometries (Lewis and Lake 1997). However, the molecular geometry data derived for this panel of coumarin substrates do not appear to fit the existing criteria, particularly for CYP2B1 substrates, and, again, this points to the involvement of other factors that regulate CYP form selectivity in this panel of coumarin substrates.

Numerous previous studies have demonstrated that 7-alkoxycoumarins undergo CYP-mediated metabolism by routes other than *O*-dealkylation (see the references in the Introduction), and Jung *et al.* (1985) have identified 3- and 6-hydroxylation as being the principal alternative routes for 7EC. The postulate that initiated these studies was that independent regulation of these alternative

pathways might impact upon the selectivity of 7-alkoxycoumarins towards CYP forms when measured as *O*-dealkylase activity. The results obtained are consistent with this thesis and suggest a novel approach to the design of form-selective substrates that may be useful for high-throughput screening of CYP-mediated drug–drug interactions.

Roser and Thomas (2000) have reported on the use of a 3,4-substituted 7-alkoxycoumarin, 3-chloro-7-methoxy-4-methylcoumarin, as a substrate for CYP-dependent metabolism in rat liver microsomes, the *O*-dealkylation of which was induced by PB but not by BNF. However, the extent of induction by PB was small (about 1.3-fold) and was associated with an increase in K_m . In addition, Renwick *et al.* (2001) evaluated 7-benzyloxy-4-trifluoromethylcoumarin and related compounds as selective probes for rat hepatic CYPs, but none of the potential probe substrates proved selective for CYP2B1 alone. The novel 4,6-disubstituted 7-alkoxycoumarins developed as selective CYP2B1 substrates in the present study thus represent a new avenue in the design of form-selective 7-alkoxycoumarin substrate probes. The finding that coumarin 6 was also a substrate for human liver microsomal *O*-dealkylation activity raises the possibility that this or similar coumarins may be useful selective substrates for human CYP forms.

Acknowledgements

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Comparative inhibition of inducible and constitutive CYPs in rat hepatic microsomes by parathion

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1. In microsomal fractions, the phosphorothioate pesticide parathion inhibits cytochrome P450 (CYP) enzymes by reversible and irreversible mechanisms resulting in the long-term suppression of drug oxidation. The present study evaluated the relative susceptibilities of constitutive and inducible CYP2 and CYP3 steroid hydroxylases to inhibition by the pesticide.

2. Enzyme kinetic analysis indicated that constitutive and dexamethasone (DEX)-induced androst-4-ene-3,17-dione (AD) 6 β -hydroxylations were similarly susceptible to inhibition by parathion (K_m/K_i ratios 1.5–1.6). However, preincubation of parathion with NADPH-fortified microsomes intensified the extent of inhibition of CYP3A-dependent 6 β -hydroxylation. Comparison of K_m/K_i ratios indicated that 6 β -hydroxylation activity in fractions from DEX-pretreated rats was about twice as susceptible as the control activity to inactivation by parathion metabolites (K_m/K_i ratio of 8.0 versus 4.0).

3. The time-dependent loss of AD 6 β -hydroxylation by parathion occurred more efficiently in fractions from DEX-induced liver than in control. Thus, half-times of 1.3 and 6.1 min, respectively, were determined for the inactivation of DEX-inducible and constitutive activities. Parathion concentrations required for half-maximal inactivation were 32 and 67 μ M in microsomes from DEX-induced and control rats.

4. In phenobarbital (PB)-induced fractions CYP2B1-mediated AD 16 β -hydroxylation was inhibited potently in a reversible fashion by parathion ($K_i = 0.37 \mu$ M; K_m/K_i ratio about 73). Inhibition was not enhanced at parathion concentrations near the K_i by a preincubation step with NADPH.

5. In control microsomes parathion elicited a type I binding interaction with oxidized CYP ($K_s = 7.7 \mu$ M, $\Delta A_{\max} = 2.2 \times 10^{-2}$ a.u. nmol CYP $^{-1}$; $\Delta A_{\max}/K_s = 2.86 \times 10^3$ a.u. nmol CYP $^{-1}$ /mM). Ligand binding was 13- and 1.6-fold more efficient in PB and DEX microsomes, respectively.

6. These findings indicate that pretreatment of rats with enzyme-inducing drugs like DEX and PB alters the profile of CYPs and their susceptibility to inhibition by parathion. Potent reversible inhibition of CYP2B1 occurred in PB-induced fractions and DEX-inducible CYPs 3A were more susceptible to mechanism-based inactivation than the corresponding constitutive CYPs from the same subfamily.

Introduction

Many drugs and chemicals are converted to reactive metabolites by cytochrome P450 (CYP)-mediated oxidation. Such bioactivation processes may lead to the covalent binding of reactive intermediates to the CYPs involved in their formation. Studies undertaken in several mammalian species have shown that phosphorothioate pesticides such as parathion undergo CYP-dependent oxidation to stable metabolic products (figure 1), but in the process generate an electrophilic sulphur metabolite that inactivates the CYP (DeMatteis 1974, Norman *et al.* 1974). It has

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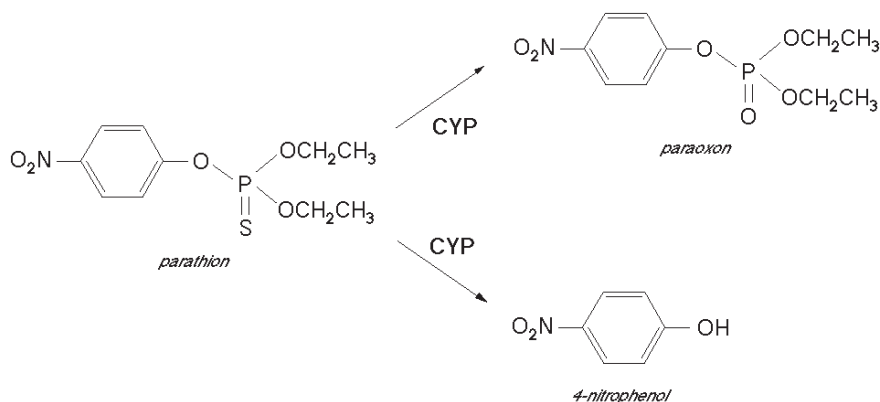


Figure 1. Role of CYP in the oxidative biotransformation of parathion to paraoxon and 4-nitrophenol.

been reported that constitutive microsomal CYPs in untreated rat liver are differentially susceptible to inactivation by parathion (Butler and Murray 1993). The significance of these findings is that exposure to parathion may exert non-uniform effects on drug oxidation pathways in mammalian liver.

Other mechanism-based inactivators of CYPs include alkenes, alkynes and dihydropyridines (Ortiz de Montellano and Mico 1980, Bocker and Guengerich 1986, Roberts *et al.* 1993). Similar processes include metabolite intermediate (MI)-complexation in which alkylamines and hydrazines are oxidized to reactive nitrogenous metabolites that sequester the CYP in a catalytically deficient state without eliciting destruction of the enzyme (Franklin 1977, Muakkasah *et al.* 1981, Reidy *et al.* 1989, Murray and Murray 2003). Pretreatment of rats with CYP-inducing drugs has been found to alter the inhibitory impact of certain mechanism-based inactivators of CYPs, including the MI complex forming alkylamines (Bensoussan *et al.* 1995, Franklin 1995, Trieu and Murray 2000). From these and other reports, it seems likely that environmental, dietary and therapeutic exposure to chemicals may alter the toxicological impact of parathion exposure by altering the profile of microsomal CYPs, which are then differentially inhibited by the pesticide. Indeed, recent studies from this laboratory and others have demonstrated that drug-mediated upregulation of CYPs modulates the incidence of drug-drug interactions as a consequence of altered formation rates of inhibitory metabolites.

The relative susceptibility of individual CYPs to inhibition and inactivation raises the possibility that CYP induction may alter the incidence of inhibitory drug interactions that impair the excretion of drugs and chemicals. The present study tested the hypothesis that altered CYP profiles in liver, modified by administration of inducing chemicals, may also alter the nature of inhibitory interactions mediated by parathion. Androgens like androst-4-ene-3,17-dione (AD) undergo regioselective oxidation by individual hepatic CYPs (Waxman *et al.* 1983). In this study, AD 6 β - and 16 β -hydroxylations in microsomes from dexamethasone (DEX)- and phenobarbital (PB)-induced rat liver were used as markers for inducible CYPs 3A and 2B, respectively. The major findings were that DEX induction promoted CYP3A inactivation by parathion and PB-inducible CYP2B activity was inhibited competitively. DEX and PB both increased the binding of parathion to oxidized microsomal CYP. These findings indicate that drug pretreatments may alter the

susceptibility of CYPs to inhibition by parathion. Reversible and irreversible inhibition processes may occur in a CYP-specific manner.

Materials and methods

Chemicals

Hyperfilm, ACS II, Hybond-N⁺ filters, [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, NSW, Australia). [Ring-¹⁴C]-parathion (specific activity 21 mCi mmol⁻¹; 95% radiochemical purity and purified by HPLC prior to use), paraoxon, 4-nitrophenol, biochemicals and unlabelled steroids were obtained from Sigma Chemical Co. (St Louis, MO, USA). Unlabelled parathion (at least 99% pure) and HPLC-grade solvents were from Rhone-Poulenc Chemicals (Baulkham Hills, Australia) and analytical grade reagents were from Ajax Chemicals (Sydney, Australia).

Animal treatments

Studies were undertaken within 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 housed under conditions of constant temperature and lighting (12 h light-dark cycle) with free access to rat diet (Glenn Forest Specialty Feeds, WA, Australia) and water. Some rats received either PB (100 mg kg⁻¹ in saline intraperitoneally (i.p.) daily for 3 days) or DEX (100 mg kg⁻¹ in corn oil i.p. daily for 3 days).

Animals were killed by exsanguination under enflurane anaesthesia, livers were removed and perfused via the portal vein with cold saline and then used to prepare hepatic microsomes by differential ultracentrifugation. Briefly, tissue was homogenized (Potter-Elvehjem) in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 100 mM sucrose, centrifuged at 10 000g for 10 min and the supernatant was centrifuged at 105 000g for 60 min. After resuspension and recentrifugation at 105 000g for 30 min, the final microsomal pellets were suspended in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA, frozen in liquid nitrogen and stored at -70°C (Murray *et al.* 1999). Microsomal protein was estimated by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard.

Assay of parathion biotransformation

Microsomal incubations (0.4 ml) contained 250 µM parathion (7×10^5 dpm) and 500 µg microsomal protein. Two min after the addition of 1 mM NADPH (final concentration), reactions were stopped by transfer to a dry ice/acetone bath. Labelled parathion, 4-nitrophenol and paraoxon (retention times 2.5, 4.5 and 9.4 min, respectively) were separated by HPLC (Ultrasphere-Si 5 µm column, 4.6 mm i.d. $\times$ 25 cm, Beckman, San Ramon, CA, USA) using a Waters Associates system. The mobile phase was dichloromethane:acetonitrile:glacial acetic acid, 93:7:0.02 and the flow rate was 1 ml min⁻¹. Aliquots (50 µl) were collected and subjected to scintillation spectrometry (ACS II, Amersham).

Microsomal steroid hydroxylation and inhibition by parathion

Microsomal AD hydroxylation activities were determined as described elsewhere (Martini *et al.* 1995). [¹⁴C]-Labelled substrates (50 µM, 0.18 µCi) were incubated with microsomal protein (150 µg from control liver, 75 µg from DEX-pretreated and 37.5 µg from PB-pretreated liver) and NADPH (1 mM) at 37°C (in 0.1 M phosphate buffer, pH 7.4; 0.4 ml). Inhibition by parathion was tested by several approaches. Reversible inhibition was tested by addition of parathion and AD to NADPH and microsomes whereas irreversible inhibition (inactivation) of CYPs by parathion metabolites was produced by preincubating parathion with NADPH-supplemented microsomes prior to addition of AD. In further experiments, parathion was incubated with NADPH-supplemented microsomes for varying periods before AD addition; corresponding control reactions were run in which NADPH-supplemented microsomes were incubated at 37°C before AD addition. Tubes were transferred to ice after 2.5 min to terminate reactions and chloroform (5 ml) was added. The organic phase was evaporated under N₂ and applied in a small volume of chloroform to TLC plates (Merck 60 F₂₅₄ silica; Darmstadt, Germany) that 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) and were quantified by scintillation counting (ACS II; Amersham). The identity of steroid metabolites was confirmed by coelution with authentic metabolites and rechromatography on TLC and HPLC. The within-assay variation was 3% (three samples analyzed in triplicate) and the interassay variation was 8% (three microsomal fractions analysed on consecutive days). Preliminary studies confirmed that metabolite formation was linear with protein concentration and time; substrate utilization in rat liver microsomes was about 15%.

Binding of parathion to oxidized CYP in rat hepatic microsomes

The interaction of parathion with ferric CYP in hepatic microsomes from control and induced rats (2 mg protein ml⁻¹ in 0.1 M potassium phosphate buffer, pH 7.4) was monitored by difference spectroscopy (Varian Cary 2300 spectrophotometer) as described (Murray 1992). Parathion was added to the sample cuvette in microlitre volumes of dimethylformamide and difference spectra were scanned between the wavelengths 380 and 500 nm. The absorbance difference (ΔA) was calculated between the apparent absorbance maximum and minimum of the binding spectrum. K_s and $\Delta A_{\max}$ values were calculated from the x- and y-axis intercepts of double-reciprocal plots of drug concentration versus ΔA (Jefcoate 1978).

The effect of parathion on CYP content in NADPH-supplemented microsomes (1 mM) was also evaluated spectrophotometrically using the ferrous-carbonyl method of Omura and Sato (1964).

Sodium dodecylsulphate polyacrylamide gel electrophoresis and immunoblotting

Rat hepatic microsomes (5 µg per lane) were heated at 100°C for 5 min with 2% sodium dodecyl sulphate and 5% 2-mercaptoethanol and then electrophoresed on 7.5% polyacrylamide gels (Laemmli 1970). Proteins were transferred to nitrocellulose sheets and incubated with CYP-specific IgGs for 120 min (3.7 µg protein ml⁻¹; responses were linear over a 10-fold protein range and fourfold range of antibody concentrations). The anti-rat CYP2B1 and CYP3A IgGs were prepared and characterized in this laboratory, as described previously (Murray 1988, Murray *et al.* 1999). Briefly, the anti-CYP IgGs recognized the homogeneous CYPs against which they were raised and were immunoinhibitory against CYP-specific steroid hydroxylation activities (anti-CYP3A and anti-CYP2B inhibited AD 6β- and 16β-hydroxylation reactions, respectively, but did not inhibit other pathways of AD hydroxylation). Immunoreactive proteins were detected by enhanced chemiluminescence and autoradiography on Hyperfilm-ECL film and the resultant signals were analysed by densitometry (BioRad GS-7000, Richmond, CA, USA).

Data analysis and statistics

Data obtained in kinetic studies of CYP inhibition by parathion were analysed according to the graphical methods of Lineweaver-Burk (1/*S* versus 1/*V*, where *S* is the concentration of steroid substrate and *V* is the rate of product formation) and Dixon (1/*V* versus *I*, the parathion concentration), followed by Dixon replot analysis (Segel 1975). Linear regression was used to obtain the lines of best fit. Kinetic constants and other data are expressed as mean ± SEM, or as means of duplicate determinations that varied by <15% from the stated values.

Results*Induction of microsomal steroid hydroxylation and CYP expression in rat liver*

Male rats were administered PB (100 mg kg⁻¹ i.p. in saline for 3 days) or DEX (100 mg kg⁻¹ i.p. in corn oil for 3 days) according to standard induction regimen (daily dosage over 3 days; animals were killed 24 h after the third injection) or received no treatment (control). Regioselective AD hydroxylation was measured in microsomal fractions isolated from each of the differently pretreated livers. PB increased CYP2B-dependent AD 16β-hydroxylation to about 33-fold of control (18.20 ± 2.89 versus 0.55 ± 0.04 nmol mg protein⁻¹ min⁻¹; *p* < 0.01; table 1), and DEX and PB administration significantly increased CYP3A-mediated AD

Table 1. AD hydroxylation in hepatic microsomes from control and induced rats.

Rat pretreatment	AD metabolite (nmol mg protein ⁻¹ min ⁻¹)				
	Testosterone	16α-Hydroxy	16β-Hydroxy	6β-Hydroxy	7α-Hydroxy
None (control)	2.80 ± 0.15	3.05 ± 0.30	0.55 ± 0.04	2.25 ± 0.07	0.27 ± 0.01
DEX	1.61 ± 0.09**	3.42 ± 0.39	1.62 ± 0.20	11.87 ± 0.81**	0.36 ± 0.02*
PB	3.09 ± 0.18	4.26 ± 0.74	18.20 ± 2.89**	8.31 ± 0.15**	0.41 ± 0.04**

Data are means ± SEM derived from measurements in microsomal fractions from individual rats (*n* = 3).

Different from control; ***p* < 0.01, **p* < 0.05.

6 β -hydroxylation, respectively, to about 5.2- and 3.7-fold of control ($p < 0.01$). In contrast with these pronounced increases, CYP2A-mediated 7 α -hydroxylation activity was increased only slightly by DEX and PB to 1.3- and 1.5-fold of that in control microsomes, and CYP2C11-dependent AD 16 α -hydroxylation activity was unchanged from control. Testosterone formation by the 17 β -hydroxysteroid oxidoreductase activity was decreased by DEX treatment to 58% of control (1.61 ± 0.09 versus 2.80 ± 0.15 nmol mg⁻¹ protein min⁻¹; $p < 0.01$; table 1) but PB was without significant effect.

Consistent with these findings, DEX increased CYP3A-immunoreactive protein expression in hepatic microsomes to about 4.6-fold of control (figure 2) and CYP2B immunoreactive protein was increased by PB administration over the 3-day treatment regimen to 40-fold of control (figure 2).

Effect of CYP-induction and preincubation with NADPH on the inhibition of microsomal CYP activities by parathion

The apparent kinetics of inhibition of CYP activities by parathion were determined in control and induced microsomes. In control microsomal fractions, the inhibition of 6 β -hydroxylation activity ($K_m = 16$ μ M, $V_{max} = 2.09$ nmol mg protein⁻¹ min⁻¹; table 2) exhibited a K_i of 12 μ M (figure 3A and table 2). When

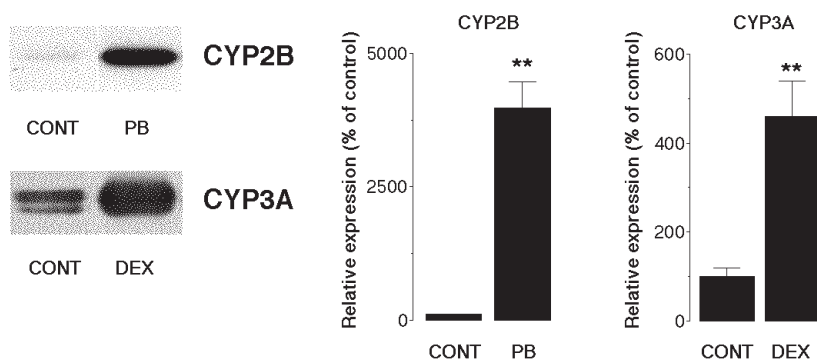


Figure 2. Western immunoblots showing induction of CYP2B and CYP3A immunoreactive protein in rat liver following induction by PB and DEX, respectively. Note the different scales on the y-axes describing the relative expression of CYPs 2B and 3A in control (CONT) and induced rat liver. **Different from control, $p < 0.01$ ($n = 4$ individual microsomal fractions per group).

Table 2. Apparent kinetic parameters for inhibition by parathion of CYP-mediated hydroxy-AD metabolite formation in microsomal fractions from control and induced rats.

Pretreatment	HydroxyAD metabolite	Preincubation*	K_m (μ M)	V_{max} (nmol mg protein ⁻¹ min ⁻¹)	K_i (μ M)	K_m/K_i	α
None (control)	6 β	no	16	2.99	12	1.5	3.5
		yes	12	2.60	3.0	4.0	2.1
DEX	6 β	no	22	20.7	14	1.6	1.5
		yes	12	17.0	1.5	8.0	2.5
PB	16 β	no	27	21.0	0.37	73	0.95

Data are means derived from independent analyses in different microsomal fractions conducted in duplicate. Data varied by $< 15\%$ from the stated values.

*Indicates whether parathion was preincubated with NADPH-supplemented microsomes before transfer to tubes containing AD, as described in the Materials and methods.

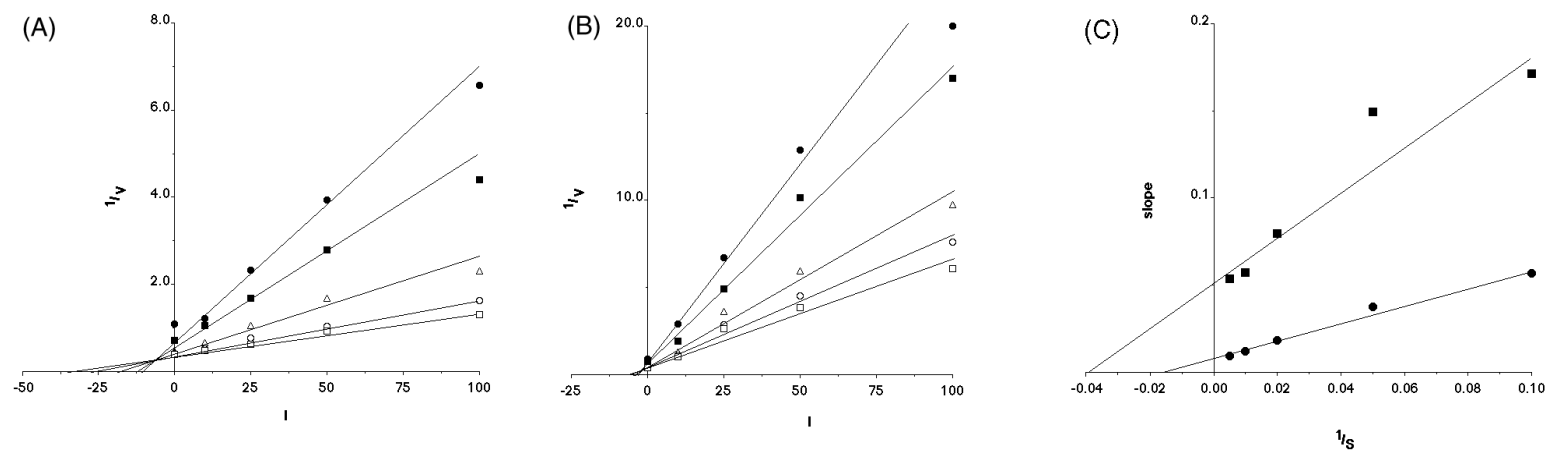


Figure 3. Kinetics of inhibition by parathion of control hepatic microsomal CYP3A-dependent AD 6β-hydroxylation. (A) Dixon plots in the absence of preincubation step, (B) Dixon plots following a 10-min preincubation between parathion and NADPH-supplemented microsomes prior to inclusion of substrate. Key to AD (substrate) concentrations: solid circles, 5 μM; solid squares, 10 μM; triangles, 20 μM; open circles, 50 μM; and open squares, 200 μM. (C) Replots constructed from Dixon slopes in (A) and (B) as a function of reciprocal AD concentration: squares, data from preincubation study; circles, data in the absence of preincubation. Units are substrate and inhibitor (μM) and reaction rate (V) (nmol mg protein⁻¹ min⁻¹).

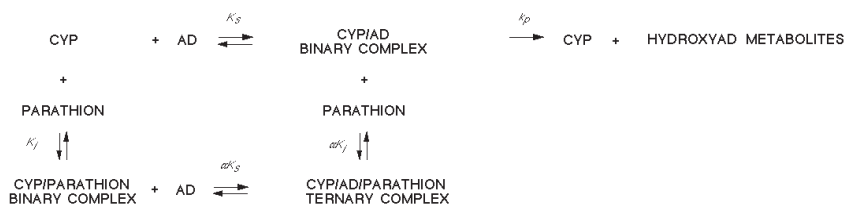


Figure 4. Kinetic equilibria showing the interaction of CYP with parathion and AD, resulting in the formation of binary and ternary complexes. K_s and K_i are the dissociation constants for the CYP-AD and CYP-parathion complexes, respectively, and k_p is the turnover number. α is the factor by which the presence of inhibitor or substrate alters the affinity of the enzyme for AD and parathion, respectively.

parathion was preincubated with NADPH-supplemented microsomal fractions before AD was introduced into the reactions, there was an increase in inhibition potency as revealed by a decrease in the apparent K_i to 3 μM (table 2 and figure 3). There was an associated increase in the K_m/K_i ratio from 1.5 to 4, reflecting a 2.7-fold increase in the relative potency of CYP3A inhibition by preincubation. From Dixon slope replot analysis, the apparent mode of inhibition in both cases was linear mixed-type (figure 3C). Values of 3.5 and 2.1 were determined for the factor α in the absence and presence of preincubation steps (figure 4). Thus, substrate and inhibitor mutually decrease each other's affinity for the catalytic CYP(s) by a factor of about 2–3 (Segel 1975).

In DEX-induced microsomes, parathion inhibited AD 6 β -hydroxylation activity ($K_m = 22 \mu\text{M}$, $V_{\max} = 20.7 \text{ nmol mg protein}^{-1} \text{ min}^{-1}$; table 2) with a K_i of 14 μM (figure 5A) that decreased after the 10-min preincubation step between parathion and NADPH-fortified microsomes to a value of 1.5 μM and increased the K_m/K_i ratio from 1.6 to 8 (figure 5B). This finding indicates a fivefold increase in potency mediated by parathion metabolites following preincubation in induced microsomes. Thus, inducible CYP3A activity was approximately twice as susceptible as control CYP3A activity to inhibition by parathion metabolites. Again, the mode of inhibition appeared to be linear mixed-type with values for α of 1.5 and 2.5, respectively, in the absence and presence of a preincubation step (figure 5C).

The time dependence of the CYP inactivation process by parathion was also studied. Several concentrations of parathion were incubated for varying times with NADPH-supplemented microsomes from untreated (figure 6A) and DEX-pretreated (figure 6B) liver before transfer to substrate. Findings were consistent with the greater susceptibility of the DEX-inducible AD 6 β -hydroxylation activity to mechanism-based inhibition. Thus, the optimal times for the loss of 50% of the initial enzyme activity were 6.1 min in control microsomes and 1.3 min in microsomes from DEX-induced rat liver. Similarly, the concentrations of parathion that effected half-maximal inactivation of AD 6 β -hydroxylase activity were 67 and 32 μM , respectively, in control and DEX-induced microsomes (figure 6C).

The inhibition of CYP2B1-mediated AD 16 β -hydroxylation in PB microsomes by parathion could only be evaluated at relatively low concentrations of parathion ($\leq 5 \mu\text{M}$) because inhibition was almost complete at higher concentrations. Inactivation of CYP was not apparent at these concentrations but was noted at concentrations around 50 μM , as judged by the loss of CO-binding capacity to reduced CYP haem (data not shown). From these studies, AD 16 β -hydroxylation

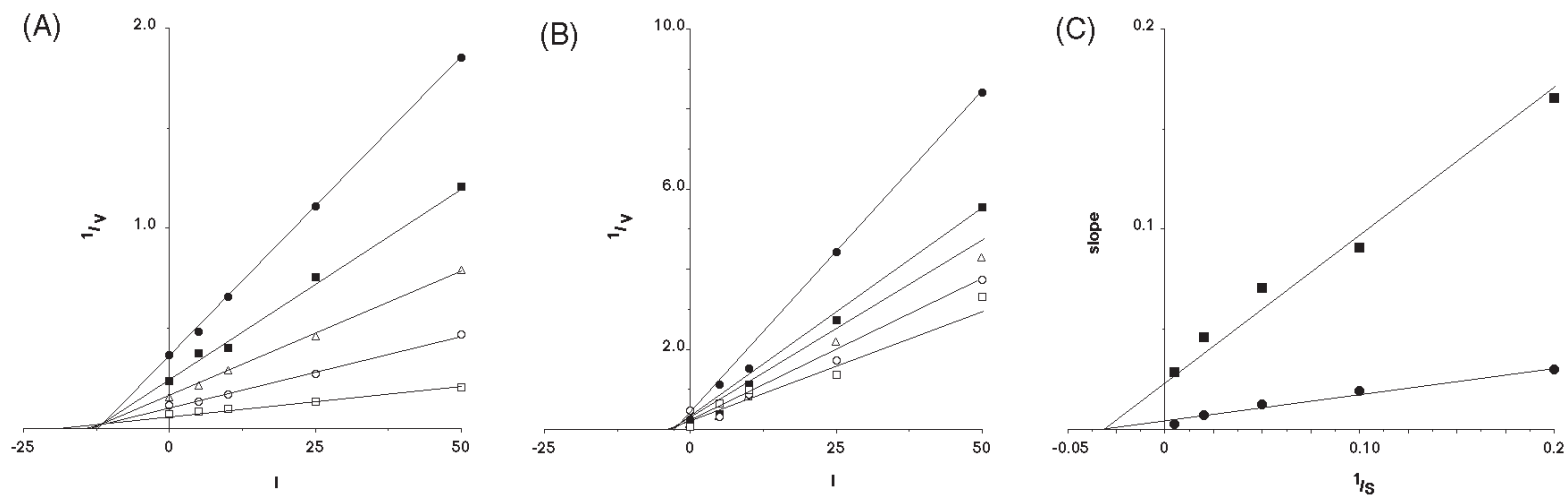


Figure 5. Kinetics of inhibition by parathion of CYP3A-dependent AD 6 β -hydroxylation in microsomes from DEX-induced rat liver. (A) Dixon plots in the absence of preincubation, (B) Dixon plots following a 10-min preincubation between parathion and NADPH-supplemented microsomes prior to inclusion of substrate. Key to AD concentrations: as shown in figure 3. (C) Replot constructed from Dixon slopes in (A) and (B) as a function of reciprocal AD concentration: squares, data from preincubation study; circles, data in the absence of preincubation. Units are as described in legend to figure 3.

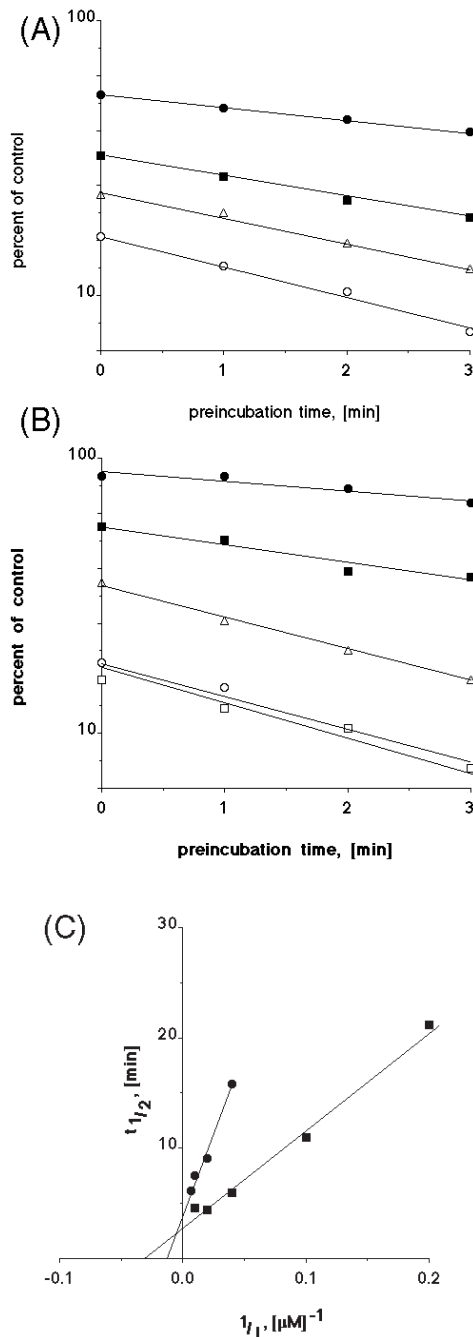


Figure 6. Semilogarithmic plots of the time-dependent intensification of CYP inhibition by parathion in NADPH-supplemented hepatic microsomes from (A) control and (B) DEX-induced rats. In (A) parathion concentrations were: solid circles, 25 μM ; squares, 50 μM ; triangles, 100 μM ; and open circles, 150 μM . In (B) parathion concentrations were: solid circles, 5 μM ; solid squares, 10 μM ; triangles, 25 μM ; open circles 50 μM ; and open squares, 100 μM . Data are means of duplicate experiments that varied by < 8% from the values shown. Control reactions: parathion (0 μM) was included at all time points in these experiments. (C) Replots of the times for loss of 50% of the zero time activity during preincubation as a function of reciprocal parathion concentration in microsomes from (circles) untreated and (squares) DEX-induced rat liver.

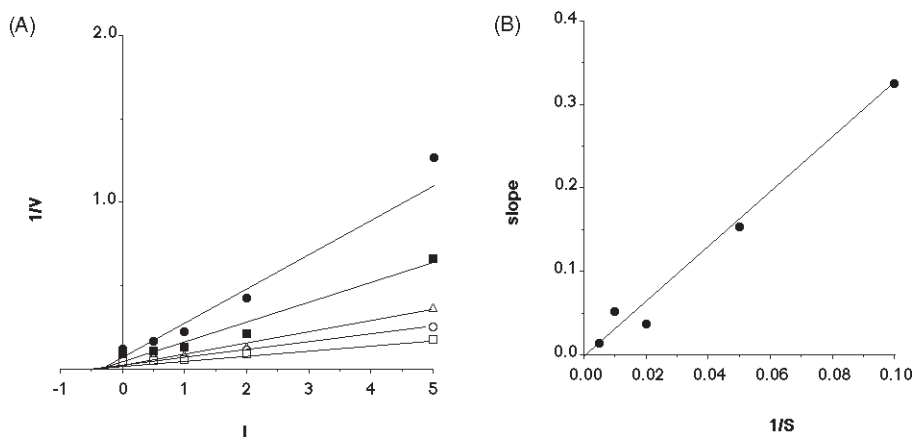


Figure 7. Kinetics of inhibition by parathion of microsomal CYP2B-dependent AD 16 β -hydroxylation in PB-induced rat liver. (A) Dixon plots, (B) replot constructed from Dixon slopes. Key to AD concentrations and units: as shown in legend to figure 3.

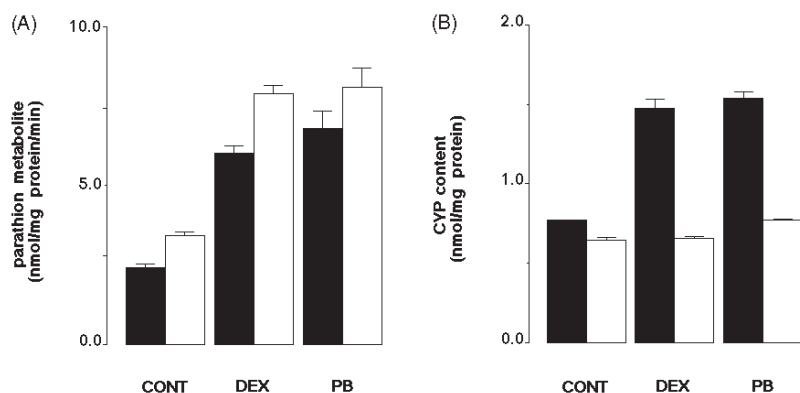


Figure 8. Effect of induction with DEX and PB on (A) the microsomal oxidation of parathion to (closed bars) 4-nitrophenol and (open bars) paraoxon in rat liver, and (B) CYP content in NADPH-supplemented microsomes in the presence (open bars) and absence (closed bars) of parathion (50 μ M); incubations were conducted for 10 min. Data are means $\pm$ SEM derived in $n = 4$ individual microsomal fractions in each treatment group.

($K_m = 27 \mu\text{M}$, $V_{\max} = 21.0 \text{ nmol mg protein}^{-1} \text{ min}^{-1}$) was inhibited with a K_i of $0.37 \mu\text{M}$ and a K_m/K_i ratio of 73 (table 2). From Dixon replot analysis, the line of best fit intercepted the origin (figure 7B), indicating that the mode of inhibition was competitive.

To explore the relationship between CYP induction and inactivation by parathion, rates of NADPH-dependent parathion (250 μM) metabolism were measured in hepatic microsomal fractions from control and differently induced rats. In control microsomes, paraoxon and 4-nitrophenol were formed at rates of 3.79 ± 0.21 and $2.45 \pm 0.27 \text{ nmol mg protein}^{-1} \text{ min}^{-1}$, respectively. Pretreatment of rats with PB increased NADPH-supported conversion of parathion to paraoxon and 4-nitrophenol to 2.7- and 2.5-fold of respective control (figure 8A), but metabolite formation was minimal at parathion concentrations near $5 \mu\text{M}$ (data not shown). Pretreatment of rats with DEX also increased the microsomal

formation of these metabolites to 2.5- and 1.9-fold of control (figure 8A). The ratio of paraoxon to 4-nitrophenol formation was similar in all fractions and the formation of paraoxon was consistently greater than that of 4-nitrophenol.

CYP loss was also measured after incubation of parathion with microsomes from differently pretreated rat liver. Thus, in control microsomes, 16% of CYP was lost on incubation with 50 μM parathion for 10 min. As shown in figure 8B, CYP degradation was enhanced by pretreatment of animals with PB (50% loss) and DEX (56% loss). Parathion (10 μM) elicited smaller CYP decreases in PB-induced microsomes (15% loss of holo-CYP), but at 5 μM CYP loss was not detected.

Spectroscopic studies of binding interactions of parathion with microsomal CYPs

The binding interactions of parathion with CYP in differently pretreated microsomes were evaluated. Parathion elicited a type I spectrum (λ_{max} about 386 nm and λ_{min} about 420 nm) in all fractions. The binding behaviour in control microsomes ($K_s = 7.7 \pm 0.5 \mu\text{M}$, $\Delta A_{\text{max}} = 2.2 \times 10^{-2}$ a.u. nmol CYP $^{-1}$; $\Delta A_{\text{max}}/K_s = 2.86 \times 10^3$) (figure 9 and table 3) was enhanced in efficiency ($\Delta A_{\text{max}}/K_s$) by PB (13-fold of control) and DEX (1.6-fold of control) pretreatment (table 3). From these findings and from application of the extinction coefficient of 110 $\text{mM}^{-1}\text{cm}^{-1}$ to the observed difference spectrum, about 20% of the available ferric-CYP was involved in the binding interaction in control microsomes (table 3). In microsomes from PB- and DEX-induced rat liver, this proportion of total CYP involved in the parathion type I interaction was increased to 56 and 72%, respectively.

Discussion

CYP-dependent oxidation of phosphorothioate pesticides to their phosphate esters and other metabolites occurs efficiently in mammalian hepatic microsomes, in isolated perfused livers and after *in vivo* exposure (Norman *et al.* 1974, Sultatos 1986, Butler and Murray 1997). The CYPs that participate in these reactions are degraded during phosphorothioate biotransformation *in vitro* by electrophilic sulphur atoms released from the thionophosphate ester moiety. The time taken for recovery of CYP activity is dependent on the synthesis of the replacement holo-enzyme, so that metabolic oxidation may be compromised for some time after exposure to the pesticide. Of potential importance in determining the toxicological impact of parathion exposure are reports that some CYPs may be especially susceptible to inhibition and inactivation during oxidation of the pesticide, whereas others are relatively resistant to inhibition (Butler and Murray 1993, Hurh *et al.* 2000). Because concurrent drug administration can alter the relative expression of individual CYPs in liver, this study tested the hypothesis that CYP induction may also modify the impact of parathion on drug oxidation capacity.

The present study establishes that certain induction pretreatments that alter the CYP profile of expression in rat liver also alter the relative susceptibility for inhibition by parathion. DEX induction enhances the susceptibility of CYP3A to inactivation whereas PB induction upregulated CYP2B1, an enzyme readily inhibited at low concentrations of the pesticide, but which is only inactivated at higher concentrations. The situation in induced liver is relatively complex in that alternate members of the CYP3A subfamily are differentially responsive to

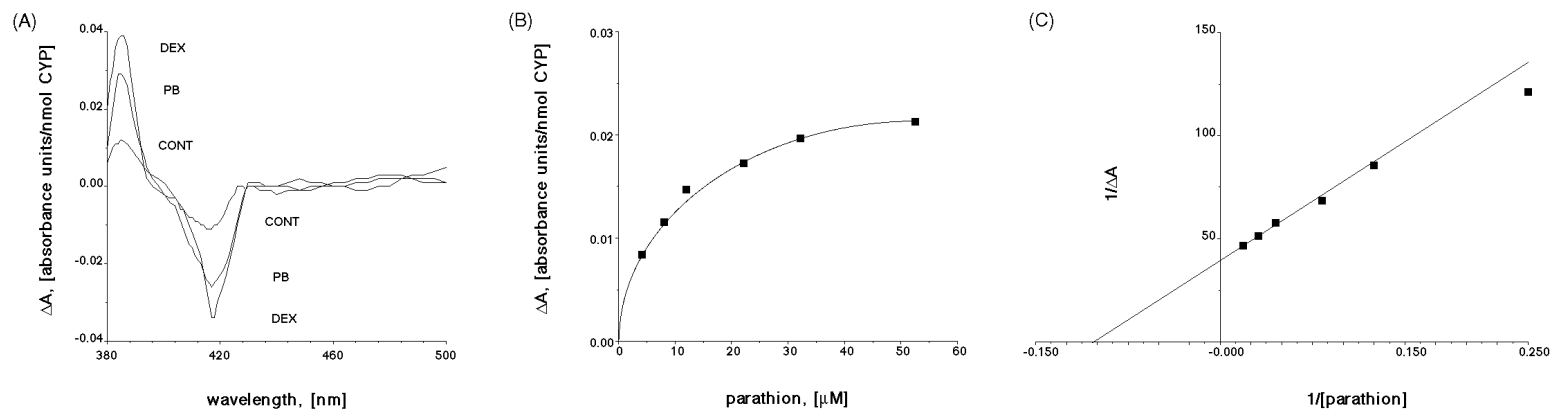


Figure 9. Type I spectra for the binding interactions of parathion with microsomal CYP. (A) Appearance of the spectra at 10 μM parathion in microsomes from control (CONT), PB-induced (PB) and DEX-induced (DEX) rat liver. (B) Absorbance change between the 386 and 417 nm wavelength pair in oxidized microsomes over a range of parathion concentrations. (C) Double reciprocal replot of the binding data obtained with parathion in (B); data are representative plots of spectral titrations performed at least in duplicate.

Table 3. Binding interactions of parathion in microsomal fractions from control and induced rats.

Rat pretreatment	K_s (μM)	ΔA_{max} (a.u. nmol CYP ⁻¹)	Ferric-CYP in binding interaction (%)	$\Delta A_{\text{max}}/K_s$ (a.u. nmol CYP ⁻¹ μM^{-1})	Fold of $\Delta A_{\text{max}}/K_s$ in control microsomes
None (control)	7.7 ± 0.5	$2.20 \pm 0.25 \times 10^{-2}$	20	2.86×10^3	1
DEX	13	6.18×10^{-2}	56	4.61×10^3	1.6
PB	2.2 ± 0.2	$7.97 \pm 0.19 \times 10^{-2}$	72	3.62×10^4	13

Data are means $\pm$ SEM of $n = 3$ individual spectral titrations, except in the case of fractions from DEX-pretreated rats, where difference spectra were obtained in duplicate; data varied by $< 8\%$ from the stated means.

agents like DEX. Thus, CYP3A1/23 is strongly induced by DEX and related chemicals, whereas the similar CYPs 3A1, 3A9 and 3A18 are considerably less inducible (Mahnke *et al.* 1997, Hoen *et al.* 2000). The present study indicates that DEX-inducible CYPs 3A (primarily CYP3A1/23) are more proficient in parathion oxidation than the constitutive CYPs 3A, including CYPs 3A2/9/18, and are more susceptible to inactivation by reactive parathion metabolites. Consistent with these findings, DEX treatment of rodents to increase parathion biotransformation has been reported (Hurh *et al.* 2000).

Inactivating metabolites of parathion are probably unable to leave the active site of the CYP in which they are formed. Thus, active site differences between CYPs probably contribute to differences in the observed extent of inactivation. CYPs 3A1/2/9/18 share 67–89% relatedness at the amino acid level, with CYPs 3A1 and 3A2 being 89% similar. From homology modelling and site-directed mutagenesis and expression studies, CYPs contain six stretches of amino acid sequence, termed substrate-recognition sequences (SRS), that appear critical for substrate–enzyme contacts within the active region of the proteins (Von Wachenfeldt and Johnson 1995). The sequences of SRS-1, 2, 3 and 5 in CYPs 3A1 and 3A2 are identical, but there are three amino acid residues that vary in each of SRS-4 and 6 (Tyr₂₉₇Ile, Ile₃₀₁Val and Pro₃₁₀Tyr in SRS-4 and Gly₄₈₂Ala, Leu₄₈₃Ile and Gln₄₈₅Glu in SRS-6). In considering SRS residues that may contribute to functional differences between the two CYPs 3A, Pro₃₁₀ in CYP3A1 is a rigid amino acid whereas Tyr₃₁₀ in CYP3A2 is a bulky aromatic residue, Gly₄₈₂ is an acidic residue in CYP3A1 and Ala₄₈₂ is a small aliphatic amino acid in CYP3A2, and the glutamine in CYP3A1 at residue 485 is replaced in CYP3A2 by a glutamic acid. Residues outside these SRS may influence the observed differences in susceptibility to parathion inactivation, but, consistent with the importance of the SRS, substrate specificities for the two enzymes are quite similar. Studies evaluating the impact of particular amino acid residues on inhibition potency are beginning to emerge. Thus, the ₂₀₅Thr residue in CYP2B1 is an important determinant of substrate specificity and inactivation by ethynyl compounds; replacement by valine markedly impaired the inactivation of the enzyme by these compounds (Lin *et al.* 2003). Similar studies are now required to account for the differential inactivation of CYPs 3A1/2 by parathion.

Inactivation is not the only mechanism of CYP inhibition that can be intensified following pretreatment of mammals with drugs. Certain drugs or their stable

metabolites have been shown to bind tightly but reversibly to the enzyme (Rein *et al.* 1979, McNeil and Murray 1996, Sutton *et al.* 1997). Thus, induction to increase the microsomal content of a CYP that is reversibly inhibited may also occur, as exemplified in the present study in which PB induced CYP2B1, which was inhibited potently and by a competitive mechanism. Inhibitory chemicals may compete with substrates and modulate CYP reduction by an electron from NADPH. This precedes the activation of molecular oxygen, which is necessary for oxidation of drug substrates. Because the inhibitory compound is not bound covalently within the CYP active centre, such reversible mechanisms are of relatively short duration. Inhibition of CYP2B1 by parathion was reversible at low concentrations and was not intensified by preincubation of the pesticide with NADPH-supplemented microsomes. Instead, CYP2B1 inactivation was observed at considerably higher concentrations that precluded the use of steroid hydroxylation, since inhibition was almost complete. Thus, the present study suggests that processes of CYP inhibition and inactivation can be dissociated.

Parathion generated a type I binding interaction with oxidized microsomal CYP in rat liver, which is consistent with a shift in the high/low-spin equilibrium of the *d*-electrons in ferrihaem toward the high-spin component (Rein *et al.* 1979). Such behaviour is usually associated with CYP substrates because reduction of the high-spin form of ferric-CYP by an incoming electron from NADPH is favoured thermodynamically over reduction of the low-spin form. The efficiency of type I binding of parathion was enhanced greatly in hepatic microsomes from PB-pretreated rats, which is consistent with the observed potent reversible inhibition of CYP2B1-mediated AD 16 β -hydroxylation. In accord with findings from direct inhibition studies, induction by PB enhanced the binding of parathion to CYP and inhibition of CYP activity. The observed high-affinity binding interactions between parathion and CYP in these microsomes is consistent with potent inhibition of reversible nature ($K_i = 0.37 \mu\text{M}$). CYPs such as CYP2B1, which are inhibited by low concentrations of parathion, could be associated with specific toxic effects in addition to the inactivation seen at higher exposure levels. Impaired clearance of xenobiotics would result in their accumulation in serum. As suggested previously, the acute sensitivity of CYP2B, an important drug oxidizing CYP, may be associated with significant effects on drug clearance at low pesticide concentrations (Murray and Butler 1995). In this regard, it is noteworthy that the human counterpart CYP2B6 has been implicated in the oxidation of parathion, but its susceptibility to inhibition remains to be determined (Butler and Murray 1997, Buratti *et al.* 2003).

The present findings that the concurrent exposure to CYP inducers can influence the mechanism and extent of CYP inhibition by parathion may have consequences for humans. Both CYP2B6 and CYP3A4 have been implicated in parathion oxidation and are both inducible. CYP3A4 expression in human liver is upregulated by ligands of the pregnane X receptor (PXR), a xenochemical sensor that forms heterodimers with the retinoid X receptor and binds at characteristic DNA elements in target genes to activate transcription (Kliewer *et al.* 1998). CYP3A4 expression is activated by a range of structurally diverse PXR ligands, including rifampicin, DEX, clotrimazole and hyperforin, a constituent of St John's wort. Induction of human CYP2B6 by PB involves the constitutive androstane receptor (CAR), which is another member of the receptor family that includes

PXR (Honkakoski *et al.* 1998). Induction of CYPs by chemicals like DEX and PB would increase the substrate flux through those CYPs, which, if susceptible to inhibition by drug metabolites, would impact on overall drug oxidation capacity. This situation is similar to, but different from, the well-described interindividual variation in expression of CYPs in human liver microsomes that results in greater than 10-fold variations in the oxidation of substrates like parathion (Butler and Murray 1997).

The present study adds to the body of knowledge that induction alters inhibitory processes that are dependent on metabolic processing rates. Enhancement of oxidative capacity toward the pro-inhibitory agent may precipitate greater inhibitory effects that are due either to the formation of CYP-destructive metabolites, or stable inhibitory products. The consequence of CYP induction by agents such as DEX or PB is to enhance the overall extent of loss of CYP activity by parathion. In some cases, such as the inhibition of CYP2B by parathion, a CYP enzyme may be extremely sensitive to the parent chemical—a process distinct from the requirement of metabolic activation.

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***In vitro* metabolism of [¹⁴C]methoxychlor in rat, mouse, Japanese quail and rainbow trout in precision-cut liver slices**

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1. The *in vitro* metabolism of [¹⁴C]methoxychlor (MXC) has been studied using precision-cut liver slices from the Sprague–Dawley male rat, CD-1 male mouse, WE strain male Japanese quail and juvenile rainbow trout (*Oncorhynchus mykiss*). The results demonstrated integrated phase I and II metabolism of MXC and species differences in the metabolic profiles were observed.

2. In rat liver slice preparations, MXC was rapidly metabolized to bis-OH-MXC by sequential *O*-demethylation followed by subsequent *O*-glucuronidation forming bis-OH-MXC glucuronide. No mono-OH-MXC glucuronide was detected. The doubly conjugated metabolite, bis-OH-MXC 4-*O*-sulphate 4'-*O*-glucuronide, was also detected as a rat-specific metabolite.

3. Formation of mono-OH-MXC and its glucuronide was the main metabolic pathway in the mouse and Japanese quail. In contrast to the rat, only minor amounts of bis-OH-MXC glucuronide were detected. A reductively dehalogenated metabolite, dechlorinated mono-OH-MXC glucuronide, was observed only in mouse preparations.

4. In rainbow trout, comparative amounts of both mono- and bis-OH-MXC glucuronide were formed as the major metabolites. Unconjugated forms of these metabolites were detected only as minor products.

5. The different metabolic profiles of MXC observed in the four animal species are possibly due to substrate specificity of contributing CYP450 monooxygenase enzyme(s) in different animal species.

Introduction

The organochlorine insecticide methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] (MXC) is a structural analogue of the environmentally persistent compound DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] and has been used as a substitute of DDT. Although MXC is biodegradable (Kapoor *et al.* 1970, Davison *et al.* 1982, 1983) and exhibits relatively lower acute toxicity in mammals compared with DDT (Kapoor *et al.* 1970), various studies have shown that it may elicit oestrogenic responses in mammals (Bulger *et al.* 1978a, Gray *et al.* 1989) and other wildlife including fish (Schlenk *et al.* 1997, Hemmer *et al.* 2001, Ankley *et al.* 2001). Such oestrogenic effects of MXC are believed to be caused by the action of its demethylated phenolic metabolites (Shelby *et al.* 1996, Gaido *et al.* 2000, Elsby *et al.* 2001), which are major phase I metabolites catalysed by the cytochrome P450 monooxygenase system (Dehal and Kupfer 1994, Kishimoto *et al.* 1995, Stresser and Kupfer 1998). Indeed, the mono- and bis-demethylated metabolites display binding activity toward oestrogen receptor *in vitro* (Bulger *et al.* 1978b, Elsby

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et al. 2001). It was additionally reported that individual metabolites have different oestrogenic activities depending on their molecular structures (Gaido *et al.* 2000). Therefore, it would be important to know the metabolic profiles of MXC in mammals and also wildlife species to understand metabolism-induced toxicity. Most of the previous reports, however, have focused on the phase I metabolic reactions of MXC, but little information is available for phase II metabolism.

Since the precision-cut tissue slice technique using an automated mechanical tissue slicer (e.g. Krumdieck Tissue Slicer) was introduced, it has been applied to drug and xenobiotic metabolism (Dogterom and Rothuizen 1993, Ekins 1996, Zalko *et al.* 1998) and toxicity studies (Lake *et al.* 1996, Thompson *et al.* 1996, Parrish *et al.* 1998, Schmieder *et al.* 2000) in recent years. The slice of liver tissue contains many intact cell layers that maintain hepatic architecture and cell-to-cell interactions. Therefore, liver slices are expected to provide integrated profiles of phase I and II metabolites that would be more similar to *in vivo* metabolism than other *in vitro* models.

In the present studies, *in vitro* metabolism of MXC by precision-cut liver slices from rat, mouse, Japanese quail and rainbow trout were conducted and the structures of phase II conjugated metabolites were elucidated. Furthermore, the different metabolic profiles obtained from the four animal species were compared.

Materials and methods

Chemicals

[Ring-U- ^{14}C]methoxychlor (5.85 MBq mg $^{-1}$, > 98% radiochemical purity) was obtained from BlyChem Ltd (Billingham, UK). Non-radiolabelled MXC (99.5% purity) was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany), mono- and bis-demethylated methoxychlor (mono-OH-MXC [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane], > 99% purity, and bis-OH-MXC [1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane], > 99% purity, respectively) from CEDRA Corporation (Austin, TX, USA), and β -glucuronidase (Type-H5, from *Helix pomatia*, also containing sulphatase activity) and D-saccharic acid 1,4-lactone from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were purchased from commercial source and were of the highest grade available.

Animals

Comparing species differences in MXC metabolism, the rodent species, rat and mouse were selected from mammals and, Japanese quail and rainbow trout were selected as models species for wildlife.

Male Sprague-Dawley rats (9 weeks old) and male CD-1 mice (18–19 weeks old) were purchased from Japan Charles River, Inc. (Kanagawa, Japan). Rats and mice were adapted to the laboratory conditions for at least 1 week. They were fed a standard diet (MF-type, Oriental Yeast Co., Tokyo, Japan) and had free access to water during an acclimatization period.

Immature rainbow trout (*Oncorhynchus mykiss*, 100–250 g body weight, female) were obtained from a fish farm at Saitama Central Fishery Co-op (Saitama, Japan) and allowed to acclimatize in dechlorinated laboratory water for at least 1 week. They were fed a commercial trout meal (Fuji Flour Milling Co., Ltd, Shizuoka, Japan) once a day.

Male Japanese quail (WE strain, 100–130 g body weight) obtained from Nippon Institute for Biological Science (NIBS; Tokyo, Japan) were allowed free access to a standard diet (NQ-1 type, NIBS) and water during acclimatization period and adapted to the laboratory conditions for at least 1 week.

All procedures carried out as part of this study were supervised by the Animal Care Committee of the Institute of Environmental Toxicology (IET).

Slice preparation

Rats, mice and quail were euthanized under anaesthesia and trout were sacrificed by a blow to the head. Their livers were immediately excised and placed in ice-chilled Krebs-Henseleit buffer (6.87 g NaCl, 0.4 g KCl, 0.25 g MgSO $_4$ ·7H $_2$ O, 0.13 g KH $_2$ PO $_4$, 2.2 g NaHCO $_3$, 0.33 g CaCl $_2$ ·2H $_2$ O, and 3.6 g fructose per 1 litre water, pH 7.4) (Dogterom 1993) previously gassed with 95% O $_2$ /5% CO $_2$ for 1 h.

Liver slices were prepared according to the method described by Barr *et al.* (1991). Briefly, liver cylindrical cores were prepared using a stainless steel 8-mm diameter tissue-coring tool (Tissue Coring Press; Alabama Research and Development Corporation, Munford, AL, USA). From the cores, tissue slices (approximately 250- μ m thickness) were immediately prepared in ice-chilled buffer using the Krumdieck tissue slicer (Alabama Research and Development Corp.).

Slice incubation

A multiwell plate culture procedure was used for slice incubation (Hashemi *et al.* 1999). Slices were placed into sterile 12-well polypropylene tissue culture plates (one slice/well) containing 1 ml incubation media. Incubations were conducted with a gyratory shaking (100 rpm) under an atmosphere of 5% CO₂/95% air at 37°C for rats and mice, 39°C for quail (the body temperature of this species was selected for the incubation temperature), and 15°C for trout (Singh *et al.* 1996), respectively. Following an 1 h pre-incubation, the medium was replaced with a fresh media and then an aliquot (5 μ l) of MXC dosing solution in dimethyl sulfoxide (DMSO) was added to 1 ml incubation media to obtain a final concentration of approximately 5 μ M (10 kBq/well, the final DMSO concentration was 0.5%, v/v).

Drug metabolizing activity of the slice preparations was validated by monitoring 20 μ M 7-ethoxycoumarin (7-EC) metabolism, forming 7-hydroxycoumarin (7-HC) and its sulphate and glucuronide conjugates, concurrently at each experimental run (Steensma *et al.* 1994, Walsh *et al.* 1995). The concentration of metabolites in the medium fraction was determined by a HPLC under the conditions described below. The amounts of 7-HC sulphate and glucuronide conjugates were estimated by enzymatic hydrolysis. An aliquot (0.2 ml) of cultured medium was incubated with β -glucuronidase (Sigma, type H-5; 500 units/assay), which also contains sulphatase activity, in 0.1 M sodium acetate buffer (pH 5.0) for 1 h at 37°C in the presence or absence of the β -glucuronidase inhibitor, D-saccharic acid 1,4-lactone (10 mM in reaction mixture). At the end of the incubation period, methanol was added to the reaction mixture to terminate the enzymatic reaction and the hydrolysate was analysed by HPLC to determine the amount of released 7-HC. The increased amount of 7-HC after enzymatic reaction in the presence of glucuronidase inhibitor was the 7-HC sulphate, and in the absence of inhibitor, the sum of 7-HC sulphate and glucuronide.

Sample preparation

At each sampling point, the slice and the medium were removed separately for metabolite analyses. The well was washed twice with 1 ml methanol to determine absorption loss of the radioactivity to the well. The medium sample was diluted with the same volume of methanol and subjected to radio-HPLC analysis. The slice was homogenized with 1 ml water using a glass-Teflon homogenizer. Duplicate aliquots (5–10 μ l) of the homogenate were subsampled for protein determination and the remainder was mixed with methanol to extract radioactive components, followed by sonication and centrifugation at 6000 rpm for 5 min at 4°C. The supernatant was analysed by a radio-HPLC. The pellet was resuspended in 0.5–1 ml water and aliquots were solubilized with tissue solubilizer (SOLUEN-350, Packard Instrument Co., Meriden, CT, USA) for radio-assay. The radioactivity in each fraction was determined by liquid scintillation counting (LSC-6100, Aloka Co., Ltd, Tokyo, Japan).

Protein was determined by the method of Bradford (1976) using a Bio-Rad (Hercules, CA, USA) protein assay kit. Bovine serum albumin was used as a standard and the procedure for protein determination essentially followed by manufacture's protocol.

Analytical procedures

Slice extracts and incubation media samples were analysed by a reversed-phase HPLC with a Shimadzu LC10AD HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a Ramona90 radioisotope detector (Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany) and a RETRIEVER II fraction collector (Isco, Inc., Lincoln, NE, USA). Chromatography was performed with a 250 $\times$ 4.6 mm Shiseido CAPCELL-PAK C₁₈ UG120 column (Shiseido Co., Ltd, Tokyo, Japan) and mobile phases consisted 10 mM ammonium acetate/acetic acid buffer (pH 4.6) in solvent (A) and acetonitrile in solvent (B). Elution was achieved with a flow rate of 1.0 ml min⁻¹ at column temperature of 40°C. A two-step gradient program was employed: 0–20 min, linear gradient from 70/30 = A/B (v/v) to 10/90 = A/B (v/v); 20–25 min, linear gradient leading 100% B. The individual HPLC peaks were separately collected into scintillation vials using a fraction collector and the radioactivity was measured by liquid scintillation counting to quantify the metabolites. The amounts of the metabolites detected in slice extracts and incubation media were summed to evaluate the formation of each metabolite.

Purified metabolites were obtained by a preparative HPLC employing the same HPLC conditions described above and the HPLC UV peaks corresponding to the MXC metabolites were separately collected.

For the study of 7-ethoxycoumarin metabolism, the same HPLC system, column and mobile phase used in MXC analyses were also applied. The quantities of 7-ethoxycoumarin metabolites were determined by ultraviolet light detection at 320 nm. The time program for gradient analysis

of 7-ethoxycoumarin metabolites was as follows: 0–20 min, linear gradient from 100/0 = A/B (v/v) to 0/100 = A/B (v/v).

Metabolite identification

The structures of the major metabolites were confirmed by either HPLC retention time comparison with the authentic standards or HPLC/ESI-MS/MS. Mass spectrometry with electrospray ionization (ESI) were performed in the negative-ion mode on a Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA, USA). The purified radioactive metabolites isolated by preparative HPLC were subjected to LC/ESI-MS and MS/MS analyses. ESI mass spectrometric conditions were as follows: electrospray voltage -4.2 kV, ion source temperature 500°C , nebulizer gas (dry air) 50 psi, heater gas (dry air) 80 psi and collision energy -25 V (for MS/MS analyses, CAD gas N_2).

To confirm the molecular structures of conjugated metabolites, enzymatic hydrolysis of purified metabolites were conducted under similar experimental conditions described above. After the enzymatic reaction with β -glucuronidase, which also contains sulphatase activity, in the presence or absence of β -glucuronidase inhibitor, the hydrolysate was analysed by the radio-HPLC.

Results

7-Ethoxycoumarin metabolism

In vitro metabolism of 7-ethoxycoumarin (7-EC) was conducted to validate the metabolic activity of the liver slice preparations from the four animal species. Table 1 shows the representative results of 7-EC metabolism after liver slices incubation and the production of 7-hydroxycoumarin (7-HC) and its sulphate and

Table 1. Metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin (7-HC), 7-HC glucuronide and 7-HC sulphate by rat, mouse, Japanese quail and rainbow trout liver slices.

	Incubation time (h)	Formation of metabolites (nmol metabolite mg^{-1} protein)		
		7-HC glucuronide	7-HC sulphate	7-HC
Rate	3	0.26 (0.08)	1.74 (0.34)	0.13 (0.01)
Mouse	3	1.62 ± 0.18	1.66 ± 0.09	0.14 ± 0.01
Japanese quail	1	0.53 ± 0.08	2.12 ± 0.64	5.27 ± 0.37
Rainbow trout	3	2.53 ± 0.40	n.d.	n.d.

Results are either means $\pm$ SEM of three replicates or means of duplicate with half range shown in parentheses.

n.d., Not detected.

The amounts of conjugated phase II metabolites were estimated by HPLC analyses of free 7-HC, before and after enzymatic hydrolysis with β -glucuronidase, which also contains sulphatase activity, in the presence or absence of the β -glucuronidase inhibitor.

Table 2. Mass spectral data of methoxychlor metabolites by negative-ESI-MS and MS/MS analyses.

Metabolites	ESI/MS		MS/MS*				
	Molecular related ions (m/z)		Fragment ions (m/z)				
	$[\text{M}-\text{H}]^{-}$ (Cl pattern)	$[\text{M}-2\text{H}]^{2-}$	(Percent relative abundance**)				
M1	571 (Cl_3)	285	395 (100)	491 (5)			
M2	491 (Cl_3)	—	85 (46)	95 (46)	113 (100)	175 (56)	455 (40)
M3	471 (Cl_2)	—	85 (56)	95 (28)	113 (84)	175 (28)	259 (100)
M4	505 (Cl_3)	—	85 (36)	95 (30)	113 (100)	175 (29)	

* Product ion scan of $[\text{M}-\text{H}]^{-}$ ion.

** Percent relative abundance to the most intense fragment ion.

M1; bis-OH-MXC 4-*O*-sulphate 4'-*O*-glucuronide, M2; bis-OH-MXC glucuronide, M3; dechlorinated mono-OH-MXC glucuronide, M4; mono-OH-MXC glucuronide.

glucuronic acid conjugates were compared. In liver slices from all four species, extensive metabolism with integrated phase I and II reactions was observed and the results from rat preparations were comparable with the ones already reported by Steensma *et al.* (1994). The percentages of 7-EC metabolism after 3 h in rat, mouse and rainbow trout liver slices were 18, 47 and 19% of the initial substrate concentration, respectively, and 77% for Japanese quail after 1 h. Formation of the metabolites was linear at least up to 3 h, except for 7-HC production in Japanese quail. Dealkylation in the quail preparations was quite rapid and the formation of 7-HC reached a maximum at 1 h and then kept constant or rather decreased after 3 h. In comparing the metabolite profiles in different animal species, it appears that there are species differences in the relative amounts of the glucuronic acid and sulphate conjugates. In rat and quail liver slices, 7-HC was preferentially conjugated with sulphate whereas the amounts of glucuronide and sulphate were nearly equal in mouse preparations. The ratio of glucuronide and sulphate metabolites in mouse liver slices was slightly differ from the results by Steensma *et al.* (1994), and it may be due to the strain or sex difference used in the studies. In the case of rainbow trout liver slices, 7-HC glucuronide was produced as the major metabolite, but no 7-HC sulphate was detected, indicating that the lack of ability to catalyse sulphate conjugation toward 7-HC by this animal species (Singh *et al.* 1996).

Methoxychlor metabolism

Distribution of radioactivity. The time course distribution of the radioactivity in rat preparations is shown in figure 1. The other species showed a similar trend to that of the rat, although the total incubation times were different. Incubations were continued for 4, 7, 8 and 8 h for rat, Japanese quail, mouse and rainbow trout,

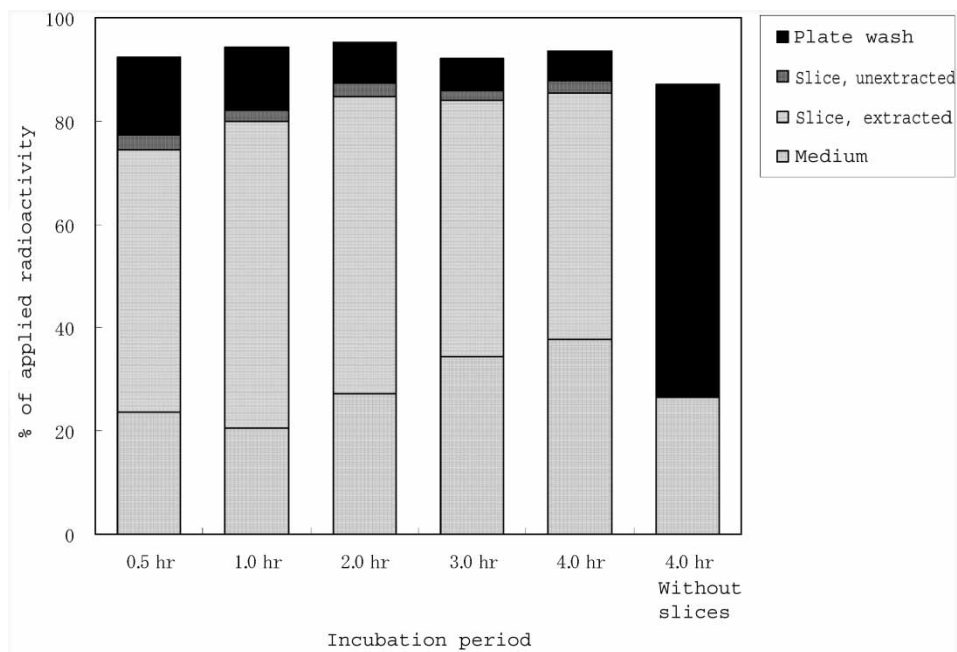


Figure 1. *In vitro* metabolism of [^{14}C]methoxychlor by precision-cut rat liver slices: time course distribution of radioactivity. Results are the mean of three replicates.

respectively, until at least more than 30% of the original MXC was metabolized. Most of the radioactivity was distributed in both medium and slice extracts fractions throughout the incubation period in any test animal preparations. Less than 17% of radioactivity was recovered from the well-washing fraction and no radioactive components other than MXC were detected from the fraction. Therefore, the amounts of metabolites formed in the liver slice preparations were calculated as the sum of the metabolite observed in the medium and in the slice extracts. Time course profiles of the distribution pattern of radioactivity seemed to be biphasic. At the beginning of the slice incubation (incubation of 0–1 h for rat), uptake of the radioactivity into the slice was observed and then the absorbed radioactivity was gradually released to the medium due to the formation of water-soluble metabolites, in the following phase.

In control samples, where MXC was incubated in the absence of slices, over 60% of radioactivity was recovered from the well-washing fraction after a 4-h incubation. No degradation products were detected in any of the fractions from the control samples, therefore MXC was considered to be stable under the incubation conditions.

Metabolites identification

Figures 2 and 3 show representative HPLC profiles of MXC metabolites by liver slices from rat (figure 2), mouse, Japanese quail and rainbow trout (figure 3). Metabolites M2 and M5 were detected as the prominent HPLC peaks for the rat preparations, M4 for mouse and quail, and M2 and M4 for trout. The identification of the metabolites was conducted by HPLC retention time comparison with the authentic standards and HPLC/ESI-MS/MS (negative-ion mode) analyses.

M5 and M6 (figure 2) were identified as bis- and mono-demethylated MXC (bis-OH-MXC and mono-OH-MXC), respectively, by comparing HPLC retention times with their corresponding authentic standards. It has been already

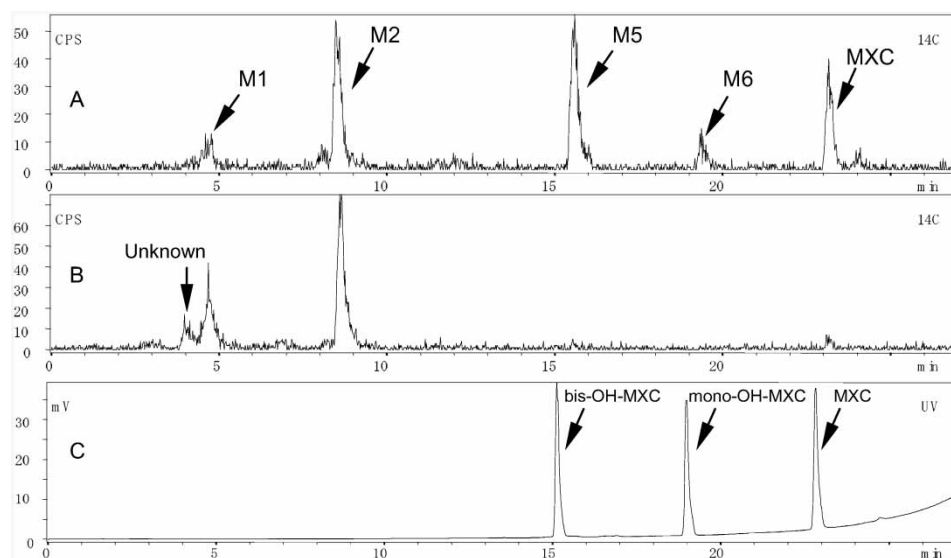


Figure 2. Chromatographic profiles of [^{14}C]methoxychlor metabolism by precision-cut rat liver slices: HPLC chromatograms of radioactive components in (A) slice extracts ($t = 0.5$ h), (B) incubation media ($t = 4$ h) and (C) authentic standards.

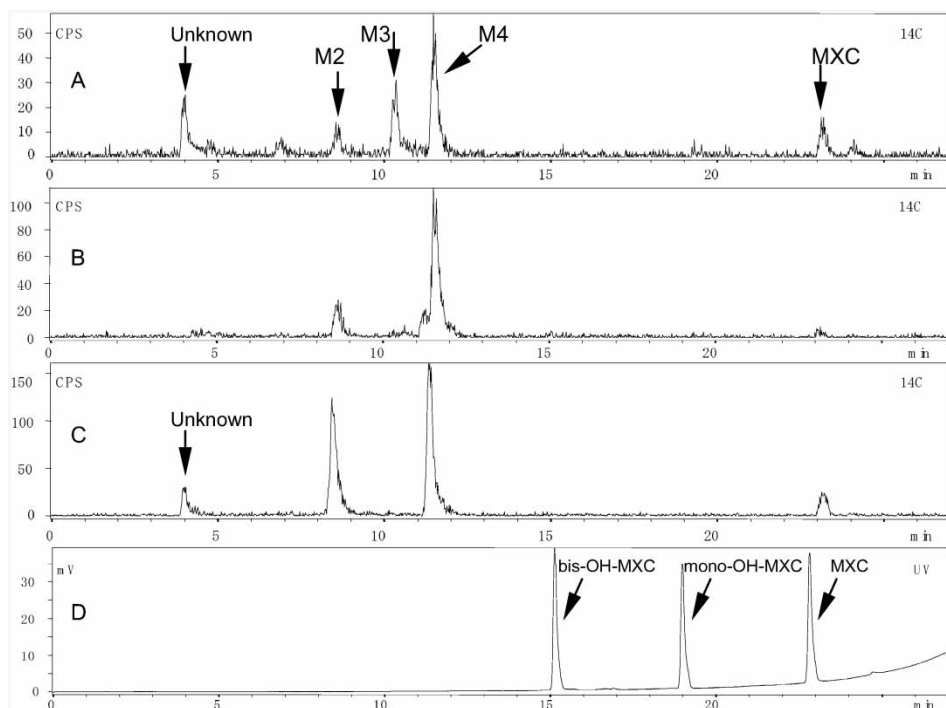


Figure 3. Chromatographic profiles of [^{14}C]methoxychlor metabolism by precision-cut mouse, Japanese quail and rainbow trout liver slices: HPLC chromatograms of radioactive components in incubation media from (A) mouse ($t=8\text{ h}$), (B) Japanese quail ($t=7\text{ h}$), (C) rainbow trout ($t=8\text{ h}$) and (D) authentic standards.

reported in several *in vitro* studies that oxidative *O*-demethylation is the predominant metabolic reaction for MXC metabolism in rat (Kurihara and Oku 1991) and human (Hu and Kupfer 2002).

ESI-MS analyses showed that the M2 and M4 had $[\text{M}-\text{H}]^-$ ions of m/z 491 and 505, respectively (table 2). MS/MS analyses revealed both M2 and M4 have a glucuronide moiety in the molecule because the specific fragment ions reflecting a glucuronide were detected at m/z 85, 113 and 175 (Ishikawa *et al.* 2004). The molecular weights of the remainder (aglycone moiety) of M2 and M4 were exactly same as those of bis- and mono-OH-MXC, respectively. The fragment ion m/z 455 detected in M2 reflects the loss of HCl from $[\text{M}-\text{H}]^-$ ion. In addition, HPLC retention times of isolated M2 and M4 peaks had shifted after enzymic hydrolysis with β -glucuronidase and had matched with those of bis- (M5) and mono-OH-MXC (M6). Therefore, M2 and M4 were identified as bis- and mono-OH-MXC glucuronide, respectively.

The rat-specific polar metabolite, designated as M1 in figure 2, had a $[\text{M}-\text{H}]^-$ ion of m/z 571 with doubly charged ($[\text{M}-2\text{H}]^{2-}$) ion at m/z 285, indicating that M1 has a molecular weight of 572 and contains two chargeable sites in the molecule. MS/MS analyses showed that M1 probably contains both glucuronide and sulphate moieties in the molecule, since observed fragment ions at m/z 395 and 491 reflect the structures for the loss of either a glucuronide or a sulphate moiety from the parent molecule, respectively and the molecular weight of the remainder of M1 is same as that of bis-OH-MXC (M5). Enzymatic hydrolysis study with

β -glucuronidase, which also contains sulphatase activity, showed that the HPLC retention time of isolated M1 had shifted to that of bis-OH-MXC (M5) after the enzymic reaction. However, when the same reaction was repeated in the presence of a β -glucuronidase inhibitor, D-saccharic acid 1,4-lactone, the HPLC peak of M1 had moved to the position of the bis-OH-MXC glucuronide (M2). These results supported the conclusions that M1 contains both glucuronic acid and sulphate moieties in the structure and the aglycone is bis-OH-MXC. Therefore, the structure of M1 was proposed as a doubly conjugated metabolite, bis-OH-MXC 4-*O*-sulphate 4'-*O*-glucuronide.

The mouse-specific metabolite, M3 in figure 3, had a $[M-H]^-$ ion of m/z 471 with the isotope pattern reflecting 2-chlorine atoms (table 2), indicating that M3 has a molecular weight of 472 and contains two chlorine atoms in the structure. The mass difference of M3 from mono-OH-MXC glucuronide (M4), which contains three chlorine atoms, was 34 amu, and corresponds to the loss of one chlorine atom. MS/MS analysis showed that M3 has a glucuronide moiety in the molecule (fragment ions reflecting a glucuronide were detected at m/z 85, 113 and 175). Therefore, the structure of M3 is proposed to be a dechlorinated form of mono-OH-MXC (dechlorinated mono-OH-MXC, [1,1-dichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane]) glucuronide. The fragment ion detected at m/z 259 reflects the loss of HCl from aglycone moiety.

The early eluting peaks with retention time of approximately 4 min, remained as unknown (figures 2B, 3A and C), and identification of the peaks are under investigation.

Metabolism of methoxychlor

As shown in figure 2, the metabolites observed in the medium fraction were only the conjugated forms (M1 and M2), and the demethylated phase I products (M5 and M6) were detected only in the slice extracts. In the case of the rat, for example, the amounts of phase I metabolites reached a maximum at 0.5 h and then decreased time dependently, and the phase II metabolites increased. These results indicated that metabolic reactions that include oxidative demethylation and subsequent conjugation were catalysed by metabolic enzymes in the liver slices and conjugated products were gradually released into the incubation medium, according to their relatively higher water solubility.

Figure 4 shows the time-course metabolism of MXC by precision-cut liver slices from the four species studied. In liver slice preparations from all test species, time-dependent metabolism of MXC was observed, and quantitative and qualitative differences were detected. The percentages of total metabolism of MXC after a 2 h incubation in rat, mouse, quail and trout were 58, 17, 23 and 27% of the original radioactivity, respectively. The percent distribution of the metabolites formed after a definitive incubation period is compared in table 3.

In rat preparations (figure 4A), a bis-demethylated compound (bis-OH-MXC, M5) and its *O*-glucuronide (M2) were the major metabolites. Formation of the bis-OH-MXC immediately reached a maximum of 24% of the original radioactivity after a 0.5 h incubation. Phase II metabolism of bis-OH-MXC subsequently followed and 40% of the radioactivity comprised the bis-OH-MXC glucuronide after 4 h (table 3). A mono-demethylated metabolite (mono-OH-

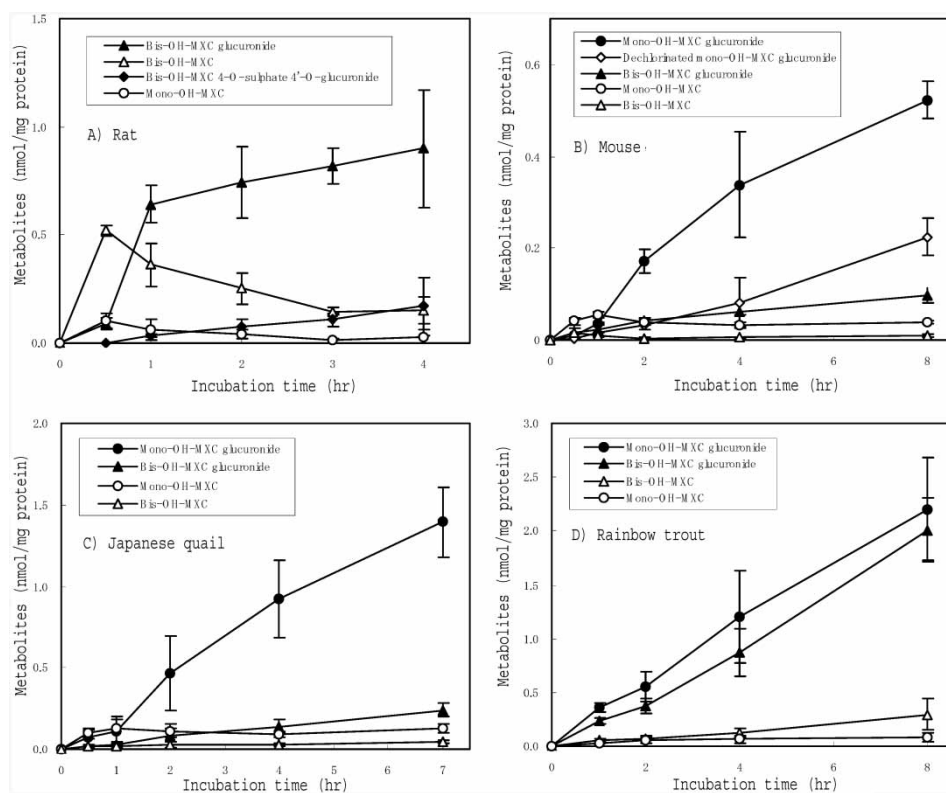


Figure 4. Metabolism of [^{14}C]methoxychlor by rat (A), mouse (B), Japanese quail (C) and rainbow trout (D) liver slices: time course of the formation of mono-OH-MXC ($\circ$), bis-OH-MXC (Δ), mono-OH-MXC glucuronide ($\bullet$), bis-OH-MXC glucuronide ($\blacktriangle$), bis-OH-MXC 4-*O*-sulphate 4'-*O*-glucuronide ($\blacklozenge$) and dechlorinated mono-OH-MXC glucuronide ($\diamond$). Results are the mean $\pm$ SEM of three replicates.

Table 3. Distribution of [^{14}C]methoxychlor metabolites in rat, mouse, Japanese quail and rainbow trout liver slices.

	Incubation time (h)*	Formation of metabolites (Percent of the original radioactivity)					
		M1	M2	M3	M4	M5	M6
Rate	4	18.0 $\pm$ 1.3	40.1 $\pm$ 3.0	n.d.	n.d.	7.1 $\pm$ 3.9	1.2 $\pm$ 0.8
Mouse	8	n.d.	3.4 $\pm$ 0.6	7.9 $\pm$ 2.4	18.1 $\pm$ 3.5	0.4 $\pm$ 0.1	1.3 $\pm$ 0.3
Japanese quail	7	n.d.	6.6 $\pm$ 1.0	n.d.	38.2 $\pm$ 4.1	1.3 $\pm$ 0.3	3.6 $\pm$ 1.0
Rainbow trout	8	n.d.	24.5 $\pm$ 2.6	n.d.	27.6 $\pm$ 1.2	5.5 $\pm$ 1.4	1.5 $\pm$ 0.4

M1, bis-OH-MXC 4-*O*-sulphate 4'-*O*-glucuronide; M2, bis-OH-MXC glucuronide; M3, dechlorinated mono-OH-MXC glucuronide; M4, mono-OH-MXC glucuronide; M5, bis-OH-MXC; M6; mono-OH-MXC.

Results are means $\pm$ SEM of three replicates.

*Metabolic profiles were compared after a definitive period of incubation.

n.d., Not detected.

MXC, M6) was detected as a transient intermediate at shorter incubation times (5% at 0.5 h), however, formation of its glucuronide was almost negligible. Therefore, MXC appeared to be rapidly metabolized to bis-OH-MXC by sequential *O*-demethylation, followed by subsequent *O*-glucuronidation. Furthermore, sul-

phation of the bis-OH-MXC glucuronide additionally occurred to form a doubly conjugated metabolite, bis-OH-MXC 4-*O*-sulphate 4'-*O*-glucuronide (M1), and this very polar metabolite was observed only in the rat preparations.

In the case of mouse and Japanese quail (figure 4B, C), metabolism of MXC was relatively slow compared to the rat. Up to an incubation time of 1 h, mono-OH-MXC was the predominant metabolite (2.7 and 4.6% of the original radioactivity for mouse and quail, respectively after 1 h) and glucuronidation to mono-OH-MXC gradually took place in both species. After a 7 (quail) or 8 h (mouse) incubation, the mono-OH-MXC glucuronide was detected as the main metabolite in both animal preparations and it reached 18 and 38% of the original radioactivity for mouse and quail, respectively (table 3). On the other hand, the bis-OH-MXC glucuronide was observed only as a minor product (3.4 and 6.6% for mouse and quail, respectively). Therefore, mono-*O*-demethylation and subsequent *O*-glucuronidation may be considered as the main metabolic reactions for these two latter species. The reductively dehalogenated metabolite, dechlorinated mono-OH-MXC glucuronide, was observed as a mouse-specific metabolite among animal species tested and reached 7.9% of the original radioactivity after a 8 h incubation.

In rainbow trout (figure 4D), nearly equal amounts of mono- and bis-OH-MXC glucuronides were produced. After an 8-h incubation, the glucuronides of mono- and bis-OH-MXC were major products (25 and 28% of the original radioactivity, respectively). Unlike the case of other animal species, unconjugated phase I metabolites were detected only as minor products throughout the incubation period, so that phase I demethylation reaction appeared to be the rate-determining step.

Discussion

Precision-cut liver slices were used to investigate the metabolism of MXC. The incubation of MXC with rat, mouse, Japanese quail and rainbow trout liver slices resulted in a time-dependent metabolism of MXC and the formation of phase II metabolites was confirmed, in addition to phase I metabolites.

In rat preparations, MXC was rapidly metabolized to bis-OH-MXC by sequential *O*-demethylation followed by subsequent *O*-glucuronidation, therefore bis-OH-MXC glucuronide was detected as the major metabolite. The sulphate conjugate of bis-OH-MXC glucuronide was additionally observed, but no observable HPLC peak of mono-OH-MXC glucuronide was detected. On the other hand, mono-OH-MXC glucuronide was the major metabolite in mouse and Japanese quail liver slices, and, hence, mono-demethylation and subsequent glucuronidation are considered the main metabolic routes for both species. A novel reductively dehalogenated metabolite, dechlorinated mono-OH-MXC glucuronide, was observed only in mouse preparations. In the case of rainbow trout, nearly equal amounts of mono- and bis-OH-MXC glucuronide were formed as final products. It appears that mono- and bis-OH-MXC are equally produced by phase I metabolism and glucuronidation subsequently follows, in this particular species.

The proposed metabolic pathways of MXC are given in figure 5. These pathways are essentially the same as those already reported as the major route of MXC metabolism in goats. Davison *et al.* (1982, 1983) found that most urinary

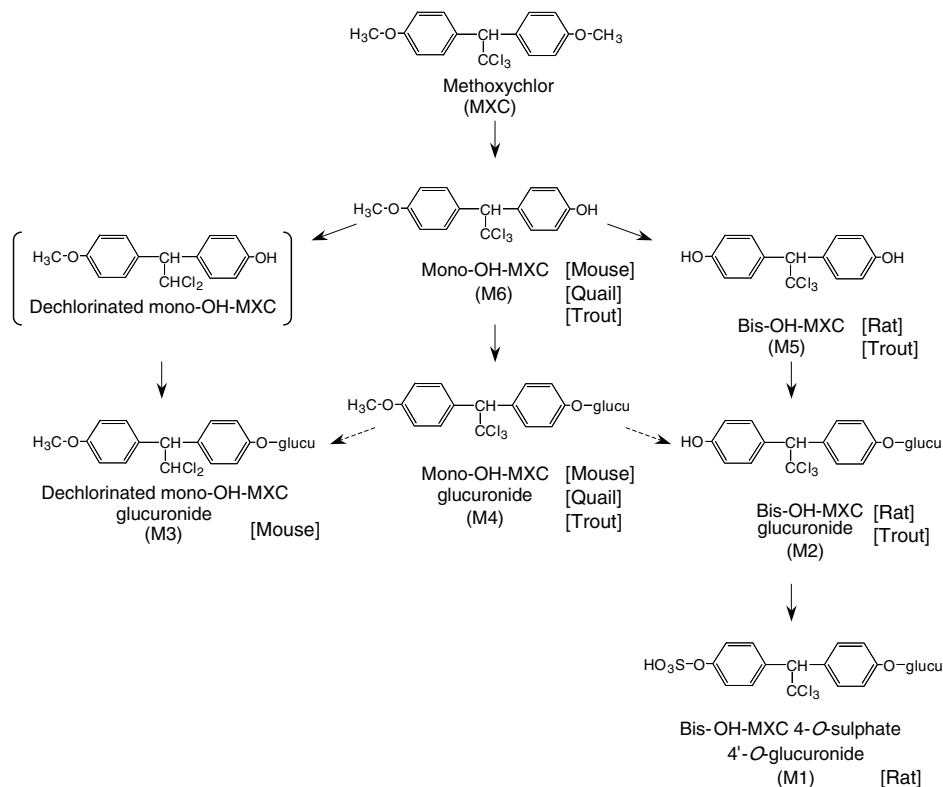


Figure 5. Proposed metabolic pathways of methoxychlor by precision-cut rat, mouse Japanese quail and rainbow trout liver slices.

metabolites in goats were completely demethylated and conjugated with glucuronic acid, and such metabolic profiles are similar to result from our *in vitro* liver slices. Therefore, the precision-cut liver slice technique has been shown to be a useful model to study bio-transformations of xenobiotics, although the direct comparison between *in vivo* and *in vitro* (liver slices) profiles in rodent species has not been made, since little information was available for phase II metabolites in a previous report which described *in vivo* MXC metabolism in rodent species (Kapoor *et al.* 1970).

The results revealed that the *O*-demethylase enzyme(s) in rat and trout can metabolize both MXC and mono-OH-MXC, whereas only MXC can be preferably demethylated by the enzyme(s) in mouse and quail. Thus, there are species differences in phase I oxidative demethylation, and such differences are probably caused by the difference in the reactivity of the contributing P450s enzymes toward MXC and its mono-demethylated metabolite. It would be necessary to compare the catalytic rate parameters for phase I metabolism of MXC (and its mono-demethylated product) in different animal species using liver microsomes in future studies.

It is already known that *O*-demethylation is the predominant reaction for MXC metabolism. Hu and Kupfer (2002) indicated that CYP1A2, CYP2C9 and CYP2C19 were the likely major enzymes contributing to MXC metabolism

in humans by using cDNA-expressed human P450 enzymes. Among these CYP enzymes, CYP2C19 and CYP1A2 catalysed the formation of both mono- and bis-demethylated metabolites, but CYP2C9 mainly contributed to the initial demethylation of MXC to mono-OH-MXC. The latter authors' results indicated that there are different substrate specificities in the different CYPs species contributing to MXC metabolism. Differences in such substrate specificities of contributing P450s in different animal species may be involved in the species differences of MXC metabolism observed in the present study, although the contribution of phase II metabolic enzyme needs also to be taken into account. Indeed, the current study has shown that demethylated phase I metabolites are extensively conjugated with D-glucuronic acid, such that the metabolites observed after longer incubation times were mostly conjugated forms of mono- or bis-OH-MXC, indicating phase II reactions also play an important role in MXC metabolism.

In summary, the present study demonstrates that MXC is extensively metabolized by rat, mouse, Japanese quail and rainbow trout precision-cut liver slices, forming mono- and bis-OH-MXC, as well as their corresponding phase II metabolites. Species differences in metabolic profiles were detected and it is likely that differences in metabolism are due to the substrate specificity of contributing CYP450 monooxygenase enzyme(s) in different animal species. The different metabolic profiles of MXC in different animal species may provide one possible explanation for the difference of MXC toxicity including oestrogenic effects toward different animal species.

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Disposition of CP-671, 305, a selective phosphodiesterase 4 inhibitor in preclinical species

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1. The disposition of (+)-2-[4-({[2-(benzo[1,3] dioxol-5-yloxy)-pyridine-3-carbonyl]-amino}-methyl)-3-fluoro-phenoxy]-propionic acid (CP-671,305), a potent and selective inhibitor of phosphodiesterase 4 (subtype D), was characterized in several animal species in support of its selection for preclinical safety studies and potential clinical development.

2. CP-671,305 demonstrates generally favourable pharmacokinetic properties in all species examined. Systemic plasma clearance after intravenous administration was low in Sprague–Dawley rats ($9.60 \pm 1.16 \text{ ml min}^{-1} \text{ kg}^{-1}$), beagle dogs ($2.90 \pm 0.81 \text{ ml min}^{-1} \text{ kg}^{-1}$) and cynomolgus monkeys ($2.94 \pm 0.87 \text{ ml min}^{-1} \text{ kg}^{-1}$) resulting in plasma half-lives $> 5 \text{ h}$. Moderate to high bioavailability in rats (43–80%), dogs (45%) and monkeys (26%) was observed after oral dosing. In rats, oral pharmacokinetics were dose dependent over the dose range studied (10 and 25 mg kg^{-1}).

3. CP-671,305 was $> 97\%$ bound to plasma proteins in rat, dog, monkey and human.

4. The principal route of clearance of CP-671,305 in rats and dogs was by renal and biliary excretion of unchanged drug. This finding was consistent with CP-671,305 resistance towards metabolism in hepatocytes and NADPH-supplemented liver microsomes from preclinical species and human.

5. CP-671,305 did not exhibit competitive inhibition of the five major cytochrome P450 enzymes, namely CYP1A2, 2C9, 2C19, 2D6 and 3A4 ($\text{IC}_{50}\text{'s} > 50 \mu\text{M}$). Likewise, no time-dependent inactivation of the five major cytochrome P450 enzymes was discernible with CP-671,305.

6. Overall, the results indicate that the absorption, distribution, metabolism and excretion (ADME) profile of CP-671,305 is relatively consistent across preclinical species and predict potentially favourable pharmacokinetic properties in humans, supporting its selection for toxicity/safety assessment studies and possible investigations in humans.

Introduction

Phosphodiesterases (PDEs) have been classified into seven major families (PDE1–7) according to their substrate sensitivity, Ca^{2+} /calmodulin requirement, and inhibitor selectivity (Beavo 1995, Manganiello *et al.* 1995). Recently, this classification has been extended to include PDE8, PDE9, PDE10 and PDE11 (Francis *et al.* 2001). PDE4, an adenosine 3',5'-cyclic monophosphate (cAMP)-specific and Ca^{2+} -independent enzyme, is a key enzyme in the hydrolysis of cAMP in mast cells, basophils, eosinophils, monocytes and lymphocytes (Doherty 1999, Dyke and Montana 2002). Recently, four human cDNA isoforms of PDE4 (PDE4-A–D) have also been identified; mRNA for all four isoforms was expressed

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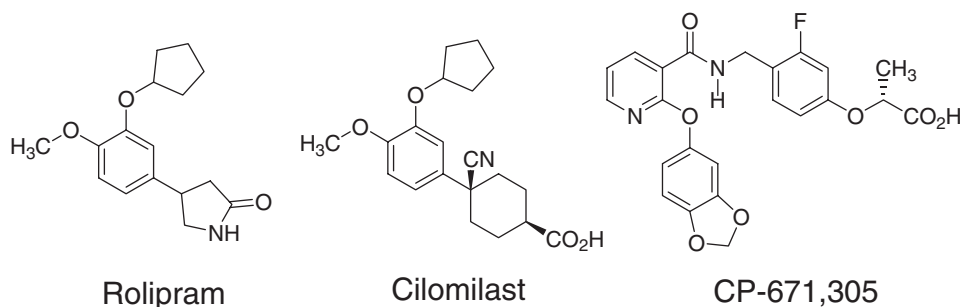


Figure 1. Structural diversity of PDE4 inhibitors.

in human lung; and PDE4-A, -B and -D were expressed in eosinophils (Muller *et al.* 1996). Inhibition of PDE4 not only leads to the down-regulation of inflammatory cell activity *in vitro*, but also it has been found generally to be anti-inflammatory and bronchodilatory in animal models (Essayan 1999, Souness *et al.* 2000). Consequently, over the past decade, many pharmaceutical companies have focused on the inhibition of PDE4 for the treatment of obstructive airway diseases including asthma and chronic obstructive pulmonary disease (COPD) (Norman 2000, Martin 2001). The first generation of PDE4 inhibitors typified by rolipram (figure 1) suffered from dose-limiting side-effects including nausea, emesis and gastrointestinal disturbances, which severely restricted their therapeutic benefits (Zeller *et al.* 1984). Two strategies for minimizing existing liabilities with PDE4 inhibitors have been extensively pursued; selectivity for the PDE4 catalytic site over the high-affinity rolipram binding site and selectivity for a specific PDE4 subtype over the other subtypes (Dyke and Montana 2002). The success of the latter strategy is evident with cilomilast (figure 1), the most advanced PDE4 inhibitor in clinical trials, which is claimed to be moderately selective towards inhibition of the PDE4-D isozyme (relative to PDE4-A-C subtypes) (Compton *et al.* 2001, Giembycz 2001, Gamble *et al.* 2003).

Towards the pursuit of PDE4 subtype specific inhibitors, the present authors recently identified a series of novel nicotinamide analogues that exhibit potent and selective inhibition of the PDE4-D isozyme as highlighted with (+)-2-[4-({[2-(benzo[1,3] dioxol-5-yloxy)-pyridine-3-carbonyl]-amino}-methyl)-3-fluoro-phenoxy]-propionic acid (CP-671,305; figure 1) (Marfat *et al.* 2004). CP-671,305 is a potent inhibitor of the PDE4-D isozyme ($IC_{50} = 3$ nM) and exhibits ≥ 96 –1286-fold selectivity for the human PDE4-D isozyme versus the -A ($IC_{50} = 310$ nM), -B ($IC_{50} = 287$ nM) or -C ($IC_{50} = 3858$ nM) subtypes. Furthermore, CP-671,305 is inactive on enzymes from the other known PDE families, i.e. PDE1-3 and PDE5-11 (IC_{50} 's > 5000 nM). CP-671,305 acts as an anti-inflammatory agent by blocking the cAMP-regulated production and release of mediators generated from neutrophils and eosinophils. As expected for a PDE4-D selective inhibitor, CP-671,305 potently inhibits the production of leukotriene E₄ from eosinophils in Sephadex bead-stimulated human whole blood with an $IC_{50} = 52$ nM. It also potently inhibits the release of leukotriene B₄ from neutrophils in Ca^{2+} ionophore-challenged blood with an $IC_{50} = 106$ nM. After subcutaneous administration to cynomolgus monkeys, CP-671,305 inhibits antigen-induced pulmonary eosinophil influx in a dose-dependent fashion.

To evaluate the potential for further development, preclinical pharmacokinetics and disposition of CP-671,305 were characterized in several laboratory animal species including rats, dogs and monkeys. Results from these studies, which are reported herein, suggest potentially favourable pharmacokinetic properties of CP-671,305 in humans, and thus, support its selection for toxicity/safety assessment studies and possible investigations in humans.

Materials and methods

Materials

CP-671,305 (chemical and isomeric purity > 99%) was synthesized at Pfizer Global Research and Development (Groton, CT, USA). Probe substrates (phenacetin, diclofenac, *S*-mephenytoin, bufuralol, testosterone, midazolam) and inhibitors (erythromycin ketoconazole, quinidine, sulfaphenazole, ticlopidine, furafylline) for individual CYP enzymes were purchased from Gentest Co. (Woburn, MA, USA). The selective and reversible CYP2C19 inhibitor (+)-*N*-3-benzyl nirvanol (Suzuki *et al.* 2002, Walsky and Obach 2003) as well as the selective and irreversible CYP2C9 inactivator tienilic acid were synthesized at Pfizer. Solvents used for analysis were of analytical or high-performance liquid chromatography (HPLC) grade (Fisher Scientific, Pittsburgh, PA, USA). Glycerol formal (75% 5-hydroxy-1,3-dioxane) was purchased from Sigma-Aldrich (St Louis, MO, USA). Microsomal fractions were prepared from male Sprague-Dawley rats (200–220 g, pool of 10–15 livers) (Charles River Laboratories, Wilmington, MA, USA), male beagle dogs (9–13 g, pool of 10 livers) and human livers (IIAM, Exton, PA, USA) using standard procedures. Pooled male cynomolgus monkey liver microsomes were purchased from Gentest. Cryopreserved hepatocytes from preclinical species and human were purchased from In Vitro Technologies (Baltimore, MD, USA). Protein concentrations were determined using the bicinchoninic acid assay method (Pierce Chemical, Rockford, IL, USA). Total cytochrome P450 (CYP) content was measured according to published protocols (Omura and Sato 1964) and human microsomes were characterized using CYP-specific marker substrate activities. Human liver microsomes were isolated from 56 individual livers and aliquots from the individual preparations were pooled based on equivalent protein concentrations to yield a representative pool of human liver microsomes. NADPH was purchased from Aldrich-Sigma (Milwaukee, WI, USA).

Plasma protein binding

The binding of CP-671,305 to rat, dog, monkey and human plasma proteins was determined using the equilibrium dialysis method. CP-671,305 (800 and 1600 ng ml⁻¹) was added to fresh plasma from animals or human. CP-671,305 concentrations examined are pharmacologically relevant based on *in vivo* efficacy studies in animals. The plasma samples (1 ml) were subjected to equilibrium dialysis (*n* = 6 or 8), using Spectra Por dialysis membranes (VWR, West Chester, PA, USA) with a molecular weight cut-off of 12.4 kDa. The resulting plasma samples were subjected to dialysis against isotonic phosphate buffer (pH 7.4) for 6 h. Following equilibration, the volume of the plasma and buffer samples removed from each equilibration cell was noted. Aliquots of dialysed plasma (100 µl) and buffer (100 µl) were transferred to 96-well blocks and acetonitrile (200 µl)

containing indomethacin as internal standard (50 ng ml^{-1}) was added to each well. Each matrix was normalized to the other by the addition of equal volumes of the opposite matrix (i.e. plasma to buffer and buffer to plasma). Samples were prepared for analysis by solid-phase extraction using a Waters Oasis[®] MAX (10 mg) extraction plate (Waters Corp., Milford, MA, USA), following the manufacturer's directions. Following extraction, the samples were then analysed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) and concentrations of CP-671,305 in buffer and plasma was determined by interpolation from a standard curve. Non-specific binding of CP-671,305 to the equilibrium dialysis device was minimal. The bound fraction of CP-671,305 was determined from the following equation:

$$\begin{aligned} \text{Fraction bound} = & [\text{CP-671,305}_{\text{Te}} - \text{CP-671,305}_{\text{F}}] \\ & \times V_{\text{pe}}/V_{\text{pi}}/[\text{CP-671,305}_{\text{Te}} - \text{CP-671,305}_{\text{F}}] \\ & \times V_{\text{pe}}/V_{\text{pi}} + \text{CP-671,305}_{\text{F}}, \end{aligned}$$

where $\text{CP-671,305}_{\text{Te}}$ and $\text{CP-671,305}_{\text{F}}$ are concentrations of CP-671,305 in the plasma side at equilibrium and in the buffer side, respectively, V_{pi} is the initial volume of plasma and V_{pe} is the plasma volume at equilibrium.

In vitro metabolism studies

The stability of CP-671,305 in liver microsomes from human, rat, dog and monkey was examined in triplicate following its incubation ($1 \mu\text{M}$) with microsomes (CYP concentration = $0.25 \mu\text{M}$) in 0.1 M potassium phosphate buffer ($\text{pH} = 7.4$) at 37°C . The reaction mixture was prewarmed at 37°C for 2 min before adding NADPH (1.2 mM). The final incubation volume was $600 \mu\text{l}$. Aliquots ($75 \mu\text{l}$) of the reaction mixture at $t = 0, 2, 5, 15$ and 30 min were added to acetonitrile ($200 \mu\text{l}$) containing indomethacin as internal standard (50 ng ml^{-1}) and the samples were centrifuged at $2500g$ for 5 min before LC/MS/MS analysis. For control experiments, NADPH was omitted from these incubations.

For the purposes of metabolite identification studies, the concentration of CP-671,305 in the microsomal incubations was increased to $100 \mu\text{M}$ and that of CYP in liver microsomes was increased to $0.5 \mu\text{M}$. Additional incubations were also conducted in the presence of glutathione (5 mM). The duration of the incubation time in the metabolite identification studies was increased from 30 to 60 min. After quenching the incubation mixtures with 2 vols acetonitrile, the solutions were centrifuged ($3000g$, 15 min) and the supernatants dried under a steady nitrogen stream. The residue was reconstituted with mobile phase and analysed for metabolite formation by LC/MS/MS.

CP-671,305 stability in hepatocytes was examined as follows. Cryopreserved hepatocytes from preclinical species and human were thawed and suspended in Williams' E media supplemented with 24 mM sodium bicarbonate and 10% foetal bovine serum at either 0.5×10^6 viable cells ml^{-1} for clearance prediction or 2×10^6 viable cells ml^{-1} for metabolite identification. CP-671,305 ($1 \mu\text{M}$ for clearance predictions or $50 \mu\text{M}$ for metabolite identification) was incubated with hepatocytes at 37°C for 4 h with gentle agitation. A gas mixture of oxygen/carbon dioxide (95:5) maintained at about 2.5 kPa for about 5 s was passed through this mixture at every 1 h of incubation. Flasks were corked immediately after gassing.

Freezing the incubation aliquots in liquid nitrogen terminated the reactions. Hepatocyte stability experiments were conducted in triplicate.

P450 inhibition studies

The ability of CP-671,305 as a competitive inhibitor of the five major CYPs, namely CYP1A2, 2C9, 2C19, 2D6 and 3A4, was examined in triplicate as follows. Phenacetin (50 μ M) (a probe substrate for CYP1A2), diclofenac (10 μ M) (a probe substrate for CYP2C9), *S*-mephenytoin (50 μ M) (a probe substrate for CYP2C19), bufuralol (10 μ M) (a probe substrate for CYP2D6), testosterone (50 μ M) or midazolam (5 μ M) (probe substrates for CYP3A4) was incubated with human liver microsomes (CYP concentration = 0.25 μ M), 3.3 mM MgCl₂, and 1.3 mM NADPH in a total volume of 0.6 ml 100 mM phosphate buffer pH 7.4, in the presence and absence of CP-671,305 at a concentration range of 0–50 μ M. The reactions were initiated by the addition of NADPH, and incubations were conducted in a shaking water bath at 37°C for 10 min in the case of diclofenac, bufuralol, testosterone and midazolam, or at 30 min in the case of phenacetin and *S*-mephenytoin. All reactions were terminated by the addition of acetonitrile (200 μ l) and the samples were centrifuged at 2500 *g* for 5 min before LC/MS/MS analysis. Procedures for the quantitation of metabolites of probe P450 substrates used in the present study have been described in detail by Obach (2000). Specific CYP enzyme inhibitors, ketoconazole (CYP3A4), quinidine (CYP2D6), sulfaphenazole (CYP2C9), (–)-*N*-3-benzylrivanol (CYP2C19) and furafylline (CYP1A2), were used as positive controls in the competitive inhibition studies.

The ability of CP-671,305 as a time- and concentration-dependent inactivator of the five major CYPs, namely CYP1A2, 2C9, 2C19, 2D6 and 3A4, was examined in duplicate as follows. Human liver microsomes (CYP concentration = 1.2 μ M) were pre-incubated with CP-671,305 at concentrations ranging from 5 to 50 μ M in duplicate, with and without NADPH (1.2 mM) in phosphate buffer (pH 7.4). The incubation volume was 200 μ l. After 0, 2, 5, 7, 10, 20 and 30 min of incubation, 10 μ l aliquots were removed added to phenacetin (100 μ M), testosterone (200 μ M), diclofenac (50 μ M), bufuralol (20 μ M) or *S*-mephenytoin (100 μ M) in 0.1M potassium phosphate with NADPH (1.3 mM). Negative control consisted of pre-incubations without CP-671,305 at each of the pre-incubation time point. Erythromycin (CYP3A4), tienilic acid (CYP2C9), ticlopidine (CYP2C19) and furafylline (CYP1A2) were included in the experiments as positive controls for specific CYP enzyme inactivation at concentrations ranging from 1 to 50 μ M. Incubations were performed in 96-well polypropylene microtiter plate in a shaking water bath at 37°C. After 15 min of additional incubation, reactions were terminated by the addition of acetonitrile (200 μ l) containing indomethacin as internal standard (50 ng ml⁻¹) and the samples were centrifuged at 2500 *g* for 5 min before LC/MS/MS analysis for the remaining CYP activity. For each time point, the peak area of metabolites from incubations containing CP-671,305 were compared with the relevant peak area of that incubation with no CP-671,305, to determine the per cent of enzyme activity inactivated by CP-671,305.

Animals

Male and female Sprague–Dawley rats (220–250 g), beagle dogs (9–13 kg) and cynomolgus monkeys (4–6 kg) were used. Bile duct exteriorized rats and

dogs were housed individually in metabolism cages during the study for 24 h for bile collection. All animals were fasted overnight before dosing.

In vivo pharmacokinetic studies

The Pfizer Institutional Animal Care and Use Committee approved all procedures. CP-671,305 was administered at 20 mg kg^{-1} intravenously (i.v.) and 10 and 25 mg kg^{-1} orally (p.o.) in rats, 5 mg kg^{-1} i.v. and 3 mg kg^{-1} p.o. in monkeys, and 3 mg kg^{-1} i.v. and 0.5 mg kg^{-1} p.o. in dogs. In bile duct exteriorized rats and dogs, CP-671,305 was also given intravenously at 20 and 3.7 mg kg^{-1} , respectively. CP-671,305 was administered via the jugular vein (rats) or via the brachial vein (dogs and monkeys). For all i.v. studies, dosing solutions of CP-671,305 were prepared in glycerol formal, whereas for p.o. studies, CP-671,305 was given as suspensions in 0.5% methylcellulose. Blood samples were taken via the jugular vein (rats and dogs) or femoral vein (monkeys) in series at appropriate times, and urine and bile were also collected from these animals for the entire study (0–24 h for rats and dogs, 0–48 h for monkeys). All samples were kept frozen until analysis.

Analytical procedures

Concentrations of CP-671,305 in microsomes, hepatocytes, plasma, urine and bile were monitored on a Sciex API model 3000 LC/MS/MS triple quadrupole mass spectrometer. Analytes were chromatographically separated using a Hewlett Packard (Palo Alto, CA, USA) Series 1100 HPLC system. An autosampler was programmed to inject $20 \mu\text{l}$ on a Phenomenex Primesphere $5 \mu\text{m}$ C18-HC $30 \times 2.0 \text{ mm}$ column using a mobile phase consisting of 10 mM ammonium acetate buffer–acetonitrile (60:40 v/v) containing 0.2% (v/v) triethylamine and 0.1% (v/v) acetic acid at a flow rate varying from 1 to 1.5 ml min^{-1} . Ionization was conducted in the positive-ion mode at the ionspray interface temperature of 400°C , using nitrogen for nebulizing and heating gas. The ion spray voltage was 5.0 kV and the orifice voltage was optimized at 30 eV. CP-671,305 and indomethacin were analysed in the multiple reaction monitoring mode using the transitions m/z 455 $\rightarrow$ 242 and 357 $\rightarrow$ 139, respectively. Calibration curves (0.01 – 10 mg ml^{-1}) were prepared by plotting the appropriate peak area ratios against the concentrations of CP-671,305 in plasma using $1/x$ weighting of CP-671,305/internal standard peak height ratios. The concentration of CP-671,305 in the plasma samples was determined by interpolation from the standard curve.

Qualitative assessments of CP-671,305 metabolism in microsomes and hepatocytes were conducted on a Sciex API model 2000 LC/MS/MS triple quadrupole mass spectrometer in conjunction with an LDC Analytical SpectroMonitor 3200 variable wavelength ultraviolet light (UV) detector. Analytes were chromatographically separated using a Hewlett Packard Series 1100 HPLC system. An autosampler was programmed to inject $50 \mu\text{l}$ of the sample on a Zorbax Rx-C₈ $4.6 \times 150 \text{ mm}$ column using a binary gradient consisting of a mixture of 10 mM ammonium formate, 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml min^{-1} . The LC gradient was programmed as follows: the solvent A to solvent B ratio was held at 100:0 (v/v) for 3 min and then adjusted from 100:0 (v/v) to 10:90 (v/v) for 20 min and from 10:90 (v/v) to 100:0 (v/v) from 20 to 25 min. The column was re-equilibrated for 5 min before the next analytical run. Post-column flow was split such that mobile phase was introduced into the mass

spectrometer via an ion spray interface at a rate of $50 \mu\text{l min}^{-1}$. The remaining flow was diverted to the UV detector positioned in line to provide simultaneous UV detection ($\lambda = 254 \text{ nm}$) and total ion chromatogram. Ionization was conducted in the positive-ion mode at the ionspray interface temperature of 150°C and using nitrogen as the nebulizing and heating gas. Ion spray voltage was 4.5 kV , and the orifice voltage was optimized at 40 eV . Initial Q1 scans were performed between m/z 50 and 1000.

Pharmacokinetic analysis

Plasma concentration–time profiles were analysed using the well-established non-compartmental method in WinNonLin v2.1 (Pharsight, Mountain View, CA, USA). The maximum plasma concentration (C_{max}) observed after p.o. dosing and the time at which it was observed (T_{max}) was determined directly from the individual plasma concentration–time profiles. Plasma clearance (CL_p) of CP-671,305 was calculated as the i.v. dose divided by the area under the plasma concentration–time curve from zero to infinity ($\text{AUC}_{0-\infty}$). $\text{AUC}_{0-\infty}$ was calculated by the linear trapezoid rule. The terminal slope of the $\ln(\text{concentration})$ versus time plot was calculated by linear least-squares regression and the half-life was calculated as 0.693 divided by the absolute slope. The steady-state volume of distribution (V_{dss}) was determined using non-compartmental method (Benet and Galeazzi 1971) as follows:

$$V_{\text{dss}} = (\text{i.v. dose} \times \text{AUMC}) / (\text{AUC})^2,$$

where AUMC is the total area under the first moment of the drug concentration–time curve from time zero to infinity.

The relative bioavailability (F) of the p.o. doses was calculated by using the following equation:

$$F = \text{AUC}_{0-\infty}^{\text{p.o.}} / \text{AUC}_{0-\infty}^{\text{i.v.}} \times \text{dose}^{\text{i.v.}} / \text{dose}^{\text{p.o.}}$$

Statistical analysis

An unpaired two-tailed Student's t -test was used to assess significance of differences in various pharmacokinetic parameters of CP-671,305 across groups. In instances where parameters possessed unequal variances, statistical analysis was performed with Welch's correction. One-way analysis of variance (ANOVA) with a Benferroni post-test comparison was used to assess difference in parameters for CP-671,305 across groups. In all cases, $p < 0.05$ was predetermined as the criterion for significance. All Statistical analysis was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Microsomal and hepatocyte stability of CP-671,305

The liver microsomal and hepatocyte stabilities of CP-671,305 were assessed following its incubation at a concentration of $1 \mu\text{M}$ with NADPH-supplemented liver microsomes and cryopreserved hepatocytes from rat, dog, monkey and human. CP-671,305 was stable during the microsomal and hepatocyte incubations (half-life $> 4 \text{ h}$) (data not shown). Even after incubation at a much higher concentration of $100 \mu\text{M}$, no metabolites of CP-671,305, including the potentially

reactive acyl glucuronide or catechol (derived from the ring scission of the 1,3-benzodioxole group), were detected in rat, dog, monkey and human hepatocyte and liver microsomal incubates (data not shown). Furthermore, no glutathione conjugate(s) was detected following incubation of CP-671,305 in NADPH-supplemented human liver microsomes in the presence of excess glutathione (data not shown).

CYP inhibition studies

The ability of CP-671,305 to function as a competitive inhibitor of the five major CYPs, namely CYP1A2, 2C9, 2C19, 2D6 and 3A4, was examined in human liver microsomes. In all instances, CP-671,305 did not inhibit the catalytic activity of the individual P450 isoforms as reflected by the IC_{50} values, which were $> 50 \mu M$ (data not shown). Under the present experimental conditions, the enzyme-specific inhibitors furafylline, sulfaphenazole, (+)-*N*-3-benzylirinanol, quinidine and ketoconazole demonstrated potent competitive inhibition of CYP1A2, 2C9, 2C19, 2D6 and 3A4 activity, respectively, in human liver microsomes. The corresponding IC_{50} values were $0.016 \mu M$ (ketoconazole), $0.058 \mu M$ (quinidine), $0.414 \mu M$ ((-)-*N*-3-benzylirinanol), $0.169 \mu M$ (sulfaphenazole) and $1.7 \mu M$ (furafylline).

CP-671,305 also did not exhibit time-dependent inactivation of individual CYP enzyme activity in human liver microsomes. In contrast, at comparable concentrations ($1\text{--}50 \mu M$), erythromycin (Mayhew *et al.* 2000), tienilic acid (Lopez-Garcia *et al.* 1994), ticlopidine (Ha-Duong *et al.* 2001) and furafylline (Racha *et al.* 1998) demonstrated potent ($> 50\%$ inactivation of enzyme activity at $10 \mu M$) time- and concentration-dependent inactivation of CYP3A4, 2C9, 2C19 and 1A2 activity, respectively, in human liver microsomes, in a manner consistent with that reported in the literature for these compounds.

In vivo pharmacokinetic studies

The pharmacokinetic parameters describing the disposition of CP-671,305 in rats are shown in table 1. The mean concentration–time profiles in rats after intravenous dosing is shown in figure 2. Following a single i.v. dose of 20 mg kg^{-1} , CP-671,305 exhibited a low CL_p ($9.60 \pm 1.16 \text{ ml min}^{-1} \text{ kg}^{-1}$) and a low V_{dss}

Table 1. Pharmacokinetic parameters defining the systemic disposition of CP-671,305 in rat after various doses and routes of administration.

Parameters	Dosing regimen			
	20 mg kg^{-1} (i.v.)	20 mg kg^{-1} (i.v. – BDC ^a)	10 mg kg^{-1} (p.o.)	25 mg kg^{-1} (p.o.)
$C_{\max}$ (ng ml ⁻¹)	–	–	1630 ± 826	5730 ± 2070
$T_{\max}$ (h)	–	–	0.8 ± 0.6	1.4 ± 1.8
$AUC_{0-\infty}$ (ng h ml ⁻¹)	$35\,200 \pm 3860$	$23\,000 \pm 6000$	7990 ± 2240	$35\,200 \pm 8300$
$t_{1/2}$ (h)	5.6 ± 1.70	1.03 ± 0.05	–	–
CL_p (ml min ⁻¹ kg ⁻¹)	9.60 ± 1.16	15.0 ± 3.41	–	–
V_{dss} (l kg ⁻¹)	0.82 ± 0.47	0.38 ± 0.19	–	–
F (%)	–	–	45	80

Data are the mean $\pm$ SD from four animals (two males, two females) and were derived from non-compartmental analysis.

^aBile duct cannulated animals.

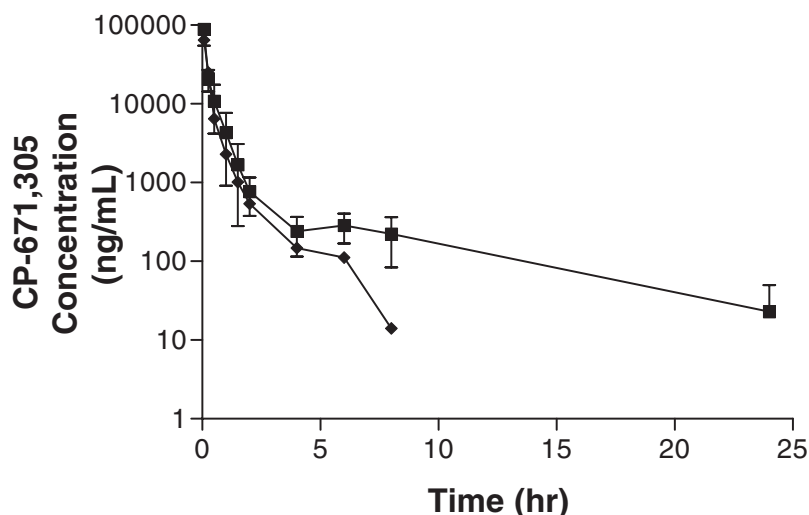


Figure 2. Plasma concentration-time profiles of CP-671,305 after intravenous (20 mg kg^{-1}) administration to normal (squares) and bile-duct (diamonds) cannulated male and female Sprague-Dawley rats (mean $\pm$ SD; $n = 4$, two males, two females).

($0.82 \pm 0.471^{-1} \text{ kg}^{-1}$) resulting in a mean elimination half-life of 5.6 h. In bile duct exteriorized rats, administered a 20 mg kg^{-1} i.v. dose of CP-671,305, the half-life of CP-671,305 was shorter (1.03 versus 5.6 h) and V_{dss} was 2.2-fold smaller; however, these parameters were not statistically significantly different compared with non bile-duct exteriorized rats (unpaired two-tailed Student's t -test, $p > 0.05$). A statistically significant 1.6-fold increase in CL_p (unpaired two-tailed Student's t -test, $p < 0.05$) was also observed after i.v. dosing to bile duct exteriorized rats, suggestive of enterohepatic recirculation of parent drug in rats. Unchanged CP-671,305 was the major component (about 50–60% of the dose) in pooled rat bile collected during the course of the study (0–24 h). Following p.o. administration of 10 and 25 mg kg^{-1} CP-671,305 to rats, a dose-dependent increase in C_{max} and $\text{AUC}_{0-\infty}$ was observed (3.5- and 4.0-fold increase in C_{max} and $\text{AUC}_{0-\infty}$, respectively, with a 2.5-fold increase in dose). The oral bioavailability of CP-671,305 at these doses was 45–80% (table 1). The mean concentration-time profiles in rats after p.o. dosing is shown in figure 3. Consistent with the *in vitro* observations in rat liver microsomes, no metabolites of CP-671,305 were discernible upon LC/MS/MS analysis of pooled plasma, urine and bile samples after i.v. and/or p.o. dosing (data not shown). Preliminary studies were also conducted to address the clearance mechanism of CP-671,305 in the rat. After i.v. administration, about 10% of the CP-671,305 dose appeared to be excreted unchanged in rat urine within 24 h. The unbound renal clearance of CP-671,305 in rats was $4.56 \pm 1.49 \text{ ml min}^{-1} \text{ kg}^{-1}$ and was obtained by multiplying CL_p by the fraction of the dose excreted in the urine and corrected for unbound fraction in plasma. The estimated renal CL_p is lower than the reported glomerular filtration rate of about $8 \text{ ml min}^{-1} \text{ kg}^{-1}$ in the rat (Till and Benet, 1979). This suggests that active tubular secretion of CP-671,305 does not occur in the kidney of the rat.

In dogs, following i.v. administration at a dose of 3 mg kg^{-1} , CP-671,305 also exhibited a low CL_p ($2.90 \pm 0.81 \text{ ml min}^{-1} \text{ kg}^{-1}$; $< 10\%$ of dog hepatic blood flow of $35 \text{ ml min}^{-1} \text{ kg}^{-1}$) and a low V_{dss} ($0.55 \pm 0.30 \text{ l kg}^{-1}$) resulting in a half-life of 5.9 h

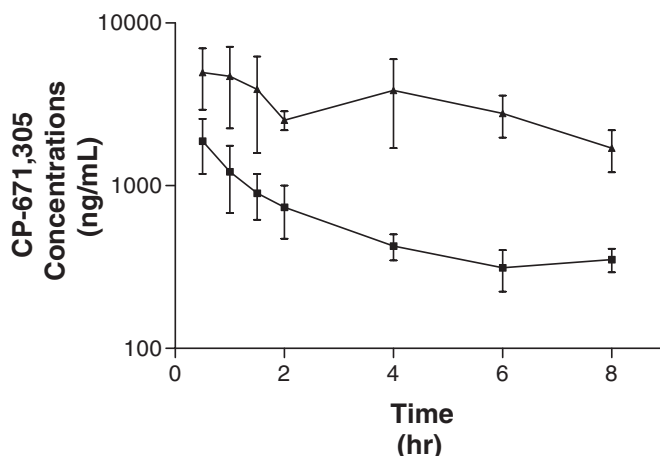


Figure 3. Plasma concentration–time profiles of CP-671,305 after oral administration at 10 mg kg^{-1} (squares) and 25 mg kg^{-1} (triangles) to male and female Sprague–Dawley rats (mean $\pm$ SD; $n = 4$, two males, two females).

Table 2. Pharmacokinetic parameters defining systemic disposition of CP-671,305 in dog after intravenous and oral administration.

Parameters	Dosing regimen		
	3 mg kg^{-1} (i.v.)	3.7 mg kg^{-1} (i.v. BDC ^{a,b})	0.5 mg kg^{-1} (p.o.)
$C_{\max}$ (ng ml ⁻¹)	–		452 ± 82.8
$T_{\max}$ (h)	–		0.5 ± 0.0
$\text{AUC}_{0-\infty}$ (ng h ml ⁻¹)	$18\ 200 \pm 5710$	$32\ 600$	1370 ± 493
$t_{1/2}$ (h)	5.90 ± 1.87	3.85	–
CL_p (ml min ⁻¹ kg ⁻¹)	2.90 ± 0.81	1.90	–
V_{dss} (l kg ⁻¹)	0.55 ± 0.30	0.21	–
F (%)	–		45

Data are the mean $\pm$ SD from three animals (two males, one female) and were derived from non-compartmental analysis.

^aBile duct cannulated animals.

^b $n = 2$ (one male, one female).

(table 2). In bile exteriorized dogs, administered an i.v. dose of 3.7 mg kg^{-1} , the observed CL_p and V_{dss} of CP-671,305 was $1.90 \text{ ml min}^{-1} \text{ kg}^{-1}$ and 0.21 l kg^{-1} , respectively, which resulted in a half-life of 3.85 h (table 2). There was no significant difference in half-life, CL_p or V_{dss} in bile exteriorized dogs compared with normal dogs (unpaired two-tailed Student's t -test, all $p < 0.05$). Approximately, 12% of the CP-671,305 dose appeared unchanged in the dog bile. Following p.o. administration at 0.5 mg kg^{-1} , systemic exposure as assessed from $C_{\max}$ and $\text{AUC}_{0-\infty}$ was 452 ng ml^{-1} and $1370 \text{ ng h ml}^{-1}$. The absolute oral bioavailability of CP-671,305 in the dog was 45% (table 2). Mean plasma concentration–time profiles for CP-671,305 after p.o. and i.v. dosing in dogs are shown in figure 4. Consistent with the *in vitro* observations in dog liver microsomes, no metabolites of CP-671,305 were discernible upon LC/MS/MS analysis of pooled plasma, urine and bile samples after i.v. and/or p.o. dosing (data not shown). As was observed in the rat, unbound renal CL_p in the dog ($0.36 \pm 0.23 \text{ ml min}^{-1} \text{ kg}^{-1}$) was estimated to be much lower than the glomerular filtration

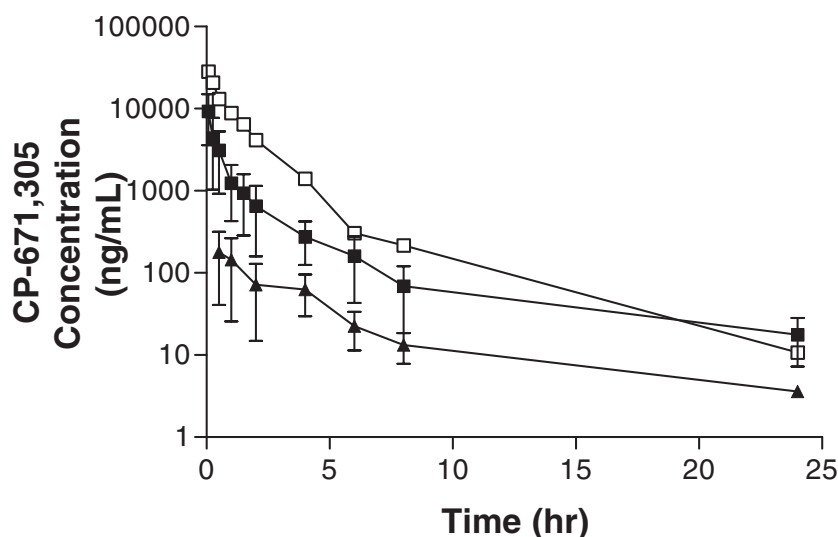


Figure 4. Plasma concentration–time profiles of CP-671,305 after intravenous (3 mg kg^{-1}) (closed squares) and oral (0.5 mg kg^{-1}) (closed triangles) administration to male and female beagle dogs (mean $\pm$ SD; $n=3/\text{route}$, two males, one female). Plasma concentration–time profiles of CP-671,305 after intravenous (3.7 mg kg^{-1}) administration to bile duct exteriorised dogs ($n=2$, one male, one female) is also depicted (open squares).

Table 3. Pharmacokinetic parameters defining systemic disposition of CP-671,305 in monkey after intravenous and oral administration.

Parameters	Dosing regimen	
	5 mg kg^{-1} (i.v.)	3 mg kg^{-1} (p.o.)
$C_{\max}$ (ng ml^{-1})	–	762 ± 184
$T_{\max}$ (h)	–	1.5 ± 0.5
$\text{AUC}_{0-\infty}$ (ng h ml^{-1})	$30\ 600 \pm 10\ 900$	4840 ± 527
$t_{1/2}$ (h)	13.1 ± 4.1	–
CL_p ($\text{ml min}^{-1} \text{ kg}^{-1}$)	2.94 ± 0.87	–
V_{dss} (l kg^{-1})	1.2 ± 0.2	–
F (%)	–	26

Data are the mean $\pm$ SD from three animals (two males, one female) and were derived from non-compartmental analysis.

rate of about $3 \text{ ml min}^{-1} \text{ kg}^{-1}$ reported in this species (Heiene and Moe 1998). This observation suggests that active tubular secretion of CP-671,305 does not occur in the kidney of dogs.

In monkeys, following i.v. administration of CP-671,305 at 5 mg kg^{-1} , CP-671,305 also exhibited a low CL_p ($2.94 \pm 0.87 \text{ ml min}^{-1} \text{ kg}^{-1}$; $< 10\%$ of monkey hepatic blood flow of $44 \text{ ml min}^{-1} \text{ kg}^{-1}$; Brown *et al.* 1997, Davis and Morris, 1993) and a low V_{dss} ($1.2 \pm 0.2 \text{ l kg}^{-1}$) resulting in a half-life of 13.1 h (table 3). Following p.o. administration at 3 mg kg^{-1} , systemic exposure as assessed from $C_{\max}$ and $\text{AUC}_{0-\infty}$ was $762 \pm 184 \text{ ng ml}^{-1}$ and $4840 \pm 527 \text{ ng h ml}^{-1}$. The absolute oral bioavailability of CP-671,305 in the monkey was 26% (table 3). Mean plasma concentration–time profiles for CP-671,305 after p.o. and i.v. dosing in monkeys are shown in figure 5. Consistent with the *in vitro* observations in monkey liver

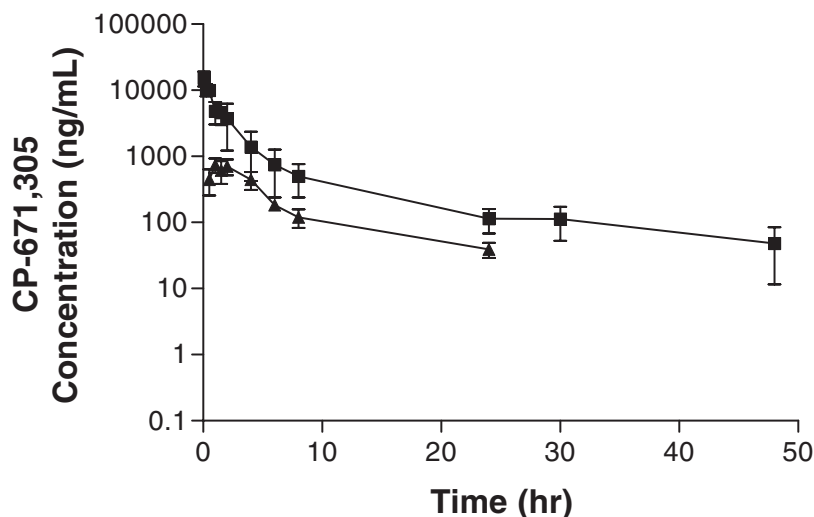


Figure 5. Plasma concentration–time profiles of CP-671,305 after intravenous (5 mg kg^{-1}) (squares) and oral (3 mg kg^{-1}) (triangles) administration to male and female cynomolgus monkeys (mean $\pm$ SD; $n = 3/\text{route}$).

Table 4. Protein binding of CP-671,305 in fresh rat, dog, monkey and human plasma.

CP-671,305 (ng ml^{-1})	Percent of fraction unbound (fu)			
	Rat	Dog	Monkey	Human
800	2.27 ± 0.34	2.06 ± 0.27	2.04 ± 0.87	2.07 ± 0.80
1600	2.99 ± 0.32	2.51 ± 0.68	1.82 ± 0.21	1.63 ± 0.31

Data are the mean $\pm$ SD ($n = 6$ or 8 determinations).

microsomes, no metabolites of CP-671,305 were discernible upon LC/MS/MS analysis of pooled plasma samples after i.v. and p.o. dosing (data not shown).

Plasma protein binding determination

In vitro plasma protein binding of CP-671,305 at concentrations of 800 and 1600 ng ml^{-1} was high in all species ($> 97\%$ bound; table 4). No evidence of concentration-dependent protein binding was observed. Cross-species comparison between rat, dog, monkey and human did not reveal a significant difference in protein binding between species at the nominal concentrations of 800 ng ml^{-1} ($p > 0.05$, one-way ANOVA).

Discussion

CP-671,305 is a novel nicotinamide-based PDE4 inhibitor that exhibits ≥ 96 –1286-fold selectivity towards inhibition of the human PDE4-D isozyme compared with the A-, B- and C-subtypes. Under experimental conditions that led to the determination of the PDE4-D potency and selectivity of CP-671,305, cilomilast demonstrated lower inhibitory potency ($\text{IC}_{50} = 37 \text{ nM}$) and selectivity for the human PDE4-D isozyme versus the A ($\text{IC}_{50} = 181 \text{ nM}$), B (141 nM) or C (573 nM) subtypes. Whether the increased inhibitory potency and selectivity

of CP-671,305 towards the PDE4-D isozyme results in decreased gastrointestinal toxicity (e.g. emesis and nausea) in humans remains to be determined. To evaluate the selection of CP-671,305 for potential clinical development, the disposition of CP-671,305 was characterized in preclinical species commonly employed in early non-clinical pharmacokinetic and safety/toxicology assessments.

From a drug metabolism/safety standpoint, the presence of the carboxylic acid and the 1,3-benzodioxole substituents in CP-671,305 was a potential concern given the susceptibility of these functional groups in forming reactive acyl glucuronides (Shipkova *et al.* 2003) and catechol/quinone (Murray 2000) intermediates, respectively. However, no evidence for acyl glucuronidation was seen in human, rat, dog and monkey hepatocyte incubations even at very high CP-671,305 concentrations. Upon incubation of CP-671,305 (100 μ M) with human liver microsomes in the presence of excess glutathione, no evidence for catechol or a glutathione adduct derived from conjugation with the reactive quinone intermediate was discernible in the incubates. Since ring scission of 1,3-benzodioxole derivatives is often accompanied by concomitant inhibition of CYP via formation of metabolic intermediate (MI) complexes (Hiramatsu *et al.* 1990, Fukuto *et al.* 1991), the ability of CP-671,305 to inhibit the five major CYPs was examined. Our findings on the lack of competitive and time-dependent (data not shown) inhibition of the major CYPs by CP-671,305 are a further indication that the 1,3-benzodioxole substituent is not susceptible to ring scission. Collectively, these *in vitro* results strongly suggest that CP-671,305 is not metabolized to reactive acyl glucuronide or catechol/quinone intermediates in liver microsomes and hepatocytes from pre-clinical species and human. Overall, CP-671,305 was stable towards metabolism in liver microsomes and hepatocytes derived from all of the species studied and these findings are consistent with (1) the observed low clearance of the compound after intravenous administration to rat, dog and monkey, and (2) the lack of detectable metabolites in pooled plasma, urine and bile from rats, dogs and monkeys following p.o. and i.v. administration.

Considering that metabolism does not appear to be a principal route of elimination for CP-671,305 after *in vivo* administration to preclinical species, and that CP-671,305 was resistant to metabolism in liver microsomes and hepatocytes from preclinical species and human, it is conceivable that CP-671,305 may also undergo excretion via a non-metabolic process in humans. Based on the excretion pattern for CP-671,305 in preclinical species, it is difficult to predict accurately the extent of potential biliary and/or renal clearance in humans. Interestingly, organic anion transporting polypeptide (OATP) and *p*-glycoprotein have been shown to mediate the cellular uptake and excretion of the carboxylic acid analogue, fexofenadine, which is principally excreted unchanged in the urine and faeces of humans (Cvetkovic *et al.* 1999). Thus, it is conceivable that uptake transporters could similarly influence the disposition of CP-671,305. The extent to which CP-671,305 will be excreted in the bile and urine will depend on its relative affinity for specific transporters in human. Studies are currently underway to examine the interactions of CP-671,305 with transport proteins that are normally associated with biliary and/or renal excretion of carboxylic acids (Kim 2002).

Despite the low CL_p, oral bioavailability of CP-671,305 was moderate at best in preclinical species. This phenomenon might be attributable to poor solubility and therefore incomplete absorption, since permeability studies in the human

Caco-2 cell line indicated that CP-671,305 is not a substrate for *p*-glycoprotein and is absorbed transcellularly by passive diffusion (data not shown). It is therefore anticipated that oral bioavailability of CP-671,305 in humans is also expected to be in the moderate range based on *F* in preclinical species, and the apparent low CL_p in human liver microsomes and hepatocytes. Estimates for human clearance of CP-671,305 were also obtained from the well-known allometric scaling method. Using this approach, the CL_p for CP-671,305 was predicted to be relatively low in humans (the projected CL_p obtained with or without protein binding correction was $1.6 \text{ ml min}^{-1} \text{ kg}^{-1}$ (allometric exponent = 0.69) and $0.4 \text{ ml min}^{-1} \text{ kg}^{-1}$ (allometric exponent = 0.38), respectively). This method has been shown to give a reasonable prediction of clearance for compounds with the allometric exponents < 0.7 (Mahmood and Balian 1996) and minimal metabolism (Sawada *et al.* 1984). Similarly, the allometric scaling method suggested a low volume of distribution for CP-671,305 in humans and the predicted V_{dss} obtained with protein binding correction (0.11 kg^{-1}) was lower than that without the correction (0.51 kg^{-1}). Estimation of the V_{dss} using the Oie-Tozer equation (Oie and Tozer 1979) led to a predicted V_{dss} in humans of 0.51 kg^{-1} . Overall, the low predicted V_{dss} in humans is consistent with the high plasma protein binding of CP-671,305 to human plasma proteins. Despite obvious structural differences, the predicted human pharmacokinetic parameters of CP-671,305 appear to be comparable with those observed with cilomilast after i.v. administration to humans (Zussman *et al.* 2001a, b). For instance, after i.v. administration at 0.06 mg kg^{-1} , cilomilast demonstrates low CL_p ($0.4 \text{ ml min}^{-1} \text{ kg}^{-1}$) and low V_{dss} (0.171 kg^{-1}), that results in a plasma elimination half-life of about 7 h.

To conclude, the study provides important information pertaining to the pharmacokinetic profile of CP-671,305, a novel PDE4 D-isozyme-selective inhibitor, in preclinical laboratory animal species. The results suggest favourable pharmacokinetic properties of CP-671,305 in humans and provide a basis for its selection for potential clinical development as an orally active agent for the treatment of COPD and asthma.

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Metabolic and structural viability of precision-cut rat lung slices in culture

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1. The principal objective was to evaluate the functional and structural integrity of precision-cut rat lung slices in culture over 72 h.
2. Lung slices metabolized 7-ethoxycoumarin in a time-dependent fashion, the major metabolites being the sulphate and glucuronide of 7-hydroxycoumarin with very low levels of the free compound. Prior treatment of rats with β -naphthoflavone elevated markedly the rate of metabolism. The optimum slice thickness, as exemplified by the metabolism of 7-ethoxycoumarin, was about 600 μ m.
3. Lung slices retained metabolic viability towards 7-ethoxycoumarin for 8 h, but after this point a marked decline in metabolic activity was noted. However, very low levels of activity were still evident following a 72 h incubation.
4. Morphological examination of lung slices revealed nuclear degeneration and loss of tissue architecture following 24 h incubation. When cellular integrity was assessed using lactate dehydrogenase, a time-dependent leakage was evident with maximum loss occurring within 24 h; longer incubations did not result in further leakage.
5. It is concluded that precision-cut rat lung slices, of 600 μ m thickness, can be maintained metabolically viable in culture for some 8 h.

Introduction

Precision-cut tissue slices are increasingly being used as an *in vitro* system in toxicology, and the advantages of this system over isolated microsomes or hepatocytes have been the subject of many reviews (De Kanter *et al.* 1999, Lerche-Langrand and Toutain 2000). Tissue slices have been employed successfully in studies to establish the metabolic pathways of xenobiotics (Ball *et al.* 1996, Ekins 1996), to assess the toxicity of chemicals (Miller *et al.* 1993, Price *et al.* 1995, 1996), to study the regulation of enzyme systems such as the cytochromes P450 (Meredith *et al.* 2003), to investigate the hepatic uptake of drugs (Olinga *et al.* 2001), and to evaluate the genotoxic and antigenotoxic potential of chemicals (Baumann *et al.* 1996, Lake *et al.* 1999). Moreover, kinetic data obtained using liver slices facilitate prediction of the *in vivo* metabolic clearance of chemicals (Worboys *et al.* 1995).

Precision-cut liver slices have been prepared successfully from a number of species such as laboratory animals (Price *et al.* 1996, Hashemi *et al.* 1999a), humans (Renwick *et al.* 2000) and food-producing animals (Sivapathasundaram *et al.* 2004). Similarly, slices have been generated from a number of tissues in addition to the liver, such as kidney, lung, prostate, spleen and heart (Parrish *et al.* 1995, 2002, Ball *et al.* 1996). Not surprisingly, precision-cut slices from the liver

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have been the most extensively investigated. Viability for as long as 5 days in culture has been reported, but this depends to a significant extent on the parameter used to assess tissue viability (Fisher *et al.* 1995). In a study emanating from the present authors' laboratory, using as a probe the metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin and its subsequent conjugation with sulphate or glucuronic acid, it was established that metabolism by liver slices in culture was maintained for some 10 h, but then declined with virtually no activity being detected following culturing for 48 h (Hashemi *et al.* 1999a). The stability of cytochrome P450 and phase II conjugation systems in rat and human liver slices in culture has been defined in some detail (Hashemi *et al.* 1999b, 2000, Renwick *et al.* 2000).

Precision-cut slices from other tissues have not received the same attention as liver slices. Successful use of precision-cut slices from extrahepatic tissues will facilitate the evaluation of tissue-specific chemical metabolism and toxicity, enable the clarification of the underlying mechanisms, and so enhance the understanding of tissue differences. Tissue slice viability in long-term culture is of major concern, especially when investigating the metabolism and toxicity of poorly metabolized chemicals, where generation of metabolites may be limited by the viability of the slices in culture. The present paper has evaluated the viability of rat precision-cut lung slices in culture over 72 h using three distinct biomarkers of structural and metabolic integrity. The viability of rat pulmonary slices has been assessed morphologically using light microscopy and correlated with structural integrity as exemplified by the leakage of lactate dehydrogenase and metabolic ability with 7-ethoxycoumarin serving as the model substrate.

Materials and methods

7-Ethoxycoumarin, LDH detection kits, sulphatase (Type H-1) and β -glucuronidase (Type B-1) (Sigma Co., Poole, UK) and Earle's balanced salt solution (EBSS) and Roswell Park Memorial Institute (RPMI) with L-glutamine culture media (Gibco-BRL Life Technologies, Paisley, UK) were purchased. Twelve-well plates were obtained from Bibby Sterilin (Helena Biosciences, Sunderland, UK).

Male Wistar albino rats (200 g) were obtained from the Experimental Biology Unit, University of Surrey. To achieve induction of the cytochrome P450 system, animals were treated with daily intraperitoneal administrations of β -naphthoflavone (50 mg kg^{-1}) for 3 days and were killed 24 h after the last injection. Rats were killed by cervical dislocation and the lungs were immediately removed and perfused intratracheally with agarose (0.75% v/w) at 37°C . Agar was allowed to solidify, and lung slices ($440\text{--}720 \mu\text{m}$) were prepared from cylindrical cores (8 mm) using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as described for rat liver (Hashemi *et al.* 1999a). The multiwell plate procedure (Dogterom 1993) using 12-well culture plates was used to culture the slices. The culture medium was essentially that described by Lake *et al.* (1993) and comprised RPMI 1640 Glutamax II containing foetal calf serum (5%), L-methionine (0.5 mM), insulin ($1 \mu\text{M}$), gentamicin ($50 \mu\text{g ml}^{-1}$) and hydrocortisone 21-hemisuccinate (0.1 mM). One slice was placed in each well in 1.5 ml culture medium. Slices were incubated under sterile conditions on a reciprocating plate shaker housed in a humidified incubator, at a temperature of 37°C and under an atmosphere of 95% air/5% carbon dioxide. The slices were initially pre-incubated for 60 min to slough off any dead cells due to slicing and were then

transferred to fresh medium and maintained at 37°C in the incubator. For incubations exceeding 24 h, the slices were placed in fresh medium every 24 h. Three slices per time point were used.

For histological examination, the 600- μm slices were briefly washed with 0.15 M KCl containing Tris-HCl (pH 7.4) and then fixed overnight in neutral phosphate buffered formalin. The fixed slice was trimmed to a central strip, which was routinely processed and wax embedded. Sections of 5 μm thickness were cut and stained with haematoxylin and eosin (Drury and Wallington 1967).

Leakage of LDH into the culture medium was determined using a kit and following the instructions provided (procedure 340-LD; Sigma). The metabolism of 7-ethoxycoumarin (50 μM) to 7-hydroxycoumarin and to its sulphate and glucuronide conjugates was monitored as described by Steensma *et al.* (1994). For the determination of the conjugates, aliquots of the media were diluted with 0.5 vol. 0.5 M sodium acetate buffer (pH 5), containing either β -glucuronidase (5000 Units ml^{-1}) or sulphatase (250 Units ml^{-1}) containing the β -glucuronidase inhibitor D-saccharic acid 1,4-lactone (17 mM). The mixtures were incubated for 16 h at 37°C, and the free 7-hydroxycoumarin was determined as follows. Aliquots (0.3 ml) of the incubation media, before and after deconjugation, were made to 1 ml with 0.154 M KCl buffer containing 50 mM Tris-HCl, pH 7.4. Following addition of 4 M HCl (0.25 ml), the samples were extracted for 30 min with chloroform (6 ml) on a rotary mixer. Aliquots (5 ml) of the chloroform layer were subsequently extracted for a further 30 min with 3 ml 0.5 M glycine-NaOH buffer, pH 10.5. The fluorescence of the aqueous layer was measured using excitation and emission wavelengths of 380 and 452 nm, respectively. Standards of 7-hydroxycoumarin were run through the same procedure. Finally, slices were homogenized in 0.154 M KCl buffer containing 50 mM Tris-HCl, pH 7.4, and total protein was determined using bovine serum albumin as standard (Bradford 1976).

Results

A time-dependent leakage of lactate dehydrogenase, indicating progressive cellular damage, was evident with maximum leakage of some 50% occurring within 24 h; incubation of the lung slices for longer periods, up to 72 h, did not appear to have further influenced the leakage of this enzyme (figure 1). Histological examination of the lung slices following staining with haematoxylin and eosin revealed that for the first 12 h of incubation, the slices maintained the normal tissue architecture, with alveolar walls remaining intact (figure 2). Incubations for 24 h or longer, however, were characterized by loss of tissue architecture and nuclear degeneration.

Lung slices metabolized 7-ethoxycoumarin in a time-dependent manner, for at least 6 h, by oxidative deethylation to generate 7-hydroxycoumarin, which is further conjugated with sulphate and glucuronic acid (figure 3A). There was no change in the level of homogenate protein during this period (figure 4A). The rate of 7-ethoxycoumarin metabolism was about 10-fold higher when the lung slices were prepared from animals pretreated with β -naphthoflavone (figure 3B).

Slices of different thickness, ranging from 440 to 720 μm , emanating from β -naphthoflavone-treated rats were incubated with 7-ethoxycoumarin for 5 h and metabolism by deethylation was determined. Metabolism was relatively low when slice thickness was below 560 μm , but a marked increase was noted when thickness

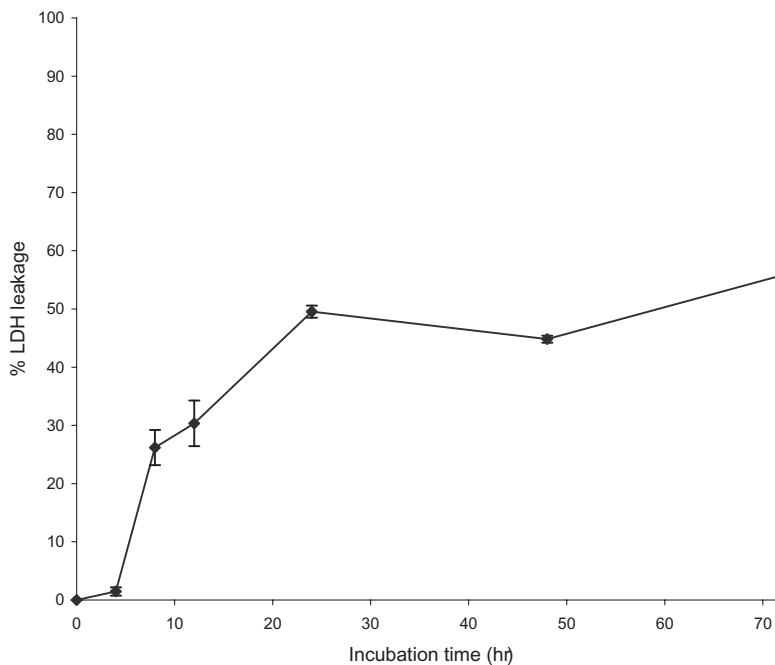


Figure 1. Leakage of lactate dehydrogenase following incubation of lung slices. Rat lung slices were incubated for up to 72 h. Lactic dehydrogenase (LDH) activity was determined in the media and is expressed as a percentage of total activity. Results are the mean $\pm$ SD for triplicate slices.

was raised to 600 μ m, which declined gradually as the thickness was increased further (figure 5).

To assess the metabolic viability of lung slices, they were pre-incubated at 37°C for various periods up to 72 h before a further 5-h incubation in the presence of 7-ethoxycoumarin. Metabolic viability, as exemplified by the total metabolism of 7-ethoxycoumarin and expressed per slice, was not substantially impaired for the first 8 h (figure 6). Following 24 h of pre-incubation, however, only about 10% of the original metabolic activity was retained but was still detectable after 72 h of pre-incubation. A similar pattern emerges when activity is expressed per mg homogenate protein (figure 6). Protein levels remained fairly constant for the first 16 h, and then declined being about 30% lower following a 72-h incubation (figure 4B).

Discussion

A major advantage of using precision-cut tissue slices is in the facile evaluation of tissue-specific metabolism and toxicity (Price *et al.* 2004). Although slices have been successfully prepared from a number of tissues (Parrish *et al.* 1995, 2002, Ball *et al.* 1996), only liver slices have been extensively studied with respect to their time-dependent viability in culture. The aim of the present study was to assess the viability of precision-cut rat lung slices.

Cellular integrity, as denoted by the leakage of lactate dehydrogenase into the media, showed gradual loss of the enzyme into the media, reaching a maximum at 24 h without further significant loss on longer incubations. A similar picture was

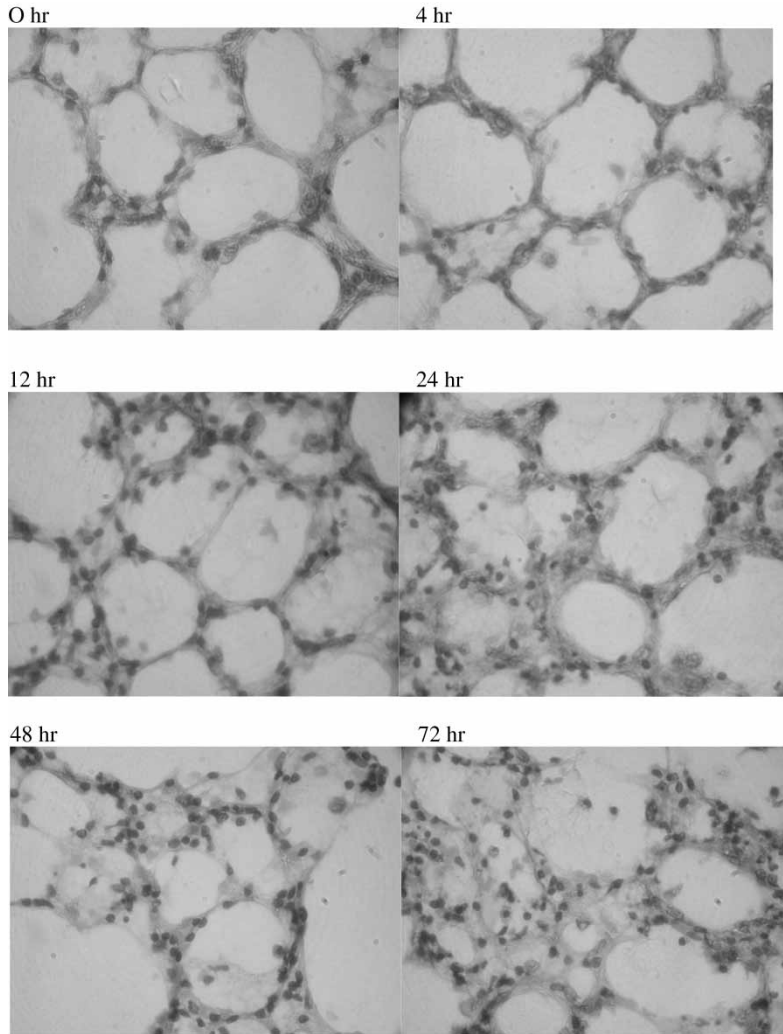


Figure 2. Light microscopy of lung slices after various culture periods. Rat lung slices (600 μm) were incubated for various times up to 72 h. They were then fixed in formalin, embedded in wax, sectioned, and stained with haematoxylin and eosin. Photomicrographs were taken using the 40 $\times$ objective.

observed when precision-cut rat liver slices were cultured under the same conditions (Hashemi *et al.* 1999a). Morphological evaluation of the lung slices indicated that the slices were viable for incubations of at least 12 h, but incubations of 24 h or longer led to degeneration of the nuclei and disruption of tissue architecture.

Metabolic viability was evaluated using 7-ethoxycoumarin as the substrate probe for a number of reasons. Its principal metabolite is 7-hydroxycoumarin, which is formed as a result of cytochrome P450-mediated oxidative dealkylation, which readily conjugates with sulphate and glucuronide, thus allowing the simultaneous monitoring of oxidation and conjugation pathways (Steensma *et al.* 1994). Moreover, 7-hydroxycoumarin can be readily determined fluorimetrically, allowing the rapid processing of a large number of samples. Lung slices metabolized this substrate in a time-dependent manner for 5 h, with a rate about half of

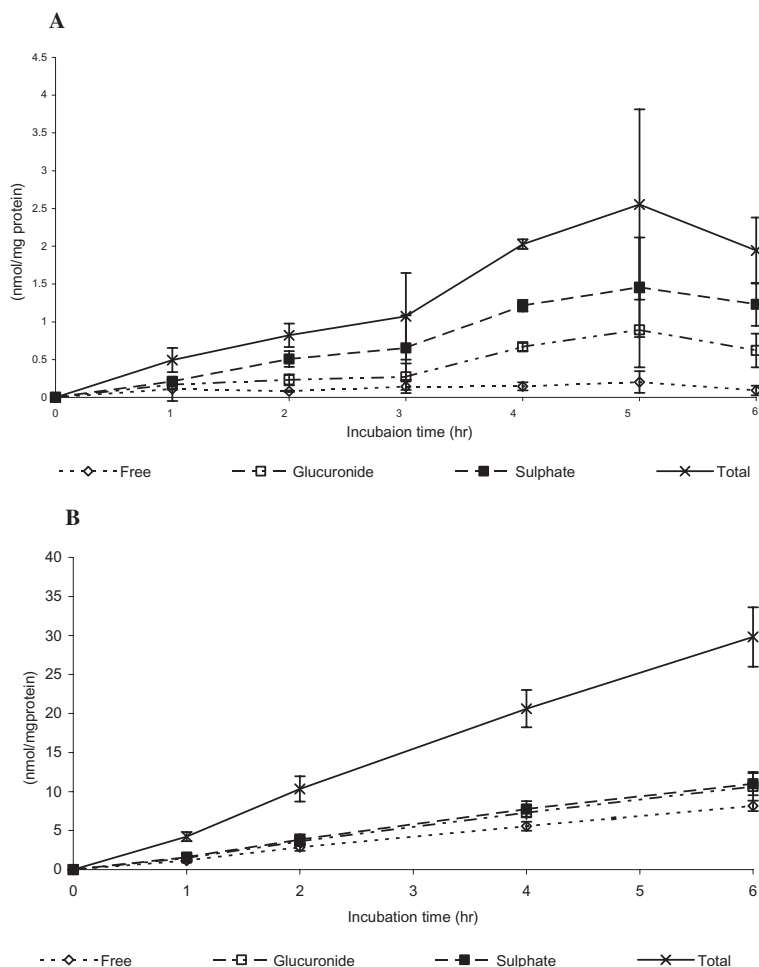


Figure 3. Cumulative metabolism of 7-ethoxycoumarin by lung slices from control and β -naphthoflavone-treated rats. Lung slices from (A) control and (B) β -naphthoflavone-treated rats were incubated with 7-ethoxycoumarin ($50\mu\text{M}$) for up to 6 h. Results are the mean $\pm$ SD for three slices.

what has been reported with liver slices from the same strain of rats (Hashemi *et al.* 1999a). In addition to the free 7-hydroxycoumarin, lung slices generated the glucuronide and sulphate conjugates to a similar extent; this contrasts with the liver where clearly the sulphate conjugate was the major metabolite. Pretreatment of the animals with β -naphthoflavone, an inducer of the CYP1 family, markedly enhanced the rate of metabolism of 7-ethoxycoumarin by about 10-fold. Rate of metabolism was clearly linear with time for at least 6 h. In recent studies, employing a 10 times higher substrate concentration and high-performance liquid chromatography-mass spectrometry analysis, the major product of 7-ethoxycoumarin in rat lungs and other extrahepatic tissues was identified as the ring-fission metabolite 4-ethoxy-2-hydroxyphenyl propionic acid (De Kanter *et al.* 2002), explaining the higher metabolic rate determined by these authors compared with the present studies. This metabolite was not monitored in the present studies.

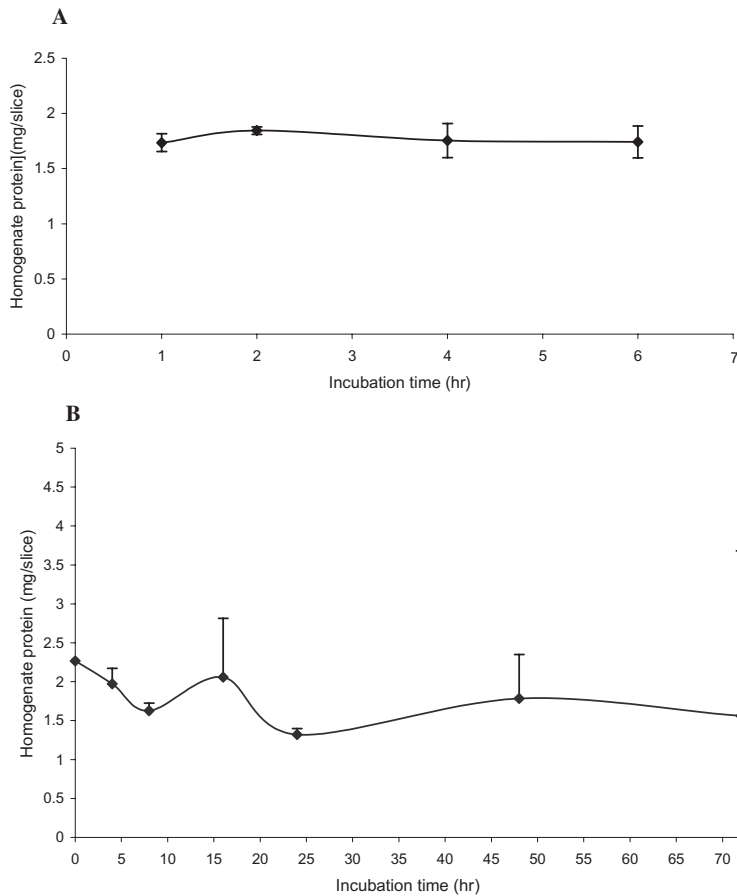


Figure 4. Effect of culturing on lung homogenate protein levels. Homogenate protein concentrations in lung slices were monitored after (A) short (6 h) and (B) long (72 h) incubation times. Results are the mean $\pm$ SD for three slices.

One of the most important factors in governing the performance of precision-cut tissue slices is slice thickness. Viability of slices is dependent on the facile diffusion of oxygen and nutrients to the cells, so that if slices are excessively thick, cells in the middle of the slice may be unable to survive. Similarly, if the slices are too thin, the ratio of damaged cells, as a result of slice preparation, to viable cells may become disadvantageous and compromise the performance of the slices. The optimum thickness for slices varies depending on the nature of the tissue (Bach *et al.* 1996). The importance of slice thickness is further highlighted in the present study where the rate of deethylation of 7-ethoxycoumarin doubled when slice thickness rose from 440 to the optimum 600 μm . Observations are consistent with previous studies where an optimum slice thickness for lungs of 500–700 μm was reported (Parrish *et al.* 1995, Bach *et al.* 1996).

To evaluate the ability of lung slices to metabolize 7-ethoxycoumarin for various periods up to 72 h, a different experimental approach was employed. The slices were incubated for a specified time, 7-ethoxycoumarin was then added and a 5-h incubation was allowed for the metabolism of the substrate. Such an approach determines the metabolic viability of the slices at a given time

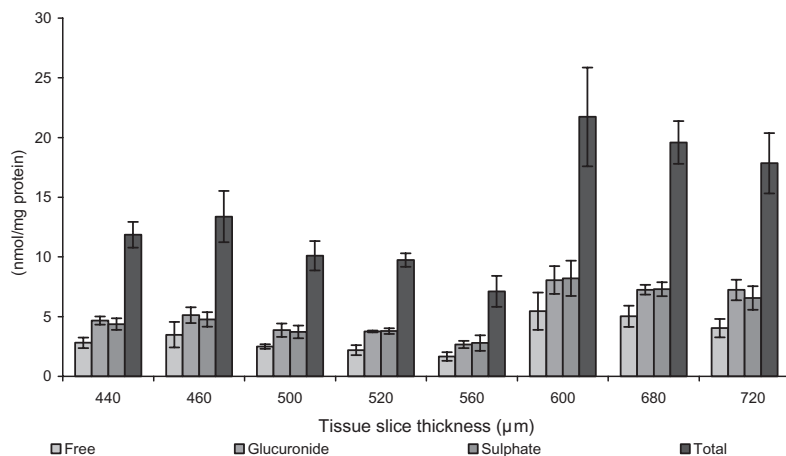


Figure 5. Effect of slice thickness on the metabolism of 7-ethoxycoumarin. Rat lung slices of different thickness (440–720 μm) from β -naphthoflavone-treated rats were incubated with 7-ethoxycoumarin for 5 h. Results are the mean $\pm$ SD for three slices.

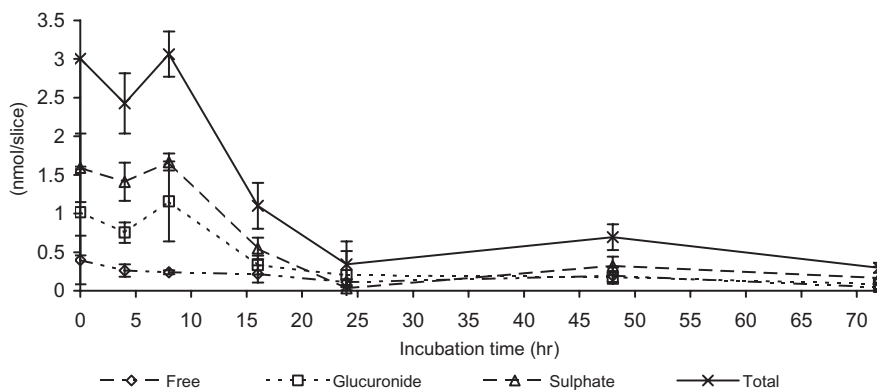
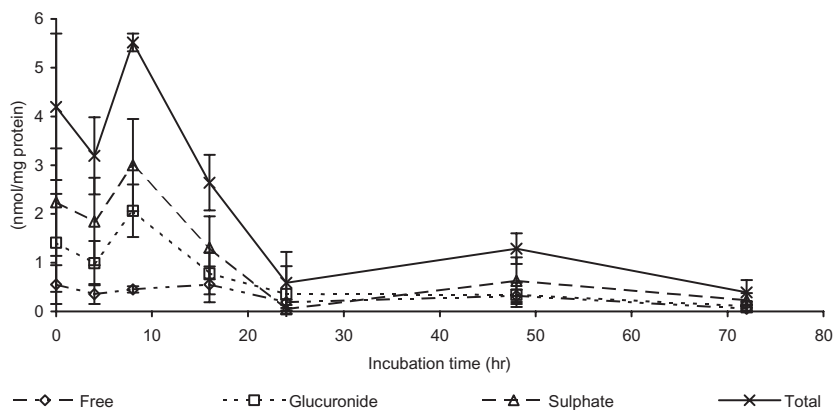


Figure 6. Effect of pre-incubation on the metabolism of 7-ethoxycoumarin by lung slices. Rat lung slices were incubated for various times up to 72 h. At the end of each incubation, 7-ethoxycoumarin (50 μM) was added and a further 5-h incubation was performed. Results are the mean $\pm$ SD for triplicate slices.

point, without the slices being compromised as a result of prior metabolism with the substrate, e.g. leading to partial depletion of essential cofactors such as UDP-glucuronic acid and 3'-phosphoadenosine-5'-phosphosulphate or suboptimal substrate concentration. For the first 8 h, slices were viable, but metabolic activity towards 7-ethoxycoumarin declined sharply after this time point. However, even after 72 h of incubation, very low levels of metabolism were still detectable. This contrasts with the liver slices where the decline in 7-ethoxycoumarin metabolism was more gradual, with the liver slices retaining some 60% of their activity following a 24-h incubation in the same media (Hashemi *et al.* 1999a). Note that the sulphate and glucuronide conjugates were produced to the same extent at all time points, implying that the viability of these enzyme systems under the current culture conditions did not differ. Since the levels of free 7-hydroxycoumarin were not significantly altered with incubation time, it is reasonable to infer that the decline in activity in the lung slices reflects impaired cytochrome P450-mediated deethylation; if the limiting enzymes were the conjugates, a rise in the free 7-hydroxycoumarin would have been observed. Indeed, at least in liver slices, phase II enzymes are better maintained in culture compared with cytochromes P450 (Hashemi *et al.* 1999b, 2000). However, cytochrome P450 viability in culture is enzyme specific, so that only the enzymes that catalyse the metabolism of 7-ethoxycoumarin in the lung have been monitored in the current studies.

In conclusion, it was demonstrated that precision-cut rat lung slices can be maintained metabolically in culture for some 8 h when slices of 600 µm thickness are used, but the activity then declines rapidly, and it should prove sufficient in most cases for metabolic and toxicological studies to be undertaken in this tissue. In contrast to lung slices, in liver slices a loss of viability is more gradual so that half of the original activity is still retained after 24-h incubations, and this difference may be attributed to the fact that the preparation of lung slices necessitates longer manipulation of the tissue that is likely to be detrimental.

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Different mechanisms of handling ingested polycyclic aromatic hydrocarbons in mammalian species: organ-specific response patterns of CYP1A1-induction after oral intake of PAH-contaminated soils

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1. Potential effects of xenobiotics on humans are largely derived from studies with animal models. However, due to species-specific processing of xenobiotics, susceptibilities to xenobiotic-dependent adverse effects are known to differ between species.

2. We analysed the basal expression of cytochrome P450 (CYP) enzymes in several organs of minipigs and rats, and their inducibility upon oral intake of soils containing polycyclic aromatic hydrocarbons (PAH). CYP-specific enzymatic activities were determined in duodenum, liver and kidney microsomes.

3. Upon ingestion of PAH-contaminated soils, CYP1A1 is differentially induced in a tissue-specific and dose-dependent manner in duodenum, liver and kidney of minipigs and rats. In the duodenum, the induction response is low in rats (about 4-fold) but it is high in minipigs (8–230-fold). By contrast, induced hepatic CYP1A1-dependent EROD-activity is higher in rats than in minipigs. The dose-response profile for renal CYP1A1 parallels that in the liver of either species but EROD-activities are 10–20 times lower than in the liver.

4. Liver microsomal CYP2E1 is only slightly modulated in its expression by ingestion of PAH-contaminated soils in both species, whereas CYP3A-dependent testosterone 2 β - and 6 β -hydroxylation is increased in liver of rats but not in minipigs.

5. The hepatic capacity for catechol oestrogen formation, i.e., the 2-hydroxylation of 17 β -oestradiol, is markedly increased in rats but not in minipigs by ingested PAH.

6. It is concluded that different metabolic and transport pathways are used by minipigs and rats to process ingested PAH. Whereas in minipigs the duodenum appears as the first efficient barrier, rats respond by efficient metabolism in the liver. What is not known is which response profile is operative in man.

Introduction

Mammalian species differ in their response to toxic compounds and in their susceptibility to chemically induced carcinogenesis (Hengstler *et al.* 1999). For inter-species extrapolation of xenobiotic effects, it is therefore important to understand the species-specificity of the underlying biochemical mechanisms of toxicant processing. In this respect, mobilization, absorption, transport and

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metabolism of the toxicants are relevant processes, which determine the toxicant concentration in several organs of the body and thus the effective site-specific internal doses. The capacity or efficacy of these processes is determined by genotype, but can also be increased or decreased by the internalised xenobiotics. In most cases, xenobiotics induce enzymes, such as members of the cytochrome P450 family, which are active in their metabolism. Due to primary processes in the tissue of contaminant entry and different subsequent transport routes, effects of xenobiotics are dependent on the exposure path, such as ingestion, inhalation or transdermal absorption (van de Wiel *et al.* 1993, Bouchard and Viau 1997, Brauze *et al.* 1997).

Intake of polycyclic aromatic hydrocarbons (PAH) by the oral pathway results in induction of the cytochrome P450 enzyme CYP1A1 in several organs of mammals (Roos 2002, Roos *et al.* 2002). CYP1A1 induction is mediated by the arylhydrocarbon receptor (Nebert *et al.* 1993), which also controls expression of CYP1B1 (Shimada *et al.* 2002). This latter enzyme is expressed at low levels in many extra-hepatic tissues but is present at higher levels in tumour tissue (Murray *et al.* 1997, 2001). Little is known, however, about the modulation of CYP1B1 levels by xenobiotics in several organs. Both CYP1A1 and CYP1B1 are involved in the oxidative metabolism of PAH, resulting in formation of reactive metabolites from ingested PAH (Guengerich 1992, Shimada *et al.* 1996, 2001). Additionally, CYP1B1 and CYP1A1 are known to catalyse the formation of catecholestrogens from oestradiol (Badawi *et al.* 2000, Spink *et al.* 2000). Thus, the level of these two CYP1-enzymes is an index of the cell's potential to produce reactive molecule species from exogenous and endogenous compounds. Adverse effects such as chemically induced carcinogenesis are often ascribed to the expression of CYP1A1 (Ioannides and Parke 1993). There are several lines of evidence that CYP1B1 is connected to various aspects of carcinogenesis (Ko *et al.* 2001, McFadyen *et al.* 2001, Murray *et al.* 2001, Thier *et al.* 2002).

It has been shown for minipigs and rats that ingestion of PAH-contaminated soil particles leads to induction of CYP1A1 in the duodenum (Roos 2002, Roos *et al.* 2002) thus indicating successful mobilization of the PAH by the gastro-intestinal juices and their subsequent absorption. The enhanced metabolic capacity of the duodenum, however, is not sufficient to prevent unmetabolised PAH from reaching the circulation and thus distribution into several organs. This is evident from CYP1A1 induction in liver (Roos *et al.* 1996), kidney, lung and spleen (Roos *et al.* 2002) after oral administration of PAH-contaminated soil particles. Due to species-specific CYP profiles, physiological characteristics of organ systems and transport mechanisms, the induction response may differ in various mammalian species. Therefore, we comparatively analysed induction of CYP1A1 in several organs of two animal models (minipigs and rats) upon oral intake of PAH-contaminated soil-particles. The two species differ in physiology and anatomy of their gastro-intestinal tract whereby that of the minipig is more similar to man (Swindle and Smith 1998) than that of the rat. Consequently, differences in basal expression levels and organ-specific induction profiles of cytochrome P450 enzymes, such as CYP1A1, can be expected between rats and minipigs. These differences may determine species-specific susceptibilities for xenobiotic induced adverse effects and have to be considered when these species are used as animal models for humans.

Material and methods

Animals and animal treatment

For the exposure experiments, three soils were used which differed largely in their PAH-content (table 1). Minipigs and rats each received daily soil doses of 0.5 g/kg body weight over a period of 28 and 7 days, respectively, by a similar administration protocol as outlined below. Daily ingested PAH and 5,6-PAH doses per kg body weight for both minipigs and rats were in the range of 33–1900 µg and 12–530 µg, respectively (table 2). As controls, non-treated animals were used. The induction of CYP1A1 was compared with the amounts of total administered PAH_{EPA} (16 PAH according to the Environmental Protection Agency USA., EPA) and with the subset of PAH with 5 and 6 aromatic rings (5,6-PAH) (Roos *et al.* 1996).

Minipigs

Sixteen Goettingen minipigs (Ellegaard Minipigs ApS, Dalmose, Denmark) were used; data on their gender, age and body weight are listed in table 3. The animals were assigned to 3 soil treatment groups (CA1, LO2, BRU) and 1 control group (CON) (4 animals each per group). Animals were fed SDS Standard Diet (Special Diets Services Ltd., Essex, UK) amounting to a daily consumption of about 3% of body weight as recommended by the supplier Ellegaard. This amount meets or exceeds the nutrient requirements of the minipigs. Half of the ration was given in the morning (8:00 am) and half in the afternoon (3:30 pm). Pigs were provided water *ad libitum*. Food consumption, body weight and health were monitored regularly throughout the experiment. Soil administration was carried out according to the following dosing protocol. Animals were administered 0.5 g soil/kg body weight (bw) per day on 28 consecutive days. Administration was performed at 1:30 pm to mimic the hand-to-mouth-activity of children, which takes places predominantly between the meals. Soil (fraction < 1 mm, see below) was mixed with 10–17.5 g standard diet, 3–6 g water and 3–6 g milk powder, to

Table 1. PAH-contents of the contaminated soils CA1, LO2 and BRU used for the exposure experiments.

PAH compound ¹	mg PAH/kg soil		
	CA1	LO2	BRU
Phenanthrene	284 ± 42	309 ± 36	5 ± 2
Anthracene	74 ± 6	38 ± 7	1 ± 0.5
Fluoranthene	949 ± 23	130 ± 54	13 ± 3
Pyrene	672 ± 35	71 ± 16	10 ± 2
Benz[a]anthracene	405 ± 20	45 ± 12	6 ± 1
Chrysene	362 ± 6	61 ± 20	8 ± 1
Benzo[b]fluoranthene	274 ± 13	34 ± 9	6 ± 1
Benzo[k]fluoranthene	154 ± 7	15 ± 4	3 ± 0.5
Benzo[a]pyrene	271 ± 16	22 ± 7	6 ± 1
Dibenzo[ah]anthracene	43 ± 6	5 ± 2	1 ± 0
Benzo[ghi]perylene	161 ± 8	19 ± 5	5 ± 0.5
Indeno[1,2,3-cd]pyrene	156 ± 8	7 ± 5	3 ± 0.5
Sum: Total PAH ¹	3805	756	67
Sum: 5- and 6-ring-PAH	1059	102	24
Number of determinations	n = 2	n = 8	n = 3

¹PAH_{EPA} (PAH analysed according to Environmental Protection Agency USA) except naphthalene, acenaphthylene, acenaphthene and fluorene (see methods). Data are shown as means ± standard deviations.

Table 2. Daily ingested doses of PAH and 5- to 6-ring PAH for minipigs and rats (mg/kg body weight).

Soil sample	CA1	LO2	BRU
µg PAH	1900	380	33
µg 5,6-PAH	530	51	12

CA1, LO2 and BRU: soil samples. Calculated values based on PAH content of the soils and amount of soil in the administered diet.

Table 3. Exposure groups, identification numbers and basic parameters of the minipigs.

Exposure group (soil)	Minipig no.	Sex	Age ¹ (days)	Weight ¹ (kg)
CA1	01	m	66	6.20
	02	m	110	7.30
	03	m	144	8.85
	04	m	165	9.75
LO2	13	f	95	5.65
	14	f	98	5.35
	15	m	99	6.05
	16	m	100	5.60
BRU	29	f	89	5.25
	30	f	89	6.05
	31	m	95	5.60
	32	m	87	5.75
CON	05	f	84	5.40
	06	f	87	5.45
	07	m	84	5.35
	08	m	87	5.65

¹Age and weight at the end of the experiment. CA1, LO2 and BRU are soil samples. CON, non-exposed control group; m, male; f, female.

a dough-like consistency. The resulting pellets were fed by hand. Soil dose was adjusted to individual pig body weight every three days. On day 29, the minipigs were sacrificed and microsomes were prepared from several tissues (see below).

Rats

Male Sprague-Dawley rats weighing about 200 g were treated for 7 days with the contaminated soils at doses equivalent to those given to the minipigs calculated on body weight (0.5 g soil/kg body weight) (table 2). Administration of the contaminated soil to the rats was carried out as follows. Rats were fasted over night and then small diet pellets of about 1 g containing the calculated daily soil doses were given to the rats. After complete consumption of the pellets, 30 g pure diet (without soil) was provided per animal until evening and then remaining diet was removed. This cycle was applied for seven days. On day 8, the rats were sacrificed and microsomes were prepared from liver, duodenum and kidney. The experiments were performed in compliance with the German Animal Protection Law (permissions 23.8720 Nr. 20.35 for minipigs and 23.8720 Nr. 24.11 for rats).

Soil samples

The soils used in the present study originate from industrial sites of the Ruhr district (CA1, LO2) and from garden plots in Baden-Württemberg (BRU), Germany. The original material was air-dried at 20°C. Prior to fractionation, larger aggregates were reduced to small pieces. For the exposure experiments, soil particles < 1 mm were prepared by sequential sieving steps so that fractions with particle sizes of > 2 mm, 1–2 mm and < 1 mm were obtained. PAH-composition and content of the soil fractions < 1 mm are shown in table 2. The soil samples were kindly provided by the Staatliches Umweltamt Duisburg (CA1), the Landesumweltamt Nordrhein-Westfalen (LO2) and the Landesanstalt für Umweltschutz, Baden Württemberg (BRU).

PAH analyses

Qualitative and quantitative analyses of soil bound PAH (table 1) were performed by reversed-phase HPLC of acetone/*n*-hexane extracts as previously described (Hack and Selenka 1996). 16 PAH according to the Environmental Protection Agency, EPA, were determined. For calculations such as dose response relationships data of the volatile compounds naphthalene, acenaphthylene, acenaphthene and fluorene were omitted because of great variation in their amounts within one and the same sample.

Preparation of microsomes

Liver and kidney microsomes were prepared as described by Guengerich (1977). For isolation of duodenal microsomes, the duodenum was cut longitudinally and washed 3 times in ice-cold 0.9% NaCl. The mucosal cells were scraped off under buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA,

250 mM sucrose, 3 U/ml Heparin and the protease inhibitor mix CompleteTM (Boehringer, Mannheim, FRG). After 20 strokes in a Dounce glass homogenizer the samples were sonified 3 times for about 5 s. Cell debris was pelleted for 25 min at 10 000 g and the resultant supernatant was centrifuged for 1 h at 105,000 g. The microsomal pellet was resuspended in about 500 μ l 10 mM Tris-HCl, pH 7.4, 20% glycerol, 0.1 mM EDTA. All microsomal preparations were immediately frozen in liquid nitrogen and subsequently stored at -70°C .

Ethoxyresorufin O-deethylation (EROD) activity

The activity was determined fluorometrically by continuous monitoring of resorufin formation in a reaction mixture containing 100 mM Tris-HCl, pH 7.8, 2.7 mM isocitric acid, 0.3 U/ml isocitrate dehydrogenase, 2 mM MgSO_4 , 50 $\mu\text{g}/\text{ml}$ bovine serum albumin, 76 U/ml catalase, 170 μM NADP⁺ and 2.5 μM ethoxyresorufin or pentoxyresorufin. After pre-incubation for 1 min at 30°C , the reaction was started with the microsomal preparation at a concentration of 1 to 100 nM P450 and followed at excitation and emission wavelengths of 530 nm and 585 nm, respectively (Burke *et al.* 1985).

Microsomal testosterone metabolism

Testosterone hydroxylation activities were determined in 1 ml reaction mixtures containing the following compounds (final concentrations in parenthesis): potassium phosphate (50 mM, pH 7.4), magnesium chloride (3 mM), sucrose (50 mM), bovine serum albumine (64 $\mu\text{g}/\text{ml}$), isocitrate (2.7 mM), isocitrate dehydrogenase (0.3 U/ml), catalase (333 U/ml), NADP⁺ (0.2 mM), testosterone (250 μM) and microsomes (1 mg microsomal protein/ml). After preincubation of the reaction mixture for 5 min at 37°C without substrate, the reaction was started by addition of testosterone and continued for 10 min at the same temperature. For termination of the reaction, samples were put on ice, supplemented with 10 μM 17α -hydroxyprogesterone (in ethanol) as internal standard and were vigorously mixed with 3 ml diethylether. The organic phase was transferred into another tube and the sample was extracted once more with 3 ml diethylether. The combined organic phases were taken to dryness under a nitrogen stream. The residue was dissolved in 50 μl methanol (HPLC-grade) and analysed by reversed phase HPLC with a Supelcosil LC-18-DB column (5 μm , 150×4.0 mm) equilibrated with 25% methanol and 75% H_2O . Elution was conducted at a flow rate of 0.9 ml/min with the following gradient obtained by mixing solvent (A) 25% methanol, 75% H_2O with solvent (B) 55% methanol, 7% acetonitrile, 38% H_2O : (1) 0–25% B in 3' 20", (2) 25–50% B in 4' 40", (3) 50–100% B in 16' and (4) 100% B for 10'. Peaks were detected by UV absorbance at 280 nm. For identification of separated metabolites, the following reference compounds were used (OHT = hydroxytestosterone): 2α -OHT, 2β -OHT, 6α -OHT, 6β -OHT, 7α -OHT, 11β -OHT, 15α -OHT, 15β -OHT, 16α -OHT, 16β -OHT, 18-OHT, 19-OHT, 6-dehydro-testosterone, androstenedione (Sigma, Taufkirchen, Germany and Steraloids, Rhode Island, USA).

Formation of 2-hydroxyestradiol

For composition of reaction mixtures, incubation conditions and metabolite extraction with diethylether, see the previous section on microsomal testosterone metabolism. The reaction was started with 250 μM 17β -oestradiol instead of testosterone. After extraction and evaporation of the organic phase (see above) the residue was dissolved in 50 μl methanol and subjected to analysis by HPLC. The same column type was used as for the separation of testosterone metabolites. Isocratic elution was performed with 33% acetonitrile/1% acetic acid at a flow rate of 1 ml/min for 35 min at 25°C . Peaks were detected by UV absorption at 280 nm. 2-Hydroxyestradiol (Sigma, Taufkirchen, Germany) was used as reference compound.

Chlorzoxazone 6-hydroxylation

6-Hydroxylation of chlorzoxazone was measured according to the method of Carrière *et al.* (1993) with slight modifications. The reaction mixture (final volume = 1 ml) contained (final concentrations in parenthesis): potassium phosphate (50 mM, pH 7.5), magnesium chloride (3 mM), sucrose (50 mM), bovine serum albumine (64 $\mu\text{g}/\text{ml}$), isocitrate (2.7 mM), isocitrate dehydrogenase (0.3 U/ml), catalase (333 U/ml), NADP⁺ (0.2 mM), chlorzoxazone (400 μM in dimethylsulfoxide) and microsomes (0.5 mg microsomal protein/ml). The reaction was started by addition of substrate and the mixture was incubated for 10 min at 37°C . The reaction was stopped with 50 μl 20% phosphoric acid and the sample stored on ice. After addition of 1.2 mM benzoxazolinone as internal standard, samples were extracted three times with 2 ml diethylether. The solvent was evaporated under a nitrogen stream and the residues dissolved in 50 μl 75% acetonitrile. Twenty μl were subjected to HPLC-analysis with a RP-C18 column (5 μm ; 250×4.6 mm; Supelco) equilibrated with 25% acetonitrile, 0.375% acetic acid. Elution was performed at a flow rate of 1 ml/min with the following solvents and solvent gradient. Solvents: (A) 0.5% acetic acid and (B) 100% acetonitrile. Gradient: 75% A, 25% B for the first 30 min followed by a linear gradient to 25% A, 75% B within the next 15 min. Peak detection was by UV absorbance at 280 nm. 6-Hydroxychlorzoxazone (Sigma, Taufkirchen, Germany) was used as chromatographic standard.

Quantitation of cytochromes P450 and protein

Total P450 was determined spectroscopically by the method of Omura and Sato (1964) by CO-binding to dithionite reduced CYP using an extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ at 450–490 nm. Protein concentrations were quantified by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Chemicals

Chlorzoxazone, isocitric acid, isocitrate dehydrogenase, catalase, ethoxyresorufin, resorufin, testosterone, 2 β -hydroxytestosterone, 6 β -hydroxytestosterone, 15 β -hydroxytestosterone, 17 β -oestradiol and 2-hydroxy-17 β -oestradiol were purchased from Sigma (Taufkirchen, FRG), benzoxazolinone from Fluka (Buchs, Switzerland). Other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical-reagent grade.

Results

We have analysed the effects of ingested PAH-contaminated soil-particles on activity levels of cytochrome P450 (CYP) enzymes in several organs of rats and minipigs. To assess the effects of contaminant ingestion, basal activity patterns of the two species were analysed and compared with levels upon ingestion of soils with different PAH-contents. Ingested PAH-doses were identical for rats and minipigs calculated on body weight and amounted to 33, 380 and 1900 μg PAH/kg body weight dependent on the soil sample (table 2).

Basal activities

Basal activities of CYP enzymes and the total microsomal CYP-content have been analysed in duodenum, liver and kidney of minipigs and rats. In the liver, constitutive microsomal CYP content is similar for both species amounting to 0.94 ± 0.08 and $1.02 \pm 0.04 \text{ nmol CYP/mg protein}$, respectively ($n=4$). CYP-dependent metabolic activities were analysed using various substrates selective for different CYP enzymes including members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2E and CYP3A-subfamilies. Some basal hepatic activities differed markedly between the two species as substantiated from microsomal testosterone metabolism. In contrast to rats, minipigs did not form significant amounts of 2 α -, 7 α - and 16 α -hydroxytestosterone (figure 1) which are dependent on CYP2C11 (2 α , 16 α) and CYP2A (7 α) in the rat (Schenkman 1992; Arlotto and Parkinson 1989). A major metabolite formed by both species is 6 β -hydroxytestosterone which is attributed to constitutively expressed CYP3A-enzymes. A further dominant activity of hepatic minipig microsomes is the testosterone 2 β -hydroxylation, which is significantly higher in minipigs than in rats. Activities of CYP1-enzymes have been determined by the ethoxyresorufin O-deethylase activity (EROD). Similar constitutive EROD activities of 194 ± 29 and $240 \pm 90 \text{ pmol resorufin mg protein}^{-1} \text{ min}^{-1}$ have been found in liver microsomes of minipigs and rats, respectively (table 4). However, CYP1A1 is not detected in liver microsomes of both species by Western blots with specific monoclonal antibodies (data not shown). Hepatic CYP2E1 was estimated by its chlorzoxazone 6-hydroxylation activity, which is significantly lower in minipigs than in rats, i.e., 201 vs. 835 pmol metabolite $\text{mg protein}^{-1} \text{ min}^{-1}$ (table 4). In duodenal and renal microsomes of minipigs and rats, constitutive EROD activities are close to zero. In Western blots, CYP1A1-protein is detectable neither in duodenum nor in kidney microsomes (data not shown). Formation of the catechol estrogen 2-hydroxyestradiol from estradiol is catalysed by several CYP enzymes, including CYP1A1 and CYP1B1.

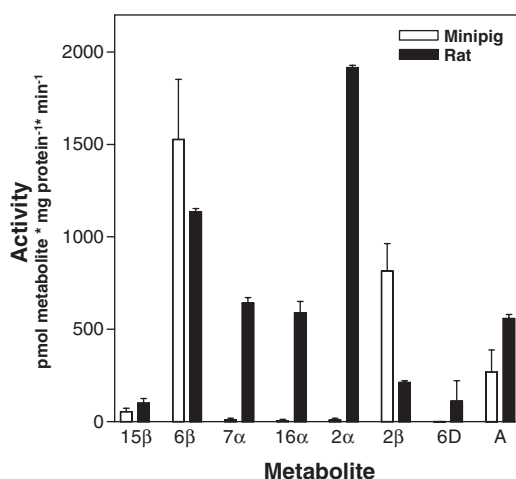


Figure 1. Formation of testosterone metabolites by liver microsomes of untreated minipigs and rats. 15β, 6β, 7α, 16α, 2α, 2β: testosterone hydroxylated at the indicated position. 6D: 6-dehydro-testosterone. A: androstenedione. Bars show means and standard deviations ($n=4$ minipigs; $n=3$ rats).

Table 4. Constitutive levels of total CYP and CYP-dependent microsomal activities in liver, duodenum and kidney of minipigs and rats.

Organ	Parameter	Minipigs ($n=5$) ⁴	Rats (varying n) ⁴
Liver	CYP-content ¹	0.90 ± 0.11	$0.86 \pm 0.07(49)$ ⁴
Liver	CYP1A1-protein ²	not detected	not detected
Liver	Ethoxyresorufin O-deethylation ³	194 ± 29	$240 \pm 90(16)$ ⁴
Liver	Chlorozoxazone 6-hydroxylation ³	201 ± 81	$835(2)$ ⁵
Liver	Testosterone 6β-hydroxylation ³	1527 ± 325	$1134(2)$ ⁵
Liver	Estradiol 2-hydroxylation ³	506 ± 150	$959(2)$ ⁵
Duodenum	Ethoxyresorufin O-deethylation ³	11 ± 5	$15.5 \pm 15.1(4)$ ⁴
Kidney	Ethoxyresorufin O-deethylation ³	4.4 ± 2.7	$14.3 \pm 4.9(3)$ ⁴

¹nmol CYP/mg protein.

²Relative CYP1A1-content analysed by Western blots. Not detected means < 5 pmol CYP1A1/mg microsomal protein.

³pmol metabolite mg protein⁻¹ min⁻¹.

⁴Means and standard deviations based on determinations obtained from the number of animals given in parenthesis.

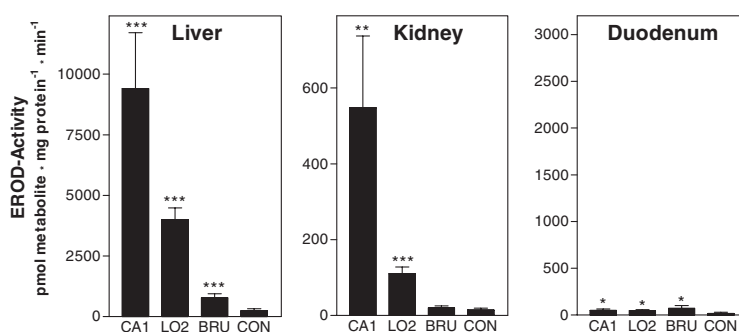
⁵Average from 2 animals (3 determinations each).

Basal liver microsomal activities are similar in rats and minipigs amounting to 960 ± 250 and 510 ± 150 pmol metabolite mg protein⁻¹ min⁻¹, respectively.

Organ- and species comparison of CYP-induction by orally administered PAH

Oral administration of PAH-contaminated soils leads to a dose-dependent induction of CYP1A1 in both minipigs and rats in a species- and organ-specific manner. The most striking result is that the response to CYP1A1-induction (EROD-activity) by the three soils is inverse with respect to induction in duodenum and liver for minipigs and rats: whilst high duodenal EROD-activities are found in the minipig, they are low in the rat. By contrast, induced hepatic EROD-activities are higher in the rat compared to the minipig (figure 2, table 5). The highly contaminated soil CA1 elicits the largest response, but significant CYP1A1 induction is also achieved with the low contaminated sample BRU in

Rats



Minipigs

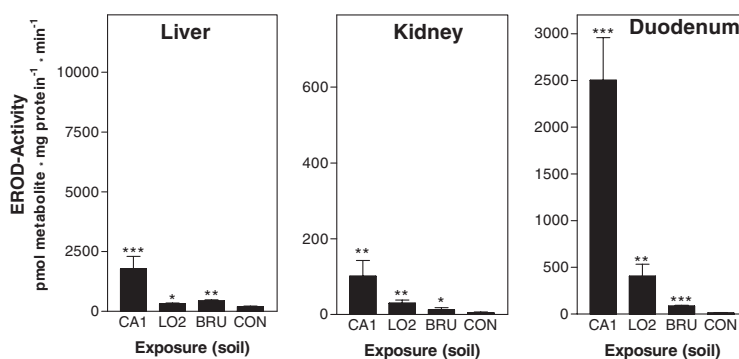


Figure 2. Differential induction of microsomal EROD-activity by the PAH-contaminated soils CA1, LO2 and BRU in liver, kidney and duodenum of rats and minipigs. CON, controls. Means and standard deviations are calculated from $n = 4$ animals (based on means of 4 determinations each). Significant differences of EROD-activities compared to the respective controls are indicated as follows: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$ (t -test).

Table 5. Statistical analysis of differences in induced EROD-activities between minipigs and rats.

Exposure	Liver	Kidney	Duodenum
CA1	0.0028 **	0.0036 **	< 0.0001 ***
LO2	< 0.0001 ***	0.0002 ***	0.0023 **
BRU	0.0192 *	0.1047	0.4799
CON	0.2789	0.0197 *	0.5893

p -values (t -test). Asterisks indicate significant differences: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$ (see figure 2).

rats and minipigs. In the rat, EROD-activities induced by CA1 amount to 9400 and 50 pmol metabolite $\text{mg protein}^{-1} \text{min}^{-1}$ for liver and duodenum, respectively. In the minipig, respective values are 1780 and 2500 pmol metabolite $\text{mg protein}^{-1} \text{min}^{-1}$. Compared to the background levels, duodenal EROD-activities are induced 4- and 230-fold in rats and minipigs, respectively. Induction of renal EROD-activity parallels that in the liver of both species but at a lower level. Maximal induced activities are 550 and 101 pmol metabolite $\text{mg protein}^{-1} \text{min}^{-1}$ in kidney microsomes of rats and minipigs.

The effect of ingested PAH-contaminated soils on liver microsomal activities other than EROD is less pronounced. CYP2E1-dependent chlorzoxazone

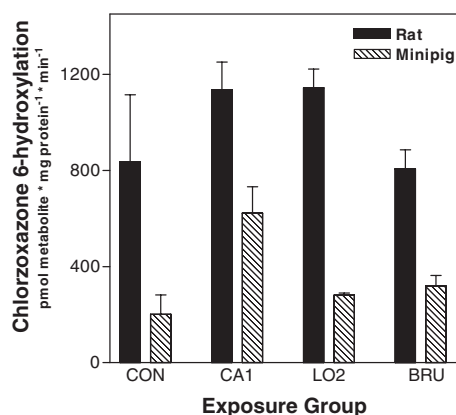


Figure 3. Chlorzoxazone 6-hydroxylation by liver microsomes of untreated (CON) and treated minipigs and rats. CA1, LO2 and BRU: activities upon ingestion of PAH-contaminated soils CA1, LO2 and BRU. Means and standard deviations are calculated from $n = 4$ animals (based on means of 2 to 4 determinations each).

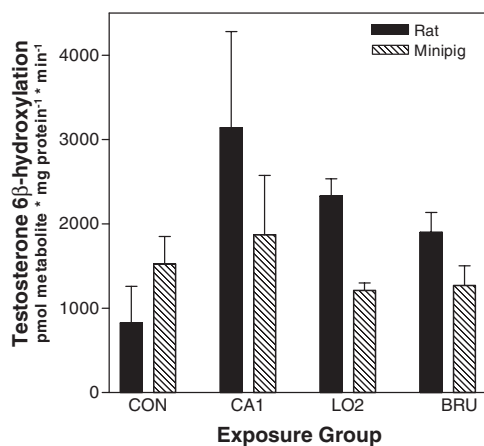


Figure 4. Testosterone 6 β -hydroxylation by liver microsomes of untreated (CON) and treated minipigs and rats. CA1, LO2 and BRU: activities upon ingestion of PAH-contaminated soils CA1, LO2 and BRU. Means and standard deviations are calculated from $n = 4$ animals (based on means of 2 to 4 determinations each).

6-hydroxylase is generally higher in rats than in minipigs and appears to be largely unaffected by contaminant ingestion in both species (figure 3). However, a slight induction effect of soil CA1 cannot be excluded for minipigs. CYP3A-catalysed 6 β - and 2 β -hydroxylation of testosterone is increased in rats by ingestion of all 3 soils, but remains mainly unaltered in minipigs (figures 4 and 5). Similarly, but more pronounced, testosterone 15 β -hydroxylation and oestradiol 2-hydroxylation are induced in the liver of rats but not in the liver of minipigs (figures 6 and 7).

Discussion

The initial defence of a mammalian organism against toxic xenobiotics is the tissues of entry, which differentially express transport proteins and enzymes active in xenobiotic metabolism. In addition to basal levels, adaptive inducibility of

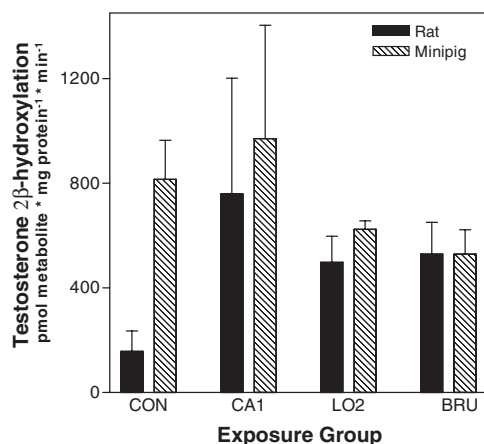


Figure 5. Testosterone 2β-hydroxylation by liver microsomes of untreated (CON) and treated minipigs and rats. CA1, LO2 and BRU: activities upon ingestion of PAH-contaminated soils CA1, LO2 and BRU. Means and standard deviations are calculated from $n = 4$ animals (based on means of 2 to 4 determinations each).

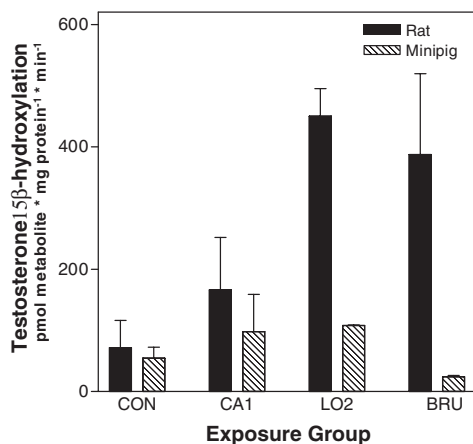


Figure 6. Testosterone 15β-hydroxylation by liver microsomes of untreated (CON) and treated minipigs and rats. CA1, LO2 and BRU: activities upon ingestion of PAH-contaminated soils CA1, LO2 and BRU. Means and standard deviations are calculated from $n = 4$ animals (based on means of 2 to 4 determinations each).

proteins, such as phase I and phase II enzymes, as a response to toxicant exposure may also differ between various tissues. The efficiency of primary processes after toxicant incorporation and first-pass effects determine the bioavailability of substances and, thus, the effective internal doses in various body compartments.

It has been suggested that the toxic effect of ingested xenobiotics is modulated by expression levels of cytochromes P450 in the small intestine (Zhang *et al.* 1997). Constitutive levels of CYP1A1 are low in duodenal mucosa cells of minipigs and rats. A marginal background EROD-activity has been found by us and also by others (Takemoto *et al.* 2003). In Western blots with specific antibodies, CYP1A1-protein was not detected (data not shown). Accordingly, in the 'ground state' without inducing factors there is only negligible duodenal CYP1A1-activity. Oral intake of particle-bound PAH and subsequent mobilization of contaminants

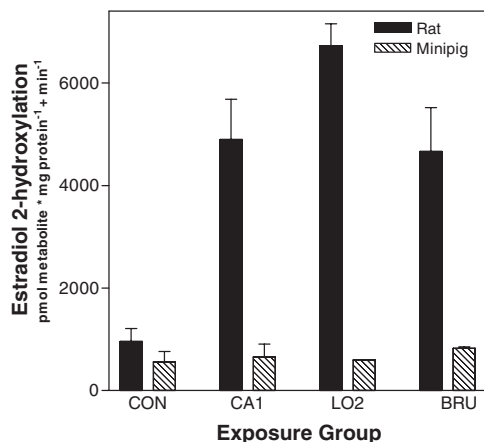


Figure 7. Oestradiol 2-hydroxylation by liver microsomes of untreated (CON) and treated minipigs and rats. CA1, LO2 and BRU: activities upon ingestion of PAH-contaminated soils CA1, LO2 and BRU. Means and standard deviations are calculated from $n=4$ animals (based on means of 2 to 4 determinations each).

in the gastro-intestinal tract leads to adaptations in the cells of the duodenum by increasing the enzymatic capacity for PAH-metabolism through induction of CYP1A1. This adaptive response differs quantitatively in minipigs and rats. Both species show an equally low constitutive level of CYP1-dependent duodenal EROD-activity. In minipigs, however, this activity is increased 230-fold by ingestion of the highly contaminated soil CA1, whereas in rats the induction is only 4-fold resulting in EROD-activities of 2500 and 50 pmol metabolite mg protein⁻¹ min⁻¹, respectively. Thus, the two species differ largely in their inducible duodenal capacity to metabolise PAH. This implies that the barrier effect of the duodenum to prevent unmetabolised PAH to enter the systemic circulation is different for minipigs and rats. It is not known, however, which portions of ingested PAH are mobilized by the intestinal fluids and which portions of subsequently absorbed PAH are metabolised by the mucosal cells, transferred to the blood or secreted back into the intestinal lumen. The kinetics of absorption, desorption and transport processes for PAH in the intestinal lumen may also have an impact on the extent of CYP1A1 induction in the duodenum. Apparently, PAH or PAH metabolites with affinity to the Ah-receptor pass the intestinal barrier in concentrations high enough to elicit induction effects in internal organs. In liver and kidney of minipigs and rats, CYP1A1 is coordinately induced upon oral intake of PAH-contaminated soils. This has been already shown by a previous study, at least for rats (Roos, 2002). However, elevated EROD-activities in the kidney are lower than in the liver of either species. In contrast to CYP1A1-induction in the duodenum, where the response is higher in minipigs than in rats, the increase in liver and kidney microsomal EROD-activity is more pronounced in rats compared to minipigs (9400 and 1780 pmol metabolite mg protein⁻¹ min⁻¹, respectively).

The findings suggest that the biochemical strategy of minipigs is to prevent toxic effects of ingested xenobiotics preferentially by a primary attack in the tissue of entry, whereas rats compensate for their low CYP1A1 metabolic capacity in the duodenum by highly induced enzymatic activities in the liver. It is noted, that the differential induction response of rats and minipigs cannot be accounted for

by the fact that we used male and female minipigs, but only male rats. We could show that male and female minipigs show similar induction profiles for CYP1A1 (Tschirbs *et al.* 2004).

The consequence of altered hepatic expression levels of cytochromes P450 upon ingestion of PAH-contaminated soils is that the metabolism of endogenous compounds, such as oestradiol, can also be increased. This effect is distinct in rats but is absent in minipigs. CYP-dependent metabolism of oestradiol leads to a number of different regio- and stereospecifically hydroxylated compounds. Among them 2- and 4-hydroxyoestradiol, so-called catecholestrogens, are of particular toxicological importance. They can be converted to reactive quinone derivatives which are able to interact with proteins and nucleic acids to form adducts or lead to DNA depurination (Samuni *et al.* 2003), a mechanism causing the tumorigenicity of 4-hydroxy-oestrogens (Cavalieri *et al.* 1997). CYP enzymes involved in formation of catecholestrogens include CYP1A1, CYP1A2, CYP1B1 and CYP3A (Lee *et al.* 2003). CYP1A1 shows a preference for 2-hydroxylation of oestradiol, whereas CYP1B1 preferentially hydroxylates the 4-position (Li *et al.* 2000, Lee *et al.* 2003). Ingestion of all soils tested here significantly increased hepatic oestradiol 2-hydroxylation activity in rats by more than 6-fold. Basal levels of this activity in minipigs are similar to those of rats and remain unaltered upon oral PAH-uptake. Taken together, the data on the altered metabolic capacities for exogenous and endogenous compounds upon oral intake of xenobiotics suggest a differential tissue selective susceptibility of minipigs or rats for toxic effects. However, there are no straightforward data available for (mini)pigs to prove this hypothesis.

As shown herein, the two animal models, minipigs and rats, differ substantially in their organ-specific CYP1A1 induction profile. Because of limited available data concerning CYP1A1 induction in humans after oral intake of xenobiotics, the toxicologically important question which animal model more closely reflects the human response pattern remains unanswered. Without drawing any conclusion it is noted that oral administration of the proton pump inhibitor and CYP1A1-inducer omeprazole to male human volunteers did not alter their capacity for oestradiol 2-hydroxylation (Galbraith and Michnovicz 1993), although omeprazole is able to induce CYP1A1 in the human duodenum (McDonnell *et al.* 1992). However, increased hepatic oestradiol 2-hydroxylase activity has been reported in rats after oral intake of indole-3-carbinol (Jellinck *et al.* 1991).

It is known that, in addition to CYP1-enzymes, oestradiol 2-hydroxylation is also catalysed by members of the CYP3A-subfamily of enzymes (Yamazaki *et al.* 1998, Cheng *et al.* 2001, Lee *et al.* 2001). In minipigs, CYP3A-dependent testosterone hydroxylations in the 2 β - and 6 β -position appear to be unaffected by oral intake of PAH-contaminated soils, whereas an induction is observed in rats. Thus, increased hepatic oestradiol 2-hydroxylase in rats may be due to CYP1-enzymes and to a minor extent to CYP3A-enzymes.

In addition to duodenum and liver, CYP1A1 is induced also in the kidney after oral intake of PAH. This shows that AhR-responsive non-metabolised PAH or non-conjugated primary PAH-metabolites reach this organ after passage through the duodenal barrier and also of the liver as the main xenobiotic-metabolising organ. Distribution of CYP1A1-inducing compounds into various organs provokes the question of the capacity of these organs to prevent site-specific adverse effects. It is known that the half-life of reactive metabolites formed in the

course of phase I-metabolism is determined by the subsequent processing by conjugating phase II-enzymes (Seaton *et al.* 1995; Sheweita 2000). Little is known, however, about the balance of the different enzyme systems in various tissues upon exposure to xenobiotics, in particular, a comparison between species is not possible due to a lack of data. Phase II activities are high in the duodenum suggesting that PAH-metabolites released by the mucosa cells are substantially conjugated. Consequently, unmetabolised PAH would be responsible for CYP1A1 induction in other organs.

The physiological or molecular mechanisms responsible for the lack of induction of CYP1A1-dependent activities in the duodenum of rats is not clear. Rat mucosal cells express the Ah-receptor (Zhang *et al.* 1997), which is involved in PAH-mediated CYP1A1 induction so that transcriptional activation of the CYP1A1-gene is possible in this tissue. Hence, the reason for the low induced activities may not occur at the transcriptional level. As Traber *et al.* (1992) pointed out, NADPH cytochrome P450 reductase can be the limiting factor for CYP-dependent activities in the duodenum because it is expressed at low levels only in intestinal cells. Another explanation may be found in factors that determine local concentrations of xenobiotics and their retention periods in tissues such as differing body size, extent of tissue surfaces and the rate of transport processes in rats compared to minipigs.

Our results demonstrate a different response of minipigs and rats with respect to effects elicited by ingested PAH on CYP1A1 expression in several organs. These results have important implications for studies on the toxic risk of substances, in particular with respect to the selection of a suitable animal model for man. To date, the human tissue-specific response pattern following oral PAH intake is not known. In future studies comparing responses on exposure to potential toxicants, there should be a focus on the tissue specificity of effects. An important aspect here may be altered susceptibility of organs to adverse effects due to modulated CYP and phase II-enzyme profiles. In order to find the most suitable animal model for the assessment of human risk, this aspect of tissue specificity and of an extended profile of phase I and phase II enzymes, receptors and transport proteins as biomarkers should be considered.

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Oxidation and rearrangements of flavanones by mammalian cytochrome P450

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1. To clarify the metabolic pathways of flavanones in mammals, the metabolism of (±)-flavanone and (±)-4'-methoxyflavanone by rat liver microsomes and recombinant human P450s in which structural changes are readily identifiable were examined.

2. The β -nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation of flavone plus (±)-2,3-*trans*-flavanonol and of 4'-methoxyflavone plus (±)-2,3-*trans*-4'-methoxyflavanonol, respectively, by rat liver microsomes was observed.

3. The same metabolites were generated by recombinant human P450s in addition to the formation of isoflavone from (±)-flavanone.

4. The kinetic isotope effects in these reactions were examined using deuterated (±)-flavanone and (±)-4'-methoxyflavanone. There was a strong isotope effect in the production of flavanonols, but the isotope effect in the production of flavones was small. The results indicated that the P450-mediated conversion of (±)-flavanone and of (±)-4'-methoxyflavanone to the corresponding metabolites proceeded via abstraction of a hydrogen radical from the C-2- or C-3-position of the flavanone skeleton.

5. The antioxidant properties of flavanone and its metabolites were examined by measuring superoxide-scavenging activity in a xanthine–xanthine oxidase–cytochrome *c* system. (±)-2,3-*trans*-Flavanonol had higher activity than that of other flavonoids.

6. Flavanones are metabolized by mammalian P450s, providing important information relevant to the metabolism and pharmacological action of dietary flavanones.

Introduction

Flavonoids are polyphenolic compounds widely distributed in plants. The daily intake of flavonoids in humans amounts to more than 1 g/person (Kuhnau 1976). A portion of these flavonoids is degraded by gut microflora and a portion is absorbed via the small intestine (Spencer *et al.* 2003). After absorption via the alimentary tract, flavonoids are distributed in body fluids and tissues either unchanged and/or in conjugated forms. Flavonoids have a broad spectrum of biological and pharmacological activities, which include antioxidant (Moridani *et al.* 2003), anti-inflammatory (Wakabayashi 1999), antimicrobial and anti-allergic (Inoue *et al.* 2002), antimutagenic (Bear and Teel 2000), and drug-metabolizing enzyme-inducing (Hodek *et al.* 2002) activities. A 5-year follow-up study by Hertog *et al.* (1993) suggested that dietary flavonoids might reduce the risk of death due to coronary arteriosclerotic diseases and that the amount of flavonoids in foodstuffs is sufficient for pharmacological activity. Moreover, Datla *et al.* (2001)

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suggested the usefulness of tangeretin, a polymethoxylated flavone derivative, in the treatment of cranial neuropathies, such as Parkinson's disease, since this compound can pass through the blood-brain barrier. Polymethoxylated flavonoids, including tangeretin, can be used as enhancers of the effect of some drugs since such flavonoids inhibit cellular excretion of drugs mediated by P-glycoprotein (Takanaga *et al.* 2000, Choi *et al.* 2002). For the evaluation of the pharmacological effects of flavonoids in vegetable foods, it is necessary to elucidate their metabolism and the mechanisms of their action *in vivo*.

After absorption via the gastrointestinal tract, flavonoids are excreted in the urine unchanged and/or as conjugates (Yasuda *et al.* 2001). Nielsen *et al.* (1998) and Breinholt *et al.* (2002) reported that dietary flavonoids are demethylated at the c-4'-position and/or hydroxylated at the c-3'-position of the B-ring of the flavone skeleton by cytochrome P450-mediated metabolic reactions (figures 1 and 2). Buset and Scheline (1979) examined the urinary metabolites of flavanone, a flavonoid, after its administration to rats, and reported that the most common metabolic reactions were reduction of the keto group at the c-4-position and hydroxylation at the c-3- or c-6-position. It is likely, therefore, that the metabolism of specific flavonoids might depend on the position and/or nature of the substituents, in spite of structural similarities with respect to the flavonoid skeleton.

In plants, naringenin, a flavanone, is biosynthesized to genistein (an isoflavone), dihydrokaempferol (a flavanone) and apigenin (a flavone). Similarly, liquiritigenin, a flavanone, generates daidzein and 7,4'-dihydroxyflavone in a reaction catalysed by isoflavone synthase (P450) (Hakamatsuka *et al.* 1991) and flavone synthase II (P450) (Martens and Forkmann 1999, Akashi *et al.* 1999), respectively. Thus, flavanones appear to be important intermediates in the biosynthesis of flavonoids in plants. Since the biosynthesis of flavonoids from flavanones is mediated by P450 in plants, it is postulated that flavanones might also be converted to other flavonoids in mammals. However, the mammalian enzymes involved in the metabolism of flavanones remain to be identified.

Most natural flavonoids include more than one hydroxy or methoxy moiety and these moieties complicate efforts to determine the structures of flavonoid metabolites. However, the structures of (±)-flavanone and (±)-4'-methoxyflavanone are much simpler than those of natural flavonoids and, thus, (1) the structures of metabolites can be readily identified by gas chromatography-mass spectrometry (GC-MS); (2) the absence of a hydroxy group eliminates the requirement for derivatization procedures for the identification of structure and/or quantitative

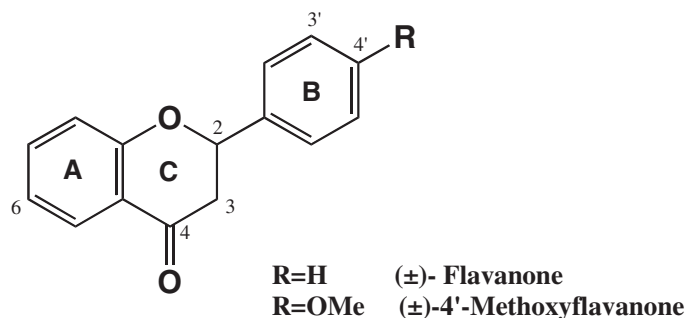


Figure 1. Structure of (±)-flavanone and (±)-4'-methoxyflavanone.

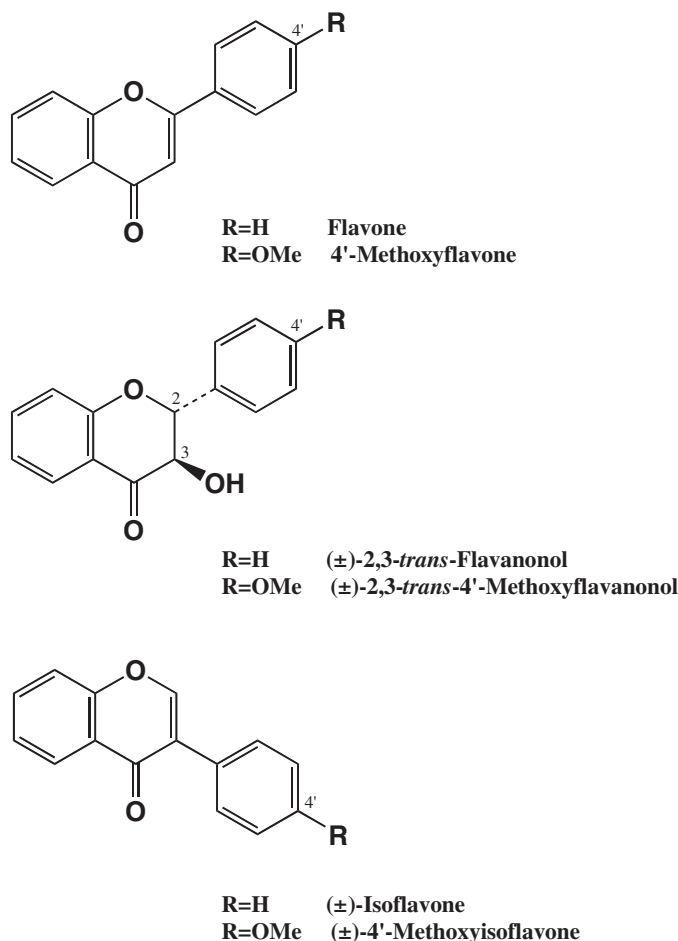


Figure 2. Structures of metabolites of (±)-flavanone and (±)-4'-methoxyflavanone.

analysis; and (3) sites of reactions, such as hydroxylation, can be readily identified. Therefore, the present study examined the metabolism of (±)-flavanone and (±)-4'-methoxyflavanone in rat liver microsomes and by recombinant human P450s in an effort to identify mammalian enzymes involved in flavanone metabolism. Canivenc-Lavier *et al.* (1996) reported that flavone increased CYP1A and 2B, whereas flavanone only enhanced CYP2B isozymes. Therefore, phenobarbital-treated rat liver and recombinant human P450, CYP1A1, 1A2 and 2B6, were examined as enzyme sources. The mechanisms of reactions with flavanones were investigated by examining kinetic isotope effects using deuterated derivatives of (±)-flavanone and (±)-4'-methoxyflavanone, both of which were prepared by a regio-selective deuteration method that the present authors had developed recently.

Materials and methods

Chemicals

(±)-Flavanone, phenobarbital and α -naphthoflavone were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Flavone was purchased

from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). (±)-4'-Methoxyflavone was purchased from Funakoshi Co., Ltd (Tokyo, Japan). Metyrapone, SKF-525A, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6P DHase) and β-nicotinamide adenine dinucleotide phosphate disodium salts (NADP⁺) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Microsomes prepared from human B lymphoblastoid cell lines expressing individual human cytochrome (CYP1A1, CYP1A2, CYP2B6) were obtained from Gentest (Woburn, MA, USA). All other chemicals were of the purest grade commercially available. (±)-4'-Methoxyflavanone, di-deuterated (±)-flavanone ((±)-3-d₂-flavanone) and di-deuterated (±)-4'-methoxyflavanone ((±)-3-d₂-4'-methoxyflavanone) were prepared as described by Kagawa *et al.* (2004). (±)-2,3-*trans*-Flavanonol and (±)-2,3-*trans*-4'-methoxyflavanonol were synthesized as described by Takahashi *et al.* (1984, 1986). Isoflavone and 4'-methoxyisoflavone were synthesized as described by Kinoshita *et al.* (1990).

Preparation of rat liver microsomes

Seven Wistar male rats (6 weeks old, each 140–150 g) were purchased from Clea Japan, Inc. (Tokyo, Japan). They were kept in a room with a 12-h light cycle and received laboratory chow (CE-2; Clea Japan) and water *ad libitum*. Each rat was given one intraperitoneal injection of sodium phenobarbital (60 mg kg⁻¹ in saline) daily for 3 days and killed 24 h after the last injection. Hepatic microsomes were prepared as described by Masumoto *et al.* (1991). The concentration of microsomal protein was determined by the method described by Lowry *et al.* (1951), with bovine serum albumin as the standard, and the concentration of cytochrome P450 was measured as described by Omura and Sato (1964).

Metabolism of (±)-flavanones in rat liver microsomes

The reaction mixture contained rat liver microsomes (6 mg protein), substrate (1 mM; dissolved in acetone), KCl (60 mM), MgCl₂ (4 mM), G6P (4 mM) and G6P DHase (5 units) in 2.5 ml 0.1 M sodium phosphate buffer (pH 7.4). After incubation for 3 min at 37°C, an inhibitor of P450 (1 mM; dissolved in acetone; see the Results) or acetone was added to the reaction mixture. The reaction was initiated by the immediate addition of NADP⁺ (to a final concentration of 0.4 mM). After incubation for 20 min at 37°C, 2 ml ice-cold ethyl acetate were added to the mixture to stop the reaction and the reaction mixture was extracted with ethyl acetate. The organic phase was separated and dehydrated with sodium sulfate. The organic layer was evaporated *in vacuo* and the residue was collected. To increase the conversion to isoflavones from 2-hydroxyisoflavanones, an important intermediate in isoflavone biosynthesis (Kochs and Grisebach 1986), methanol (1 ml) was added to the residue. After reflux for 1 h, the solution was allowed to stand overnight at room temperature. After evaporation of the solvent, the residue was dissolved in ethyl acetate (500 μl) and an aliquot of this solution was subjected to GC-MS/selected ion monitoring (SIM) analysis.

Metabolism of (±)-flavanone and (±)-4'-methoxyflavanone by human P450s

The incubation mixture contained microsomes from cell lines expressing human cytochrome P450 (CYP1A1, CYP1A2 or CYP2B6; 1 mg protein) or control microsomes from cell line including the empty vector, substrate (1 mM), KCl (60 mM), MgCl₂ (4 mM), G6P (4 mM) and G6P DHase (2 units), in 2.5 ml 0.1 M

sodium phosphate buffer (pH 7.4). After incubation of this mixture for 3 min at 37°C, the reaction was initiated by addition of NADP⁺ to a final concentration of 0.4 mM. After incubation for 20 min at 37°C, the mixture was treated in the same manner as the mixture obtained with rat liver microsomes and analysed by GC-MS/SIM.

Kinetic isotope effects on flavanone metabolism by rat liver microsomes

Flavanone and 4'-methoxyflavanone that had been di-deuterated at the C-3-position were synthesized as described by Kagawa *et al.* (2004). The extent of di-deuteration of these compounds was determined by ¹H-nuclear magnetic resonance (NMR) to be 91.4 and 92.3%, respectively. Di-deuterated compound or non-deuterated compound (each 1 mM) was incubated with rat liver microsomes and subjected to analysis by GC-MS/SIM as described above. K_H and K_D are defined as the amounts of protonated and deuterated products, respectively. (±)-2,3-*trans*-3-d₁-flavanonol derived from (±)-3-d₂-flavanone and non-deuterated (±)-2,3-*trans*-flavanonol derived from (±)-flavanone were monitored as the molecular ions m/z : 241.1 and 240.1, respectively. In flavone formation, 3-d₁-flavone and non-deuterated flavone were monitored as the molecular ions m/z : 223.1 and 222.1, respectively. In the case of (±)-4'-methoxyflavanone, (±)-2,3-*trans*-3-d₁-4'-methoxyflavanonol and (±)-2,3-*trans*-4'-methoxyflavanonol were monitored as the molecular ions m/z : 271.1 and 270.1, respectively. 3-d₁-4'-Methoxyflavone and 4'-methoxyflavone was monitored the molecular ion, m/z : 253.1, 252.1, respectively. The amounts of monodeuterium-labelled metabolites derived from di-deuterium-labelled flavanones were corrected from the deuterium content of substrates. Therefore, the kinetic isotope effects defined that K_H/K_D were calculated from the ratios of amounts of these protonated and deuterated products.

Analysis by GC-MS/SIM

GC-MS/SIM analysis was performed using a model JMS-AX505 HA mass spectrometer from Jeol (Tokyo, Japan), a gas chromatograph (5890 series II; Hewlett Packard, Palo Alto, CA, USA), a 30-m capillary column (DB-5MS) for flavanone and 60-m capillary column for 4'-methoxyflavanone (i.d., 0.25 mm; J. & W. Scientific, Palo Alto, CA, USA). When the 30-m capillary column was used, the injection temperature, interface temperature, ionizing voltage, ionizing current, accelerating voltage, temperature of the ion source and the flow rate of helium were set at 250°C, 280°C, 70 eV, 300 μA, 3 kV, 250°C and 30 ml min⁻¹, respectively. The initial temperature of the column was 100°C. After 1 min, it was raised at 15°C min⁻¹ to 220°C. After 4 min at 220°C, it was raised at 5°C min⁻¹ to 250°C and then the temperature was maintained at 250°C. When the 60-m capillary column was used, the injection temperature, interface temperature and flow rate of helium were set at 270°C, 290°C and 70 ml min⁻¹ respectively. The ionizing voltage, ionizing current, accelerating voltage and temperature of the ion source were the same as described above. The column was maintained at 100°C for 1 min and then the temperature was raised at 50°C min⁻¹ to 270°C and held. Metabolites of (±)-flavanones, i.e. (±)-2,3-*trans*-flavanonol, isoflavone and flavone, were monitored as the molecular ions m/z : 240.1, 222.1 and 222.1, respectively. In the case of (±)-4'-methoxyflavanone, (±)-2,3-*trans*-4'-methoxyflavanonol,

4'-methoxyisoflavone and 4'-methoxyflavone were monitored as the molecular ions m/z : 270.1, 252.1 and 252.1, respectively.

Antioxidant activities of flavonoids

Antioxidant activities were measured by the method of Tamura *et al.* (2000). The reaction mixture, prepared in 50 mM potassium phosphate buffer (pH 7.4), contained 10 μ M cytochrome *c* and 50 μ M xanthine. The reaction was started by the addition of xanthine oxidase at 25°C and the reduction of cytochrome *c* was monitored at 550 nm. The amount of xanthine oxidase added was adjusted to give a rate of change of absorption at 550 nm ($\Delta A_{550\text{ nm}}$) of 0.025 min⁻¹ in the absence of flavonoids. Antioxidant activity was determined in terms of the concentration of flavonoid that halved the rate of reduction of cytochrome *c*.

Results

Metabolism of (±)-flavanone and (±)-4'-methoxyflavanone by rat liver microsomes

We combined NADPH and either (±)-flavanone or (±)-4'-methoxyflavanone (figure 1) with microsomes prepared from the livers of rats treated with phenobarbital for 3 days and incubated the mixture at 37°C for 20 min. The analysis of the resultant metabolites by GC-MS/SIM allowed the identification of two major metabolites, flavone and (±)-2,3-*trans*-flavanonol, which, it was postulated, had been produced by oxidation of the C-2/C-3 bond and hydroxylation at the C-3-position of flavanone, respectively (figures 2 and 3). These metabolites of (±)-flavanone were consistent with both the retention times and the mass spectra of authentic samples by GC-MS. The yields of these metabolites decreased considerably if no NADP⁺ was added to the reaction mixture, or when an inhibitor of

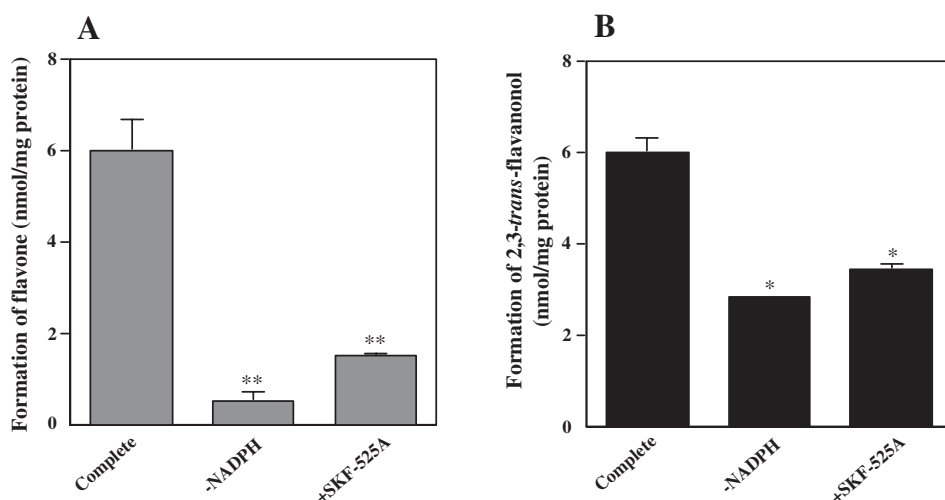


Figure 3. Formation of flavone (A) and (±)-2,3-*trans*-flavanonol (B) from (±)-flavanone by rat liver microsomes. Each column and bar represent a mean $\pm$ SD ($n=3$). Each complete reaction mixture contained microsomes (6.2 mg protein), 1 mM (±)-flavanone, 4 mM MgCl₂, 60 mM KCl, 0.4 mM NADP⁺, 4 mM G6P and 2 units G6P DHase in 0.1 M sodium phosphate buffer (pH 7.4) with modifications as indicated. Mixtures were incubated at 37°C for 20 min. Significant differences from the result for the complete reaction mixture: * $p < 0.05$, ** $p < 0.01$.

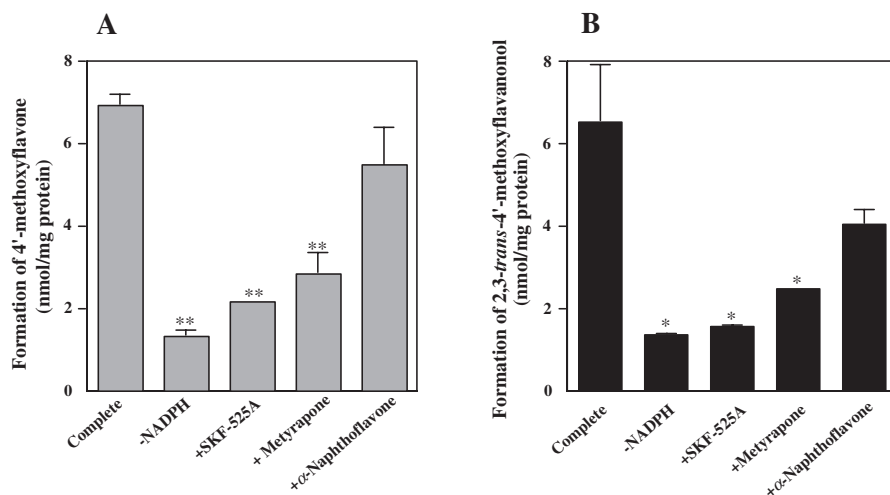


Figure 4. Formation of 4'-methoxyflavone (A) and (±)-2,3-*trans*-4'-methoxyflavanonol (B) from (±)-4'-methoxyflavanone by rat liver microsomes. Each column and bar represent a mean $\pm$ SD ($n=3$). Each complete reaction mixture contained microsomes (6.2 mg protein), 1 mM (±)-4'-methoxyflavanone, 4 mM MgCl_2 , 60 mM KCl, 0.4 mM NADP^+ , 4 mM G6P and 2 units G6P DHase in 0.1 M sodium phosphate buffer (pH 7.4) with modifications as indicated. Mixtures were incubated at 37°C for 20 min. Significant differences from the result for the complete reaction mixture: * $p < 0.05$, ** $p < 0.005$.

P450, SKF-525A, was added to the mixture (figure 3). The results suggested that the rat liver microsomal enzymes involved in the conversion of (±)-flavanone to flavone and (±)-2,3-*trans*-flavanonol might be P450s.

It was found that (±)-4'-methoxyflavanone was similarly metabolized to 4'-methoxyflavone and (±)-2,3-*trans*-4'-methoxyflavanonol by NADPH-dependent oxidation in rat liver microsomes (figures 2 and 4). These metabolites of (±)-4'-methoxyflavanone were consistent with both the retention times and the mass spectra of authentic samples by GC-MS. The effects of inhibitors of P450 on this reaction were also examined and it was found that whereas α -naphthoflavone had no effect, both SKF-525A and metyrapone significantly inhibited the formation of both methoxy metabolites (figure 4).

Metabolism of (±)-flavanone and (±)-4'-methoxyflavanone by recombinant human P450s

The experiments with rat liver microsomes suggested that P450s might be involved in the metabolism of (±)-flavanone. Therefore, the effects of three recombinant human P450s (CYP1A1, CYP1A2, and CYP2B6) were then examined. As anticipated, that all three P450 isozymes, namely CYP1A1, CYP1A2 and CYP2B6, could convert (±)-flavanone to flavone and (±)-2,3-*trans*-flavanonol (figure 5A, B). Among the P450 isozymes examined, CYP1A1 had the highest activity with respect to the yield of flavone and CYP2B6 had the lowest activity. By contrast, CYP2B6 had the highest activity with respect to the yield of (±)-2,3-*trans*-flavanonol. Furthermore, a small amount of isoflavone was produced both by CYP1A1 and by CYP2B6, a reaction not observed in the experiments with rat liver microsomes (figure 5C). When (±)-4'-methoxyflavanone was used as the substrate, both CYP1A2 and CYP2B6 generated 4'-methoxyflavone, whereas

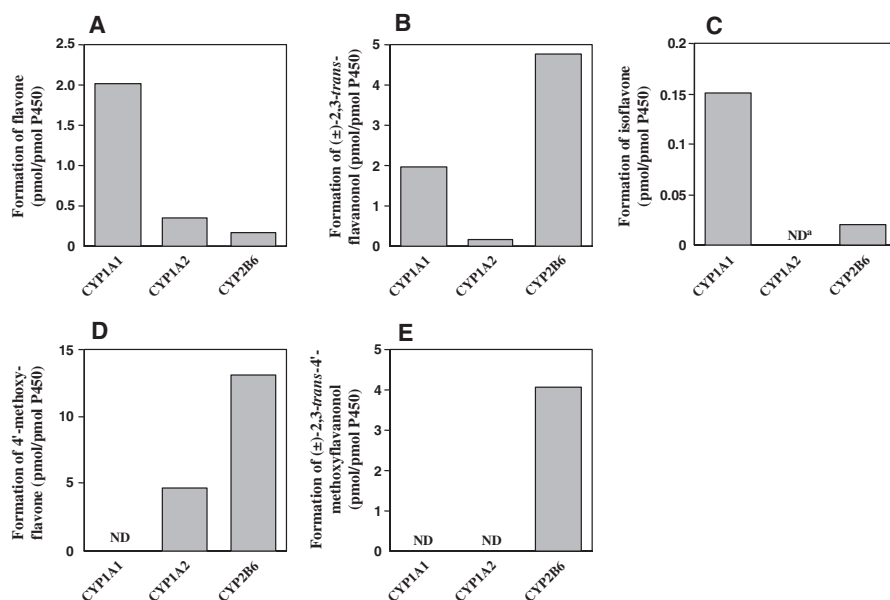


Figure 5. Formation of flavone (A), (±)-2,3-trans-flavanonol (B), isoflavone (C), 4'-methoxyflavone (D) and (±)-2,3-trans-4'-methoxyflavanonol (E) by human cytochrome P450s. Reaction mixtures contained 1 mg human cytochrome P450 as indicated, 1 mM (±)-flavanone or (±)-4'-methoxyflavanone, 4 mM MgCl₂ 60 mM KCl, 0.4 mM NADP⁺, 4 mM G6P and 2 units G6P DHase in 0.1 M sodium phosphate buffer (pH 7.4). The mixtures were incubated at 37°C for 20 min. Data are derived from three determinations. ^aNot detected.

CYP1A1 did not, unlike the results when (±)-flavanone was the substrate (figure 5A, D). In addition, (±)-2,3-trans-4'-methoxyflavanonol was generated only by CYP2B6 (figure 5E), and none of the P450s tested could generate 4'-methoxyisoflavone. These findings indicated that mammalian P450s could catalyse the metabolism of flavanones.

Kinetic isotope effects on the reaction catalysed by rat liver microsomes

To determine the molecular mechanisms of the reactions of (±)-flavanone and (±)-4'-methoxyflavanone with P450s, the corresponding derivatives were prepared, in which the C-3-position of the flavanone skeleton was selectively deuterated, by a method established by Kagawa *et al.* (2004). (±)-3-d₂-Flavanone and (±)-3-d₂-4'-methoxyflavanone were converted by rat liver microsomes to (±)-2,3-trans-3-d₁-flavanonol or (±)-2,3-trans-3-d₁-4'-methoxyflavanonol plus 3-d₁-flavone or 3-d₁-4'-methoxyflavone, respectively. In the case of both the (±)-2,3-trans-flavanonol and the (±)-2,3-trans-4'-methoxyflavanonol produced as metabolites, the non-deuterated form was formed at a substantially higher relative level than the corresponding deuterated metabolite, demonstrating a clear kinetic isotope effect ($K_H/K_D > 1$; table 1). A kinetic isotope effect in the formation of flavone and of 4'-methoxyflavone was also observed, but the effect was much smaller than that associated with the formation of flavanonols. The results implied that the P450-mediated reactions proceeded via abstraction of a hydrogen radical from the C-2- or C-3-position of the flavanone skeleton.

Table 1. Kinetic isotope effects (K_H/K_D) on the metabolism of ($\pm$)-flavanone and ($\pm$)-4'-methoxyflavanone metabolism by rat liver microsomes. Reaction mixtures contained microsomes (6.2 mg protein), a 1 mM di-deuterated or non-deuterated flavanone, 4 mM $MgCl_2$, 60 mM KCl, 0.4 mM $NADP^+$, 4 mM G6P and 2 units G6P DHase in 0.1 M sodium phosphate buffer (pH 7.4).

K_H/K_D	($\pm$)-Flavanone		($\pm$)-4'-Methoxyflavanone	
	Formation of flavone	Formation of ($\pm$)-2,3- <i>trans</i> -flavanonol	Formation of 4'-methoxyflavone	Formation of ($\pm$)-2,3- <i>trans</i> -4'-methoxyflavanonol
	1.5	5.9	1.6	9.9

Data are derived from three determinations.

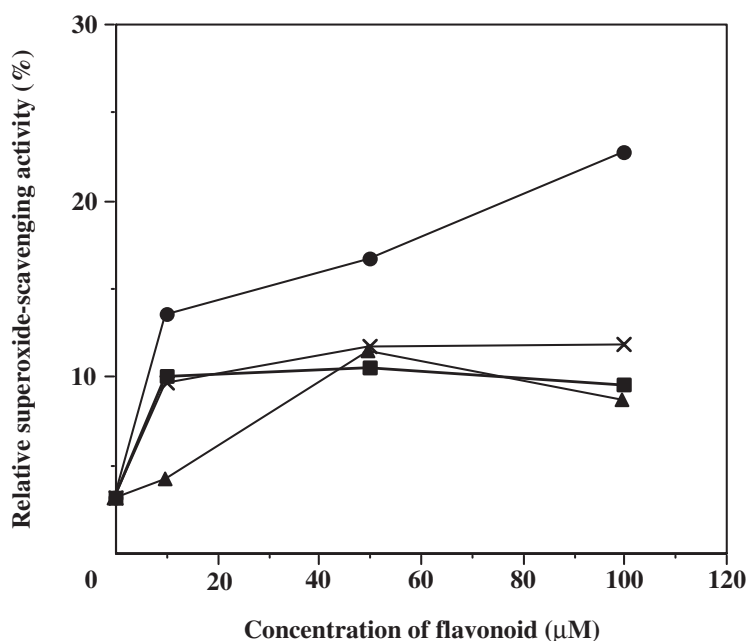


Figure 6. Superoxide-scavenging activity of ($\pm$)-flavanone and its metabolites, as determined by the xanthine–xanthine oxidase–cytochrome *c* method. ■, ($\pm$)-Flavanone; ×, flavone; ●, ($\pm$)-2,3-*trans*-flavanonol; ▲, isoflavone. Each reaction mixture was prepared in 50 mM potassium phosphate buffer (pH 7.4) and contained 10 μ M cytochrome *c* and 50 μ M xanthine. The reaction was started by the addition of xanthine oxidase at 25°C and the reduction of cytochrome *c* was monitored at 550 nm. Data are derived from two determinations.

Antioxidant activities of flavanone and its metabolites

The antioxidant activities of flavonoids are believed to play a role in prophylaxis of various diseases. Breinholt *et al.* (2002) reported that flavonoid metabolites, generated by P450, had markedly different biological activities from those of the parent compound. Therefore, the antioxidant properties of flavanone and its metabolites were examined by measuring superoxide-scavenging activity in a xanthine–xanthine oxidase–cytochrome *c* system. Flavone and isoflavone had antioxidant activities similar to that of the parent compound, ($\pm$)-flavanone, whereas ($\pm$)-2,3-*trans*-flavanonol had higher activity than that of other flavonoids (figure 6).

Discussion

It was revealed that ($\pm$)-flavanone and ($\pm$)-4'-methoxyflavanone were converted by mammalian P450s to flavone plus ($\pm$)-2,3-*trans*-flavanonol and to 4'-methoxyflavone plus ($\pm$)-2,3-*trans*-4'-methoxyflavanonol, respectively. Thus, the formation of flavanonol as a urinary metabolite of flavanone, as reported by Buset *et al.* (1979), might be mediated by P450. The formation of ($\pm$)-2,3-*trans*-flavanonol from ($\pm$)-flavanone by rat liver microsomes was inhibited by SKF-525A, an inhibitor of CYP2B (Knickle and Bend 1994) (figure 3B). In human, CYP2B6 had the highest catalytic activity of the recombinant P450s examined (figure 5B). Therefore, it is likely that the main P450 involved in the present experimental system was CYP2B in this study. By contrast, CYP1A1 was about 10-fold more active than CYP2B6 in the formation of flavone (figure 5A). The formation of flavone by rat liver microsomes was also inhibited by SKF-525A, but considerable catalytic activity was detected even in the presence of this inhibitor, remaining significantly higher than the activity observed in the absence of NADPH (figure 3A). Considering these results, one can conclude that the formation of flavone from ($\pm$)-flavanone is catalysed by both CYP1A and CYP2B (figure 5A).

In comparison with CYP1A1 and CYP1A2, CYP2B6 had markedly higher activity in the reactions that generated 4'-methoxyflavone and ($\pm$)-2,3-*trans*-4'-methoxyflavanonol from ($\pm$)-4'-methoxyflavanone (figure 5D, E). In the microsomal reaction, the formation of these metabolites was markedly inhibited by two inhibitors of CYP2B, SKF-525A and metyrapone (Huang and Waxman 2001), whereas the inhibitor of CYP1A, α -naphthoflavone (Liu *et al.* 2003) had no significant inhibitory effect (figure 4). These results suggest that CYP2B is mainly involved in the conversion of ($\pm$)-4'-methoxyflavanone to 4'-methoxyflavone and ($\pm$)-2,3-*trans*-4'-methoxyflavanonol.

It was demonstrated that human CYP1A1 and CYP2B6 catalysed the conversion of ($\pm$)-flavanone to isoflavone (figure 5C). In plants, isoflavone synthase catalyses the conversion of flavanone to isoflavone, and the mechanism of this reaction has been investigated in detail (Hakamatsuka *et al.* 1991). Rearrangement of the phenyl group is necessary for the conversion of flavanone to isoflavone and, thus, the mechanisms of reactions involving flavanone and mammalian P450s are of great interest.

As part of our efforts to elucidate the mechanism of the P450-mediated metabolism of ($\pm$)-flavanone and ($\pm$)-4'-methoxyflavanone, we recently reported a method for regio-selective deuteration at the C-3-position of ($\pm$)-flavanone and ($\pm$)-4'-methoxyflavanone (Kagawa *et al.* 2004). Using deuterated derivatives of ($\pm$)-flavanone and ($\pm$)-4'-methoxyflavanone prepared by our method, we examined the kinetic isotope effects in the respective reactions. We observed a strong isotope effect ($K_H/K_D = 5.9$ – 9.9) in the formation of ($\pm$)-2,3-*trans*-flavanonol and of ($\pm$)-2,3-*trans*-4'-methoxyflavanonol from the corresponding flavanone, as well as weak isotope effects ($K_H/K_D = 1.5$ – 1.6) in the formation of flavone and of 4'-methoxyflavone, respectively (table 1). The isotope effects suggest that the metabolic reactions that generate flavanonols and flavones might be initiated by abstraction of a hydrogen radical by P450 (Urano *et al.* 1996). A putative molecular mechanism for the metabolic reactions is shown in figure 7. Referring to the extent of each kinetic isotope effect, we postulate that abstraction of a hydrogen radical from the C-3-position is a major feature of the conversion of

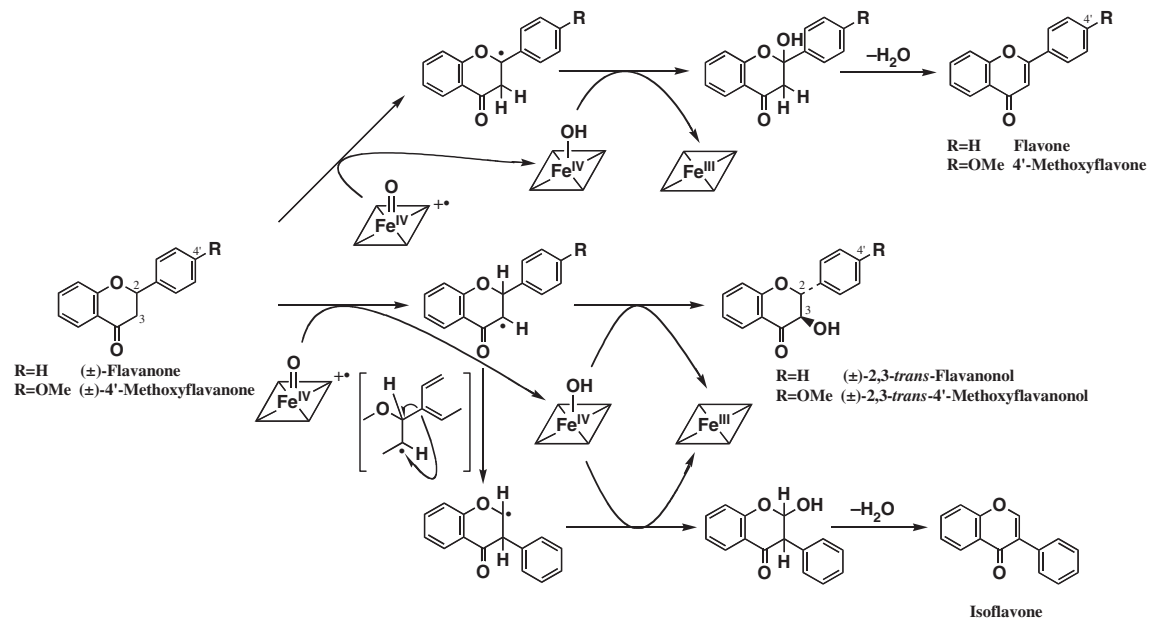


Figure 7. Proposed mechanism of the metabolism of ($\pm$)-flavanones by P450.

($\pm$)-flavanone and ($\pm$)-4'-methoxyflavanone to ($\pm$)-2,3-*trans*-flavanonol and ($\pm$)-2,3-*trans*-4'-methoxyflavanonol, respectively. It is believed that flavanonols are produced by bonding of a hydroxy radical, derived from the hydrogen radical abstracted by P450, to the carbon radical at the C-3-position that has been generated by the abstraction of a hydrogen. The weak isotope effect observed in the formation of flavones can be interpreted as follows: an initial inefficient abstraction of a hydrogen radical by P450 occurs at the C-2-position to generate a carbon radical at this position, and a hydroxy radical, derived from the abstracted a hydrogen radical, bonds to the carbon radical to produce a 2-hydroxy intermediate, which is finally dehydrated to flavone. The weak isotope effect probably occurs at the dehydration step. Furthermore, the following pathway might account for the formation of isoflavone: after the initial abstraction of a hydrogen radical at the C-3-position, the phenyl ring at the C-2-position moves to the C-3-position, with generation of a carbon radical at the C-2-position and subsequently bonding of a hydroxy radical to the carbon radical to produce an unstable β -hydroxyketone intermediate, which is readily dehydrated to isoflavone.

The present study suggests that a pathway for the oxidation of flavonoids, similar to that found in plants, exists in mammals, providing useful information with respect to the biological significance of the formation of oxidized flavonoids. Scavenging of reactive oxygen radicals is one of the many bioactivities of flavonoids and such activity is enhanced as the number of hydroxy moieties increases (Bors *et al.* 1990). We confirmed this relationship in the present study: ($\pm$)-2,3-*trans*-flavanonol, with a hydroxy group, had the highest antioxidant activity of all tested metabolites of ($\pm$)-flavanone (figure 6).

Canivenc-Lavier *et al.* (1996) reported the selective induction of expression of CYP2B by ($\pm$)-flavanone in the rat liver, and the present study revealed that CYP2B6 selectively catalysed the conversion of ($\pm$)-flavanone to ($\pm$)-2,3-*trans*-flavanonol. Together, these results suggest that a mechanism exists for the efficient conversion of flavanones, which are ingested by mammals as plant foodstuffs, to more biologically active flavanonol derivatives, which play a role in prophylaxis in mammals.

In plants, 2-oxoglutarate dioxygenase has been characterized as an enzyme that is involved in the biosynthesis of flavonoids, but no species of P450 that mediates hydroxylation at the C-3-position of the flavanone skeleton has been identified in plants. Sawada *et al.* (2002) suggested the presence of unknown P450 type flavanone 3-hydroxylase. The present investigation demonstrated that mammalian P450s can catalyse the conversion of flavanones to flavanonols, implying the presence of as yet unidentified molecular species of P450 in plants that catalyse hydroxylation of flavanones at the C-3-position.

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Metabolism of human cytochrome P450 marker substrates in mouse: a strain and gender comparison

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1. The aim was to characterize mouse gender and strain differences in the metabolism of commonly used human cytochrome (CYP) P450 probe substrates.
2. Thirteen human CYP probe substrates (phenacetin, coumarin, 7-ethoxy-4-trifluoromethyl coumarin, amiodarone, paclitaxel, diclofenac, *S*-mephenytoin, bufuralol, dextromethorphan, chlorzoxazone, *p*-nitrophenol, testosterone and lauric acid) were used in activity measurements. The metabolism of the probe substrates was compared in liver microsomes from male and female NMRI, CBA, C57bl/6, 129/SvJ and CD1 strains. The expression of proteins identified on Western blots with commonly available antibodies selective for specific human and rat CYP enzymes were compared in the different mouse strains.
3. Males had higher metabolism than corresponding females for phenacetin *O*-deethylation (human marker for CYP1A2 activity), and a high correlation was found between phenacetin activity and immunoreactivity in Western blots produced with rat CYP1A2 antibodies.
4. Protein detected by antibodies cross-reacting with human CYP2B6 and rat CYP2B1/2 antibodies was female specific except for the 129/SvJ strain, where it was absent in both genders.
5. Females generally had a higher metabolism of bufuralol 1'-hydroxylation and dextromethorphan *O*-demethylation (human markers for CYP2D activity).
6. Bufuralol 1'-hydroxylation correlated with a female-dominant mouse CYP, which was detected with antibodies against rat CYP2D4.
7. *p*-Nitrophenol 2-hydroxylation correlated better than chlorzoxazone 6-hydroxylation with the protein detected with antibodies against rat CYP2E1, indicating that *p*-nitrophenol is a more specific substrate for mouse CYP2E1.

Introduction

The cytochrome P450 (CYP) monooxygenase enzyme system present in the liver plays a central role in the oxidation of a wide variety of structurally unrelated compounds, including exogenous compounds such as pharmaceutical agents, chemical carcinogens and other lipophilic xenobiotics, as well as endogenous steroids, fatty acids, prostaglandins and vitamin D₃ (Gonzalez 1990, Ryan and Levin 1990). Considerable differences exist between humans and mice in the expression and catalytic activities of different CYP orthologues (Bogaards *et al.* 2000). Although CYP enzymes usually make xenobiotics less toxic, they are also frequently involved in the formation of reactive intermediates or allow leakage of free oxygen radicals capable of causing toxicity (Guengerich 1997, 2002).

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Therefore, differences in the levels of catalytically distinct CYP enzymes significantly contribute to interspecies differences in the toxicity of chemicals. Nevertheless, drug safety assessments, including pharmacological and toxicological studies, are usually performed in rodents. The use of mice within the pharmaceutical industry is rapidly increasing, partly due to rapid development of new transgenic disease models. To date, 93 functional CYP genes and 21 pseudogenes have been described in the mouse (<http://www.drnelson.utmem.edu/mouse.master.table.html>, accessed 7 January 2004) with 82 members from the four major drug-metabolizing families in mouse liver (table 1).

Gender differences in xenobiotic metabolism and sex-dependent CYP expression have been observed in some mouse strains (Harada and Negishi 1984, Macleod *et al.* 1987, Wong *et al.* 1987, Henderson *et al.* 1990, Yoshioka *et al.* 1990). In spite of the extensive use of different mouse strains, information about mouse-specific metabolism and strain and gender differences is still scarce. This causes problems in early drug development when trying to extrapolate pharmacokinetic parameters as well as pharmacological effects and toxicity, which depend on exposure to drug candidates and their metabolites, from mice to humans.

Table 1. CYP enzymes of the four major drug-metabolizing CYP families in humans, mice and rats.^a

Family	Subfamily	Human	Mouse	Rat
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2
	B	1B1	1B1	1B1
CYP2	A	2A6, 2A7, 2A13, 2A18	2A4, 2A5, 2A12, 2A22	2A2, 2A3
	B	2B6, 2B7	2B9, 2B10, 2B13, 2B19, 2B20x, 2B23	2B1, 2B2, 2B3
	C	2C8, 2C9, 2C18, 2C19	2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50, 2C51x, 2C54, 2C55, 2C65, 2C66, 2C67, 2C68, 2C69, 2C70	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23
	D	2D6	2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40	2D2, 2D3, 2D4, 2D5, 2D18
	E	2E1	2E1	2E1
	F	2F1	2F2	2F4
	G	2G1	2G1	2G1
	J	2J2	2J5, 2J6, 2J7, 2J8, 2J9, 2J11, 2J12, 2J13	2J3
	R	2R1	2R1	2R1
	S	2S1	2S1	2S1
	T		2T4	
	U	2U1	2U1	
	W	2W1	2W1	
CYP3	A	3A3X, 3A4, 3A5, 3A7, 3A43	3A11, 3A13, 3A16, 3A25, 3A41, 3A44, 3A57, 3A59	3A1, 3A2, 3A9, 3A18, 3A23
CYP4	A	4A11, 4A20X, 4A22	4A10, 4A12a, 4A12b, 4A14, 4A29, 4A30b, 4A31, 4A32	4A1, 4A2, 4A3, 4A8
	B	4B1	4B1	4B1
	F	4F2, 4F3, 4F8, 4F11, 4F12, 4F22,	4F13, 4F14, 4F15, 4F16, 4F17, 4F18, 4F37, 4F39, 4F40	4F1, 4F4, 4F5, 4F6, 4F19
	V	4V2	4V3	
	X	4X1	4X1	4X1
	Z	4Z1		4Z1

^a<http://drnelson.utmem.edu/mouse.master.table.html> (accessed 7 January 2004).

Currently, many different strategies are employed for the identification of CYP enzymes responsible for the biotransformation of therapeutic agents in humans. These include the use of selective CYP substrates, inhibitors or inhibitory antibodies as well as cDNA-expressed human CYP enzymes. Correlation analysis of metabolic rates of drugs with immunoquantified protein levels or predetermined metabolic rates of enzyme selective substrates in kits of characterized liver microsomes are also used. Since mouse-specific probe substrates and antibodies were not commercially available, the current study was performed with selective probe substrates for human CYP enzymes as a first step to characterize strain and gender differences *in vitro*. Antibodies against human and rat CYP enzymes were evaluated as possible tools to show expression of different mouse CYP subfamilies.

Few studies have been made where activities and/or CYP expression have been investigated simultaneously in many different mouse strains. In the present study, the metabolism of 13 compounds used as selective probe substrates for 10 different human CYP enzymes were examined in five different mouse strains widely used in different laboratory models. The protein expression of mouse CYPs in liver microsomes from both genders was also measured in all five strains using Western blot with some commercially available antibodies produced against some human and rat CYPs from all major drug-metabolizing families.

Materials and methods

Animals

Both males and females from five different mouse strains widely used in drug development studies were chosen: NMRI, CBA, C57bl/6, 129/SvJ and CD1 strains. On the Western blots with antibodies towards members of the CYP2C subfamily, liver microsomes from male and female C57bl/CBA and male MutaTM Mouse (CD₂-lac Z80/HazfBRstrain) were included. All animals (except the CD1 strain) were supplied by M&B, Denmark. CD1 animals were supplied from Charles River, Sweden. Livers were excised from animals that had been fed *ad libitum* with standard pelleted diet. After excision, the livers were put into liquid nitrogen and stored at -70°C .

The animals from the CBA, C57Bl/6, C57bl/CBA and 129/SvJ strains were all 8 weeks old, the NMRI mice 4–5 weeks, the MutaTM Mouse mice 12 weeks and the CD1 mice 24 weeks old.

Chemicals

All the following chemicals were obtained from Sigma Chemicals Co. (St Louis, MO, USA): amiodarone hydrochloride; bovine serum albumin (BSA); chlorzoxazone; coumarin; cytochrome C; dextromethorphan; diclofenac; 7-ethoxy-4-trifluoromethylcoumarin (7-EFC); 7-hydroxycoumarin; lauric acid; β -NADPH; *p*-nitrophenol; phenacetin and testosterone; Ultrafine Chemicals (Manchester, UK) was the source for the following metabolite standards: ($\pm$)-bufuralol hydrochloride salt; dextrophan; ($\pm$)-hydroxybufuralol maleate salt; 12-hydroxy-lauric acid and 6 β -hydroxytestosterone. 6-Hydroxychlorzoxazone was supplied by Research Biochemicals International (Natick, MA, USA). 4-Hydroxydiclofenac and 7-hydroxy-4-trifluoromethyl coumarin (HFC) was supplied by Gentest Corp. (Woburn, MA, USA). Acetaminophen (paracetamol) was supplied by AstraZeneca (Södertälje, Sweden). Paclitaxel was supplied by Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA) and *S*-mephenytoin was supplied by Toronto Research Chemicals (North York, Canada). All other chemicals were of analytical grade and obtained from Merck. (Darmstadt, Germany).

The sources of primary antibodies used in Western blots and corresponding cDNA expressed CYP enzymes used as positive controls on the blots are listed in table 2.

All primary antibodies were produced in rabbit except those purchased from Daiichi (Tokyo, Japan), which were produced in goat (anti-rat CYP2B1, CYP2C6, CYP2C11, CYP2C13) and anti-rat CYP4A (Amersham Pharmacia Biotech, Uppsala, Sweden) that was produced in sheep. The anti-rabbit-IgG secondary antibodies were linked to horseradish peroxidase (HRP) while the anti-sheep/goat IgG secondary antibodies were biotinylated. All secondary antibodies were supplied from Amersham.

Table 2. Antibodies and positive control microsomes used in Western blot analysis of mouse liver microsomes.

Primary antibody (polyclonal)	Dilution of primary antibodies	Source (company) ^a	Positive controls	
			cDNA-expressed enzyme ^b	Induced rat microsomes ^c
Anti-rat 1A1	1/2500	Chemicon	rat 1A1	β-naphthoflavone
Anti-rat 1A2	1/375	Amersham	rat 1A2	β-naphthoflavone
Anti-human 1B1	1/500	Gentest	human 1B1	
Anti-human 2A6	1/500	Puracyp	2A6	β-naphthoflavone
Anti-rat 2B1	1/2500	Daiichi	2B1 + b5	β-naphthoflavone
Anti-human 2B6 and anti-rat 2B1/2	1/2000	Chemicon	2B6	phenobarbital
Anti-rat 2C6	1/5000	Daiichi	2C6 + b5	phenobarbital
Anti-human 2C8/9/19 and anti-rat 2C12	1/2000	Chemicon	2C19	arochlor 1254
Anti-rat 2C11	1/1000	Daiichi	2C11 + b5	arochlor 1254
Anti-rat 2C13	1/5000	Daiichi	2C13 + b5	saline
Anti-rat 2D1	1/2500	Chemicon	2D1	arochlor 1254
Anti-rat 2D4	1/1250	Chemicon	2D1	
Anti-human 2D6	1/1250	Chemicon	2D6	arochlor 1254
Anti-rat 2E1	1/2500	Amersham	human 2E1 + b5	isoniazid
Anti-rat 3A	1/250	Amersham	3A1 + b5	dexamethasone
Anti-rat 4A	1/1250	Amersham	4A11	clofibrate
Anti-human 4A11	1/50	Puracyp	4A11	clofibrate

^aBD Gentest (Woburn, MA, USA), Chemicon International, Inc. (Temecula, CA, USA), AmershamPharmaciaBiotech (Uppsala, Sweden), Daiichi (Tokyo, Japan), Puracyp (San Diego, CA, USA).

^bBD Gentest. All cDNA-expressed enzymes contained NADPH-cytochrome P450 NADPH reductase.

^cXenoTech, LCC (Kansas City, KS, USA).

Methods

Microsomal preparation. Microsomes were prepared both from male and from female animals (except MutaTM Mouse where only males were available). Livers were pooled for each strain and sex separately. Pools prepared from CBA, C57bl/CBA and C57bl/6 mice contained 10 animals per gender; CD1 pools contained livers from five animals per gender. The 129/SvJ and NMRI pools were prepared from nine males and 10 females, respectively. The MutaTM Mouse pool contained 11 males.

Livers were homogenized in 2–4 vols homogenization buffer (10 mM sodium/potassium-phosphate/1.14% KCl, pH 7.4) at 0–4°C. Microsomes were prepared by ultracentrifugation (105 000 *g* for 70 min) of the post-mitochondrial supernatant (10 000 *g* for 10 min). The microsomal pellet was resuspended in homogenization buffer and again centrifuged for 70 min at 105 000 *g*. The washed microsomes were suspended in 50 mM potassium phosphate buffer (0.4 ml *g*⁻¹ original liver weight) and stored at approximately -80°C until use.

Protein concentration. The protein content of the liver microsomes was determined using a CARY 400 UV-VIS spectrophotometer and analysed with Cary Win UV Concentration Application, v.02.00 (25) by a method scaled down from the original method by Lowry *et al.* (1951), using bovine serum albumin as a standard.

Total CYP content. The total cytochrome P450 content in the liver microsomes was quantified spectrophotometrically. The concentration of cytochrome P450 was determined from the CO-difference spectra of dithionite-reduced samples using an extinction coefficient of 91 mM⁻¹cm⁻¹ between 450 and 490 nm (Omura and Sato 1964). Spectra for determination of total P450 were recorded using a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer.

CYP reductase. The NADPH-cytochrome P450 reductase activities were determined spectrophotometrically as the rate of reduction of cytochrome C at 550 nm and 30°C, in 0.33 M potassium phosphate buffer, pH 7.6. Purified NADPH-cytochrome P450 reductase was used as a reference and recorded by a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer.

NADPH oxidation. Microsomal protein, 0.5 mg from each sample, was diluted to 2 ml with 50 mM phosphate buffer, pH 7.4. The mixture was divided between two cuvettes, and pre-incubated at 37°C for 3 min before the reaction was started by the addition of 10 μ l 10 mM NADPH solution. The rate of NADPH oxidation at 37°C was measured by continuously recording the absorbance at 340 nm (loss of reduced NADPH) with a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer. The extinction coefficient used was 6.2 mM⁻¹cm⁻¹ and the result reported as pmol oxidized NADPH mg⁻¹ microsomal protein min⁻¹.

Analysis of CYP activities. Classical markers for human CYP enzymes were incubated with mouse liver microsomes. The activities measured and human enzymes they usually assess are as follows: phenacetin *O*-deethylation (CYP1A); coumarin 7-hydroxylation (CYP2A6); 7-EFC deethylation (CYP2B6); diclofenac 4'-hydroxylation (CYP2C9); bufuralol 1'-hydroxylation (CYP2D6); dextromethorphan *o*-demethylation (CYP2D6); chlorzoxazone 6-hydroxylation (CYP2E1); *p*-nitrophenol *O*-hydroxylation (CYP2E1); testosterone 6 β -hydroxylation (CYP3A) and lauric acid 12-hydroxylation (CYP4A). In addition the rate of disappearance of paclitaxel, amiodarone and *S*-mephenytoin in incubations with mouse liver microsomes were measured.

Microsomes, substrate and buffer were mixed and pre-incubated at 37°C for 3–5 min before starting incubations with addition of NADPH. Incubation volumes were 0.2 ml for all assays except coumarin 7-hydroxylation, 7-EFC, dextromethorphan *O*-demethylation, chlorzoxazone 6-hydroxylation and *p*-nitrophenol, where the final volumes were 0.1, 0.1, 0.5, 0.5 and 1.0 ml, respectively. NADPH concentrations were 1 mM for all incubations except coumarin 7-hydroxylation, 7-EFC and *p*-nitrophenol where the NADPH concentrations were 0.1, 0.1 and 0.36 mM, respectively. Potassium phosphate buffer, 50 mM, pH 7.4, was used for all incubations except the *p*-nitrophenol assay where 100 mM potassium phosphate buffer, pH 6.8, was used. Incubations without NADPH and incubations with boiled mouse liver microsomes were used as negative controls. Time curves, with the human cytochrome P450 substrates, were run and the rates estimated from the linear part using standard curves. The standard curves were run with at least four different concentrations of metabolite or parent compound, covering the range of concentrations measured in the assays. Incubation conditions are listed in table 3.

Fluorescence was measured in real time. When activities were measured with HPLC or LC/MS a stop solution was added (table 3) and precipitated proteins removed by centrifugation and the supernatant analysed.

Western blot analysis. Solubilized microsomal protein (2.5–10 μ g/well) was applied to each well of a 0.75 mm thick 8.7% polyacrylamide gel. The microsomal proteins were separated by SDS-PAGE for 45 min at 200 V in a Bio-Rad Miniprotean cell (Hercules, CA, USA). The proteins were transferred to PVDF Membranes (PolyScreen, NEN Life Science Products, Inc., Boston, MA, USA) electrophoretically using a Bio-Rad Trans-Blot SD (semi-dry transfer cell) apparatus with 1.5 mA cm⁻² membrane. The transfer time was set to 50 min.

The PVDF membranes were blocked with TBS buffer (50 mM Tris-HCl, pH 7.4; 200 mM NaCl) containing 5% non-fat dry milk and 0.05% Tween-20 for 1 h at 30°C, and thereafter kept in blocking solution over night (at 6°C). The blocking solution was discarded and the primary antibodies (table 2), diluted in blocking solution, were added and the membranes incubated at 30°C for 2 h. The membranes were washed with TBS containing 1% non-fat dry milk and then incubated with secondary antibodies in blocking solution (diluted 1/2500) for 1 h at 30°C. PVDF membranes were washed with TBS containing 1% non-fat dry milk and the antibody complexes detected with the ECLTM detection kit and Hyperfilm-ECL as described by the manufacturer (Amersham). The anti-rabbit-IgG secondary antibodies were linked to horseradish peroxidase (HRP) while the anti-sheep/goat IgG secondary antibodies from Amersham were biotinylated, and detection was made adding also streptavidin-HRP conjugate. The films were developed and scanned using a Model GS-700 Imaging densitometer from Bio-Rad and analysed with Molecular Analyst Software, Version 1.4. The optical density over the total band area was designated as the band volume. Five measurements were made of background density and the average subtracted. The result is given in arbitrary units and compared only within the same blot.

Statistical methods

Spearman rank correlations (R_s and p) were calculated using Sigma Stat (version 2.03) in correlation analysis between activities and Western blot densities. Co-correlation analysis was also performed, for all activity-activity pairs as well as Western blot-Western blot pairs.

The coefficient for the correlation between the metabolism of a substrate S and any protein I was called X_{SI} (activity-Western blot). The highest X_{SI} and all correlations with $p < 0.001$ for a substrate were called a_{SP} . The protein giving this correlation coefficient was called P_S and was considered to be the major metabolizing enzyme(s) for substrate S. The coefficient for the correlation between protein P

Table 3. Summary of incubation conditions for *in vitro* activity measurements.

Activity measured commonly used as a probe for (human CYP)	Initial probe concentration (μM)	Microsomal protein concentration (mg ml^{-1})	Reaction time (min)	Final solvent concentration	Termination/stop solution (ml)	Analytical method			
						Column	Mobile phase	Flow rate (ml min^{-1})	Detection system
Phenacetin <i>O</i> -deethylation (CYP1A2)	1	0.50	5	–	acetonitrile (100 μl)	Symmetry C ₁₈ 50 $\times$ 2.1 mm, 3.5 μm	gradient 2–95% CAN in 0.1% HAc	0.400	LC/MS
Coumarin 7-hydroxylation (CYP2A6)	4	0.25	10	–	fluorescence recorded in real time	–	–	–	fluorescence plate reader; excitation: 390 nm; emission: 460 nm; cut-off: 435 nm
7-Ethoxy-4- trifluoromethyl- coumarin deethylation (CYP2B6)	10	0.20	15	0.2% Acetonitrile	fluorescence recorded in real time	–	–	–	fluorescence plate reader; excitation: 405 nm; emission: 535 nm; cut-off: 475 nm
Diclofenac 4'-hydroxylation (CYP2C9)	5	0.25	10	–	acetonitrile: glacial acid (47:3, 50 μl)	Symmetry C ₈ 50 $\times$ 2.1 mm, 3.5 μm	gradient 2–95% ACN in 0.1% HAc	0.400	LC/MS
Bufuralol 1'-hydroxylation (CYP2D6)	5	0.25	10	–	acetonitrile (100 μl)	Symmetry C ₁₈ 50 $\times$ 2.1 mm, 3.5 μm	gradient 2–95% ACN in 0.1% HAc	0.400	LC/MS
Dextromethorphan <i>O</i> -demethylation (CYP2D6)	5	0.50	15	–	70% perchloric acid (100 μl)	Symmetry C ₈	30 mM ammonium acetate, pH 4: acetonitrile 75:25	1.0	fluorescence excitation: 270 nm; emission: 312 nm

Chlorzoxazone 6-hydroxylation (CYP2E1)	40	0.50	15	0.6 mM KOH	70% perchloric acid (100 µl)	LiChroCART 250-4	gradient 30–60% ethanol	0.8	ultraviolet light absorbance: 280 nm
<i>p</i> -Nitrophenol <i>O</i> -hydroxylation (CYP2E1)	1200	0.20	40	–	0.6 M perchloric acid (500 µl)	–	–	–	visible light-photometry absorbance: 546 nm
Testosterone 6-β-hydroxylation (CYP3A4)	80	0.50	30	0.4% DMSO	acetonitrile (400 µl)	LiChroCART 250-4	gradient 30–60% ethanol	0.8	ultraviolet light absorbance: 254 nm
Lauric acid 12-hydroxylation (CYP4A)	50	0.50	30	–	acetonitrile: 1 M H ₃ PO ₄ (1:1, 20 µl)	AquaSil C18 30 × 2.1 mm, 3 µm	gradient 2–80% ACN in 0.1% HAc	0.30	LC/MS
Amiodarone disappearance	1	0.50	10	1% Acetonitrile	acetonitrile (200 µl)	Atlantis 30 × 2.1 mm, 3 µm	0.1% formic acid	0.35	LC-MS/MS
Paclitaxel disappearance	10	0.50	10	0.1% Ethanol	acetonitrile (100 µl)	Atlantis 30 × 2.1 mm, 3 µm	0.1% formic acid	0.35	LC-MS/MS
<i>S</i> -mephenytoin disappearance and 4-hydroxylation (CYP2C19)	10	0.50	40	0.1% Methanol	acetonitrile (100 µl)	Symmetry C ₈ 50 × 2.1 mm, 3.5 µm	gradient 2–80% ACN in 0.1% HAc	0.400	LC/MS

LC/MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry detection.

and any other protein I was defined as b_{PSI} (Western blot–Western blot). A correlation X_{SI} was considered to be due to co-correlation at the protein level if:

$$Y_{\text{SI}} > X_{\text{SI}},$$

where

$$Y_{\text{SI}} = a_{\text{SP}} \times b_{\text{PSI}} \times 1.25$$

(to allow for a 25% error in correlation coefficients).

The average (male and female) activities and protein expressions were determined for each strain (Ave_s). Each average (Ave_s) was compared with the corresponding total average for the rest of the mouse pools ($\text{Ave}_{\text{tot-s}}$). A strain value was considered as deviating from the other strains if its average (Ave_s) was outside the limits:

$$(\text{Ave}_{\text{tot-s}}/2) \quad \text{and} \quad (\text{Ave}_{\text{tot-s}} \times 2).$$

If a protein expression or an activity should be considered as generally gender dominant in mice, at least four strains out of the five examined had to have at least 25% higher activity (or protein expression) for one gender, when comparing males and females from the same strain.

When a sex-dependent difference occurs in a mouse strain, the greatest degree of sexual dimorphism is usually about twofold (Mugford and Kedderis 1998). Therefore, if a protein expression or an activity was to be considered as gender dominant for a specific mouse strain in this study, one gender had to have at least a twofold higher activity (or protein expression) than the other gender. The average activities and protein expressions were determined for all strains (Ave_{tot}). Each average (Ave_{tot}) was compared with the corresponding average reported in the literature (Ave_{lit}). A value in this study (Ave_{tot}) was considered to be in good agreement with literature data (Ave_{lit}) if the value (Ave_{tot}) was between the limits:

$$(\text{Ave}_{\text{lit}}/2) \quad \text{and} \quad (\text{Ave}_{\text{lit}} \times 2).$$

To compare data from the present study with data from the literature, recalculations of metabolic rates were performed to get metabolic rates at the K_m reported in the literature. When different K_m values were reported in different articles, the data from the present study were recalculated separately for each article to be comparable with the reported literature metabolic rates. When metabolic rates and K_m values were reported separately for each gender within one article, as in Bogaards *et al.* (2000), an average of their rates as well as an average of their K_m values was used.

All metabolic rates from the literature were assumed to be V_{max} values. Metabolic rates from the present study were designated as V_{max} values, when the probe concentration used was greater than or equal to 2.5-fold the K_m reported in the literature. Rates at K_m were calculated as follows:

$$\text{Rate at } K_m = V_{\text{max}}/2.$$

When the concentration in the present study (c_s) was lower than the K_m reported in the literature, linear kinetics was assumed and rates at K_m were calculated as follows:

$$\text{Rate at } K_m = (v_s \times K_m)/c_s,$$

where v_s is the average rate measured in the present study.

Results and discussion

When comparing strains, no significant differences were found in the total cytochrome P450 levels, cytochrome P450 reductase activities or NADPH oxidation rates. Within the CBA strain the females had twofold higher NADPH-oxidation rate and cytochrome P450 reductase activities than the males (table 4). There was a significant correlation ($R=0.84$, $p<0.001$) between the total CYP levels and the CYP reductase activities.

The metabolism of 13 human cytochrome P450 marker substrates was investigated (figure 1 and table 5) in liver microsomes from both genders from the different strains in order to investigate strain and gender differences. The results are discussed below together with results from Western blot analysis (figures 2 and 3). Antibodies against human and rat CYP enzymes were evaluated as tools to measure mouse enzyme expression (Shimada *et al.* 2003).

Table 4. Mouse microsome characteristics.

	CBA		129/SvJ		NMRI		CD1		C57bl/6	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
NADPH-oxidation ($\text{pmol min}^{-1} \text{mg}^{-1}$)	7700	17200	13300	13200	9000	10300	8500	6500	7700	8400
P-450 reductase ($\text{U mg}^{-1} \text{min}^{-1}$)	184	403	202	204	221	243	152	146	118	197
Total P450 (pmol mg^{-1})	476	629	418	430	479	592	339	331	404	354

For assay conditions, see the Materials and methods.

Data are means from multiple measurements performed with pools of five to 10 animals.

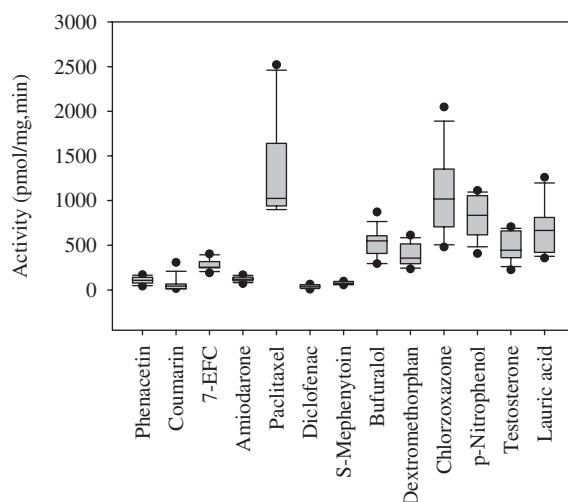


Figure 1. Mouse microsomal activities with human cytochrome P450-specific probe substrates. ($\text{pmol min}^{-1} \text{mg}^{-1}$ microsomal protein). Activity measurements in the present study were performed with liver microsomes from both genders of the NMRI, CBA, CD1, C57bl/6 and 129/SvJ strains, as specified in table 3. Data was combined and displayed as a box plot representing statistical values. The boundary of the box closest to zero indicates the 25th percentile; a line within the box marks the median; and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. In addition, the maximum, and minimum, values are shown.

Antibodies against human CYP1B1 could not detect any proteins in mouse liver microsomes, probably due to insignificant expression in the mouse liver as in other species (Muskhelishvili *et al.* 2001, Choudhary *et al.* 2003, Shimada *et al.* 2003).

Phenacetin O-deethylation

For the phenacetin O-deethylation measurements, all strains showed higher activities in male microsomes than in the corresponding female microsomes, with the exception of the NMRI strain where the activity was higher in the females than in the males (figure 4 and table 5). Male microsomes from the CBA, 129SvJ, NMRI and C57bl/6 strains gave more intense bands in Western blots with rat CYP1A2 antibodies than the corresponding female microsomes (table 6), but the difference in the NMRI strain was only 20%. In the microsomes from female CD1 the immunoreactive protein expression was higher than in the CD1 males, although the expression was low in both. The amino acid identity between rat

Table 5. Mouse strain activities (pmol min⁻¹ mg⁻¹ microsomal protein) for human cytochrome P450 specific probe substrates.

Substrates ^a	CBA		129/SvJ		NMRI		CD1		C57bl/6		Average
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Phenacetin	169	103	142	78	109	124	54	42	161	96	108
Coumarin	14	67	111	307	14	13	50	42	42	19	68
7-EFC	303	387	403	253	253	317	257	191	222	249	284
Diclofenac	8	23	42	52	56	59	19	63	40	40	40
Bufuralol	554	871	525	661	544	607	292	409	298	597	536
Dextromethorphan	293	613	355	558	364	336	253	356	235	515	388
Chlorzoxazone	706	2046	1731	1144	1246	1353	754	533	890	478	1088
<i>p</i> -Nitrophenol	561	1055	1113	1080	864	808	406	617	870	670	804
Testosterone	463	706	428	674	632	662	223	297	381	363	483
Lauric acid	550	780	1135	1260	398	480	420	355	810	780	697
Amiodarone	94	144	129	138	169	161	71	111	103	103	122
Paclitaxel	1210	940	940	1640	2400	900	2520	1010	1040	900	1350
<i>S</i> -Mephenytoin	72	96	97	89	65	74	55	57	80	69	75

^aSubstrate used in activity measurements. For incubation conditions and activities measured, see table 3.

Data are calculated rates from the linear part of time curves run with each substrate.

CYP1A2 and mouse CYP1A2 is considered to be 93% (<http://www.ncbi.nlm.nih.gov/UniGene>, accessed 1 October 2003). Therefore, not surprisingly, a high correlation ($R=0.96$, $p<0.001$) was found between immunoreactivity in the blots and the phenacetin activity (figure 4), which suggests that the polyclonal antibody against rat CYP1A2 could be specifically detecting mouse CYP1A2 microsomes from all strains.

Phenacetin *O*-deethylation also showed correlation (table 7 and figure 4) with the blots with human CYP4A11 antibodies ($R=0.62$, $p<0.05$). However, this correlation is possibly due to co-correlations ($R=0.60$, $p=0.06$) between CYP1A2 and CYP4A11 at the protein level.

Antibodies against rat CYP1A1 could not detect any protein in mouse liver microsomes, probably due to low expression in the liver (Shimada *et al.* 2003) as observed in other species. In spite of the low expression of CYP1A1, mRNA for CYP1A1 has been found in mouse liver (Choudhary *et al.* 2003).

Coumarin 7-hydroxylation

Coumarin was most efficiently metabolized in the 129/SvJ strain and to a lesser extent in the NMRI and C57bl/6 strains (table 5). In the CBA and 129 strains, the females metabolized coumarin twice as fast as the males while the opposite was observed in the C57bl/6 strain, where the males metabolized coumarin faster than the females. Mouse CYP2A5 has previously been found to exhibit a high coumarin 7-hydroxylase activity (Lavery *et al.* 1999). No significant correlations between human CYP2A6 Western blot densities (table 7) and coumarin activity was found ($R=0.24$) indicating that the bands detected might not represent only CYP2A5. The CYP2A6 antibodies are thus not suitable for measuring expression of CYP2A5. No other proteins detected correlated with coumarin activity (table 7).

Two distinct bands were detected with human CYP2A6 antibodies (figure 2), but only the upper band was detected at a molecular weight in the expected P450 range. The CD1 and NMRI strains showed low expression of the protein

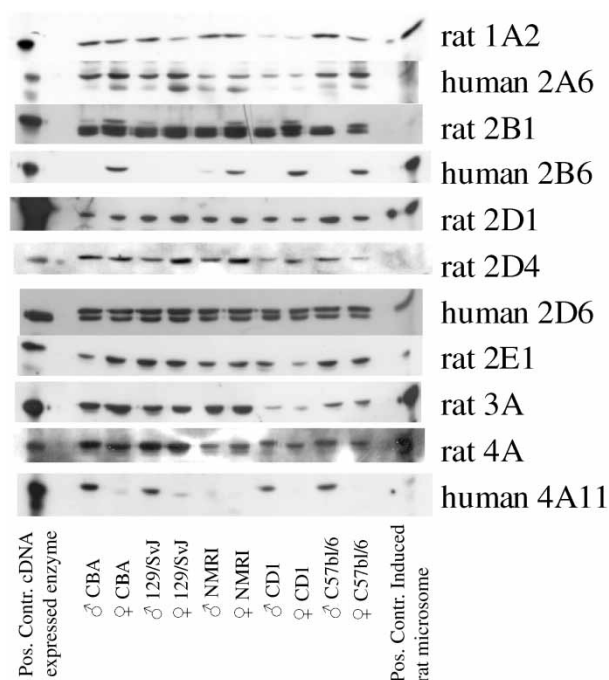


Figure 2. Strips show western blot results from gels run with 5 µg/well (except with CYP1A2, where 10 µg/well was used) of mouse liver microsomes. The gels were run and blotted as described in Materials and methods. Strips are labelled on their right-hand side with the antibody used. Two positive controls were included on each gel: cDNA-expressed human or rat CYP and induced rat liver microsomes; as specified in table 2.

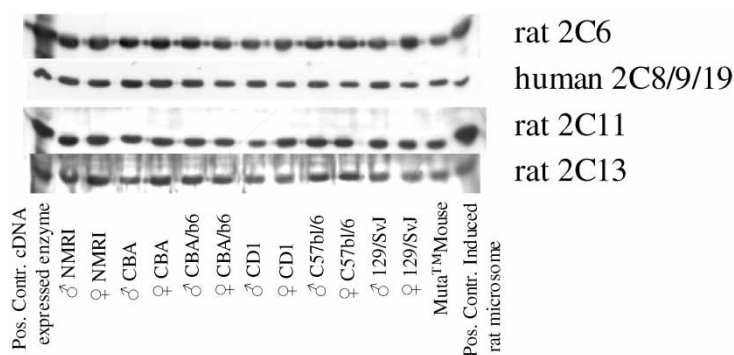


Figure 3. Strips shows western blot results for the CYP2C family from gels run with 2.5 µg/well (except with CYP2C8/9/19 antibodies, where 5 µg/well was used) of mouse liver microsomes. The gels were run and blotted as described in Materials and methods. The strips are labelled on their right-hand side with the antibody used. Two positive controls were included on each gel: cDNA-expressed human or rat CYP and induced rat liver microsomes, as specified in table 2.

compared with the other strains, and the females showed higher expression in all strains except CD1, where the males had higher expression.

7-Ethoxy-4-trifluoromethyl coumarin deethylation

No systematic strain or gender differences could be detected for 7-EFC deethylation (table 5). When comparing the activities with 7-EFC and Western

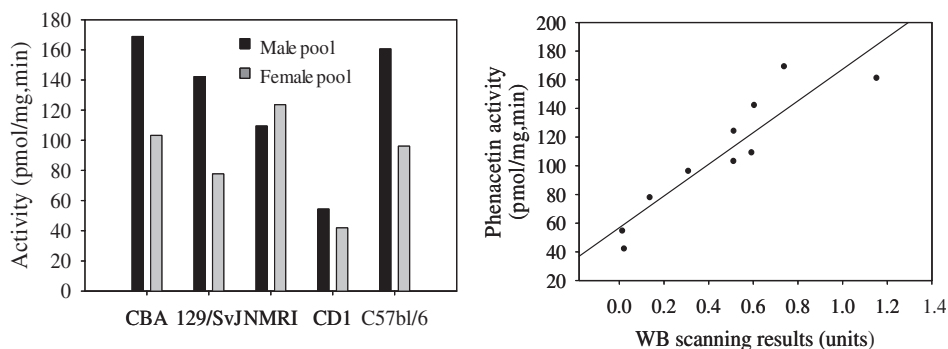


Figure 4. Phenacetin activities (table 7) in the different strains/genders (left). Males had generally higher phenacetin activity than the corresponding females (except for the NMRI strain). Incubation conditions were as described in the Material and methods and in table 3. Phenacetin activity correlated well ($R_s=0.96$, $p<0.001$) with band intensities in Western blots with antibodies against rat CYP1A2 (right). Western blots were performed and the scanning results (table 6) were obtained by densitometric measurements as described in Material and methods.

blots (table 6), no correlations were found with any of the CYP2B bands. Therefore, 7-EFC does not appear to be a good probe for any of the proteins detected on the CYP2B Western blots. Interestingly, the protein detected as the lower band on the CYP2D6 blot was the protein that showed the best correlation (table 7) with 7-EFC activity ($R=0.77$, $p<0.01$), possibly indicating the involvement of mouse protein detected by this antibody in the metabolism of 7-EFC. The upper band on the blots with rat CYP3A antibodies also showed correlation (table 7) with 7-EFC deethylation ($R=0.66$, $p<0.05$). The involvement of this protein in the metabolism of 7-EFC cannot be excluded, although the correlation between the bands detected as the lower band on the human CYP2D6 blot and the upper band on the rat CYP3A blot was not significant ($R=0.53$, $p=0.11$).

Since 7-EFC deethylation has been used as a marker for human CYP2B6 two separate gels were run and blotted with antibodies against members of the CYP2B subfamily (figure 2). One membrane was incubated with antibodies against rat CYP2B1 and the other against human CYP2B6 (cross-reacting with rat CYP2B1/2). For the positive controls (rat CYP2B1 and human CYP2B6 respectively) only one band per gel could be detected. Striking differences were detected between the two membranes. On the blot with rat CYP2B1 antibodies, three separate bands were detected in lanes with mouse microsomes. The two lower bands had higher intensity than the upper band. The upper band showed high expression in the CBA strain and no expression in the C57bl/6 strain. For the NMRI, CBA and CD1 strains the difference between the genders was more than twofold with the females having the higher expression. The two lower bands did not show any systematic strain or gender differences. The membranes with human CYP2B6 antibodies showed a marked gender-specific pattern (figure 2). The males expressed almost no immunoreactive proteins in any strain. In the 129/SvJ strain there was no detectable expression in any gender. CYP2B9 is known to be down-regulated in male mice from the age of 3–4 weeks to reach a zero level at the age of approximately 13 weeks (Jarukamjorn *et al.* 2002). Since only a weak band could be seen for male NMRI mice (age 4–5 weeks) on the gel with antibodies against

Table 6. Western blot band intensities^a.

	Band detected by antibodies against the following CYPs ^b																		
Mouse strain (gender)	r1A2	h2A6 ^c	r2B1 ^c	r2B1 ^d	h2B6	r2C6 ^d	h2C8/9/19	r2C11	r2C13	r2D1	r2D4	h2D6 ^c	h2D6 ^d	r2E1 ^c	r3A ^c	r3A ^d	r4A ^c	r4A ^d	h4A11
CBA (male)	0.74	2.01	0.22	4.31	0.03	3.36	4.36	4.36	4.36	2.10	3.54	3.43	2.61	1.25	3.80	0.33	2.66	0.00	4.87
CBA (female)	0.51	2.72	1.00	5.92	1.89	3.54	5.02	5.02	5.02	1.91	5.19	2.71	2.09	3.08	5.04	0.60	0.00	2.80	0.58
129/SvJ (male)	0.61	1.48	0.32	4.71	−0.01	2.23	2.95	2.95	2.95	2.71	2.28	3.66	2.95	3.63	2.67	0.41	3.76	0.00	3.54
129/SvJ (female)	0.14	2.49	0.29	6.67	−0.02	3.05	1.85	1.85	1.85	3.57	3.96	4.13	2.91	2.85	3.07	0.00	0.00	3.97	0.68
NMRI (male)	0.59	0.56	0.12	5.10	0.06	3.81	3.94	3.94	3.94	2.13	2.16	2.81	1.86	1.30	3.60	0.27	0.84	0.00	0.40
NMRI (female)	0.51	0.82	0.63	7.16	1.00	3.31	3.76	3.76	3.76	2.49	3.09	2.91	2.14	1.47	4.02	0.14	0.00	1.79	0.08
CD1 (male)	0.02	0.38	0.18	4.64	−0.01	2.20	2.82	2.82	2.82	1.60	0.11	1.80	1.96	1.02	0.27	−0.01	1.31	0.00	2.59
CD1 (female)	0.02	0.23	0.79	5.71	1.99	2.26	2.16	2.16	2.16	0.88	0.23	2.10	1.54	0.10	−0.05	0.15	0.00	1.49	0.04
C57bl/6 (male)	1.15	1.64	−0.04	4.13	−0.01	2.42	2.95	2.95	2.95	3.65	1.51	2.59	1.84	1.88	0.87	0.05	2.11	0.00	3.51
C57bl/6 (female)	0.31	2.56	0.00	2.93	1.48	2.39	2.06	2.06	2.06	1.77	0.23	2.36	1.37	1.54	0.81	0.17	0.00	1.66	0.08
C57bl/CBA (male)	−	−	−	−	−	3.27	3.936	3.856	2.706	−	−	−	−	−	−	−	−	−	−
C57bl/CBA (female)	−	−	−	−	−	2.452	2.663	3.01	2.235	−	−	−	−	−	−	−	−	−	−
Muta TM Mouse (male)	−	−	−	−	−	1.836	2.13	3.009	1.336	−	−	−	−	−	−	−	−	−	−

^aValues were obtained by scanning optical densities from hyperfilm ECL as described in the Material and methods.

^bAntibodies used as specified in table 2.

^cUpper band on Western blot.

^dLower band on Western blot.

Table 7. Correlations according to Spearman rank between Western blot band intensities and activities measured.

Substrate ^b	Band detected by antibodies against the following CYPs ^a																	
	r1A2	h2A6 ^c	r2B1 ^c	r2B1 ^d	h2B6	r2C6 ^d	h2C8/ 9/19	r2C11	r2C13	r2D1	r2D4	h2D6 ^c	h2D6 ^d	r2E1 ^c	r3A ^c	r3A ^d	r4A ^e	h4A11
Phenacetin	0.96***	0.27	-0.25	-0.27	-0.25	0.38	0.61	0.38	0.39	0.61	0.39	0.50	0.37	0.33	0.53	0.41	0.33	(0.62*)
Coumarin	-0.26	0.24	0.24	0.13	-0.43	-0.41	-0.38	-0.68	-0.50	0.15	0.17	0.15	0.35	0.54	-0.21	-0.06	0.57	0.30
7-EFC	0.26	0.24	0.46	0.32	-0.14	0.16	0.56	-0.19	0.23	0.17	0.57	0.51	0.77**	0.47	0.66*	0.50	0.45	0.42
Diclofenac	-0.26	-0.46	0.29	0.57	0.26	0.02	-0.30	0.36	-0.02	0.11	-0.08	0.11	-0.13	-0.09	-0.12	-0.15	-0.19	-0.68
Bufuralol	-0.02	0.71*	0.43	0.52	0.24	(0.62*)	0.20	0.18	0.53	0.15	0.80**	0.53	0.32	0.47	(0.73*)	0.37	0.50	-0.23
Dextro-methorphan	-0.35	0.49	0.41	0.42	0.36	0.37	-0.09	-0.05	0.14	-0.15	0.44	0.22	0.02	0.38	0.29	0.39	0.25	-0.46
Chlorzoxazone	0.31	0.16	0.46	0.59	-0.14	0.37	0.53	0.13	0.43	0.48	(0.63*)	0.50	0.60	0.65*	0.69*	0.37	0.42	0.18
p-nitrophenol	0.34	0.42	0.19	0.33	-0.30	0.19	0.02	0.08	0.15	(0.71*)	0.56	(0.62*)	0.46	0.90***	0.36	0.30	(0.67*)	0.17
Testosterone	0.28	0.54	0.41	0.64*	-0.02	(0.78**)	0.46	0.22	0.61	0.48	0.94***	0.71*	0.54	0.55	0.90***	0.37	0.55	0.04
Lauric acid	0.35	(0.71*)	-0.12	-0.09	-0.58	-0.07	-0.21	-0.18	0.00	(0.69*)	0.47	0.58	0.49	0.86***	0.20	0.05	0.86***	0.50
Amiodarone	0.07	0.04	0.40	0.73*	0.23	0.61	0.29	0.46	0.52	0.32	0.51	0.44	0.20	0.35	0.58	0.30	0.08	-0.43
Paclitaxel	-0.10	-0.36	-0.33	-0.08	-0.53	0.02	-0.05	-0.49	-0.29	0.01	-0.16	-0.05	0.09	-0.38	-0.20	-0.40	-0.25	0.38
S-mephenytoin	0.49	(0.62*)	0.30	0.25	-0.28	0.20	0.22	0.04	0.21	(0.69*)	(0.74*)	(0.70*)	(0.62*)	0.93***	0.54	0.42	0.89***	0.40

^aAntibodies used as specified in table 2.^bSubstrate used in activity measurements. For incubation conditions and activities measured, see table 3.^cUpper band on Western blot.^dLower band on Western blot.^eMost intense band on Western blot, with apparent different molecular weight between males and females.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For statistically significant correlations (R_s), values within parentheses refer to a co-correlation existing between the protein with the highest correlation coefficient and the protein detected by the antibody mentioned in the header of the column (see the Statistical methods).

human CYP2B6, and no bands were detected for males of other strains (all ≥ 8 weeks), the protein detected may well be mouse CYP2B9.

Diclofenac 4'-hydroxylation

When measuring diclofenac metabolism, the females generally had higher activity than the corresponding males, except for the C57bl/6 strain where the males and females had similar activities (table 5). The difference between males and females was less than twofold for the NMRI and 129/SvJ strains. The CBA strain had lower diclofenac metabolism than the other strains. Diclofenac metabolism did not correlate (table 7) with any immunoreactivities measured (table 6).

On the four gels run with antibodies against members of the CYP2C family only minor differences were detected between the strains (figure 3). When comparing the band intensities on the membranes with rat CYP2C13 antibodies, the CD1 had low expression compared with the other strains. On the blots with antibodies against rat CYP2C11, the female pool from the CD1 strain had twofold higher expression than the male pool.

Amiodarone disappearance

There were no evident strain or gender differences in amiodarone metabolism (table 5). The only significant correlation between amiodarone and a protein detected by Western blot (table 7) was with the lower band CYP2B1 immunoreactivity (table 6) ($R=0.73$, $p<0.05$). Even though amiodarone and paclitaxel both are substrates for human CYP2C8, no correlation could be found ($R=-0.23$) between the disappearances of these substrates. However, amiodarone metabolism correlated with diclofenac 4'-hydroxylation ($R=0.65$, $p<0.05$), which could possibly imply that the same enzyme is involved in the metabolism of both these substrates.

Paclitaxel disappearance

Paclitaxel was metabolized at a higher rate in males than in females, except for the 129/SvJ strain, where the female activity was almost twice as high as that in males (table 5). However, the C57bl/6 males had less than 25% higher metabolic rate of the females. Paclitaxel disappearance did not correlate (table 7) with any activities or immunoreactivities measured (table 6).

S-mephenytoin disappearance

No gender or strain differences were observed in *S*-mephenytoin metabolism (table 5). The highest correlation (table 7) with this activity was with CYP2E1 ($R=0.93$, $p<0.001$) and CYP4A ($R=0.89$, $p<0.001$) immunoreactivities (table 6). *S*-mephenytoin hydroxylation was the activity with the highest correlation to CYP2E1 immunoreactivity, which suggests that *S*-mephenytoin might be a good substrate for the mouse enzyme detected by rat CYP2E1 (see the discussion under *p*-nitrophenol 2-hydroxylation). The involvement also of the protein detected by rat CYP4A antibodies in the metabolism of *S*-mephenytoin cannot be excluded.

None of the bands detected with any of the CYP2C antibodies correlated with *S*-mephenytoin activity, which suggests that at least mouse CYP2C enzymes are not solely responsible for the metabolism of this substrate, or that the rat and human CYP2C antibodies used are not good tools for detecting mouse CYP2C members.

Bands on blots with human CYP2A6 antibodies, rat CYP2D1 antibodies, rat CYP2D4 antibodies and human CYP2D6 antibodies (both bands) showed correlation (table 7) with *S*-mephenytoin metabolism ($R=0.62, 0.69, 0.74, 0.70$ and 0.62 , all $p>0.05$). However, these correlations could just as well be explained by co-correlation between these proteins and the protein detected by rat CYP2E1 (with CYP2A6; $R=0.67, p<0.05$, CYP2D1; $R=0.64, p<0.05$, CYP2D4; $R=0.56, p=0.08$, upper CYP2D6; $R=0.55, p=0.09$ and lower CYP2D6; $R=0.46, p=0.17$) and/or rat CYP4A antibodies (CYP2A6; $0.65, p<0.05$, CYP2D1; $R=0.56, p=0.08$, CYP2D4; $R=0.74, p<0.05$, upper CYP2D6; $R=0.72, p<0.05$ and lower CYP2D6; $R=0.71, p<0.05$).

Bufuralol 1'-hydroxylation

Females had considerably higher bufuralol 1'-hydroxylase activity than males in all strains (table 5), but for the NMRI strain the difference was only 10%. Bufuralol activity showed correlation (table 7) with the band intensities on the rat CYP2D4 blot ($R=0.80, p<0.01$) (table 6). The bands detected with antibodies against rat CYP2D4 showed low protein expression in the CD1 and C57bl/6 strains, while the CBA strain showed high expression. The females had higher expression than the corresponding males for all strains except C57bl/6 (figure 2). For the C57bl/6 strain the males showed more than sixfold higher expression than the females.

The protein detected as the upper band with human CYP2D6 antibodies correlated (table 7) with the band detected by rat CYP2D4 antibodies ($R=0.78, p<0.01$) and the band detected by rat CYP2D1 antibodies ($R=0.70, p<0.05$), and might be identical. However, the correlation between the upper band detected by rat CYP2D1 antibodies and the bands detected by rat CYP2D4 was low and not significant ($R=0.46, p=0.17$).

The Western blot bands detected with antibodies against rat CYP2D1 gel showed low expression in the CD1 strain, and the male C57bl/6 showed twofold higher expression than the females.

Bufuralol 1'-hydroxylation also correlated significantly (table 7) with the bands detected with human CYP2A6 antibodies ($R=0.71, p<0.05$). The involvement of the protein detected with human CYP2A6 antibodies in the metabolism of bufuralol could not be excluded, although the correlation between the bands detected with human CYP2A6 antibodies and the bands detected by rat CYP2D4 was only 0.60 ($p=0.06$).

The blots with rat CYP2C6 antibodies and the upper band on the blots with rat CYP3A antibodies showed correlation (table 7) with bufuralol 1'-hydroxylation ($R=0.62, p<0.05$ and $R=0.73, p<0.05$). However, this correlation may possibly be due to significant co-correlations with CYP2D4 ($R=0.67, p<0.05$ and $R=0.87, p<0.001$, respectively) at the protein level.

Dextromethorphan O-deethylation

Females had higher dextromethorphan activity than males from the same strain, except for the NMRI strain where the genders had similar activities (table 5). Dextromethorphan metabolism did not show significant correlation (table 7) with any proteins detected on Western blots (table 6), but correlated with bufuralol 1'-hydroxylation ($R=0.75, p<0.05$).

Chlorzoxazone 6-hydroxylation

In chlorzoxazone 6-hydroxylation the female CBA had twofold higher metabolism than the males (table 5). Chlorzoxazone correlated with the NADPH oxidation ($R=0.86$, $p<0.001$), as did Western blot bands detected with rat CYP2E1 antibodies (table 6) ($R=0.65$, $p<0.05$). It is known that human and rat CYP2E1 show a higher rate of NADPH oxidation nmol^{-1} cytochrome P450 than most other isoforms (Gergel *et al.* 1997) which here also appears to be the case in mice. The CYP2E subfamily is considered one of the most conserved CYP-subfamilies with only one member in most species (including mouse). Differences were nevertheless found between strains and genders on the anti-rat CYP2E1 blot (figure 2); the CD1 strain showed lower expression than the other strains and the females expressed only about one-tenth as much protein as the males. The 129/SvJ strain showed higher expression than the other strains and female CBA had more than twofold higher expression than male CBA.

The highest correlation coefficient between hydroxylation of chlorzoxazone and any immunoreactivity (table 6) was with the protein detected as the upper band on the CYP3A gel ($R=0.69$, $p<0.05$) thus indicating the involvement of this mouse protein in chlorzoxazone metabolism. Involvement of members of the CYP3A subfamily in the metabolism of chlorzoxazone has also been shown in humans (Gorski *et al.* 1997).

The blots produced with rat CYP2D4 antibodies also showed correlation with chlorzoxazone 6-hydroxylation ($R=0.63$, $p<0.05$). However, this correlation could be explained by co-correlation between CYP2D4 and the upper band on the CYP3A gel ($R=0.87$, $p<0.001$).

p-Nitrophenol 2-hydroxylation

p-Nitrophenol and chlorzoxazone activities (table 5) both showed significant correlations (table 7) with the bands on the gels blotted with rat CYP2E1 antibodies (table 6) ($R=0.90$, $p<0.001$ and $R=0.65$, $p<0.05$, respectively). The bands on the Western blot with rat CYP2E1 antibodies thus most likely corresponded to mouse CYP2E1. The higher correlation for *p*-nitrophenol with rat CYP2E1 indicates that this reaction is more specific than chlorzoxazone 6-hydroxylation. This has also been suggested for human CYP2E1 (Tassaneeyakul *et al.* 1993). *p*-Nitrophenol, as well as chlorzoxazone, correlated with the NADPH oxidation ($R=0.69$, $p<0.05$).

The upper band on the blots with human CYP2D6 antibodies showed correlation (table 7) with *p*-nitrophenol ($R=0.62$, $p<0.05$). Co-correlation between human CYP2D6 and the upper band on the gel with CYP2E1 ($R=0.55$ but $p=0.09$) could possibly explain this correlation.

The blots with rat CYP4A antibodies and on blots with rat CYP2D1 also showed correlation (table 7) with the metabolism of *p*-nitrophenol ($R=0.67$, $p<0.05$ and $R=0.71$, $p<0.05$, respectively). However, the correlations between these proteins and *p*-nitrophenol may also be due to co-correlations with CYP2E1 immunoreactivity ($R=0.76$, $p<0.01$ and $R=0.64$, $p<0.05$, respectively).

Testosterone 6 β -hydroxylation

On immunoblots using rat CYP3A antibodies at least two bands were detected (figure 2) with the upper band showing the highest intensity (table 6). The CD1 and C57bl/6 strains showed low intensity of the upper and lower band

compared with the other strains and the CBA strain showed higher intensity on both bands compared with the other strains. The lower band did not show any significant correlation (table 7) with any of the activities measured (table 5), and the female 129/SvJ and male C57bl/6 and CD1 did not express this protein. Female CD1 showed no expression of the upper band detected by rat CYP3A antibodies.

Testosterone 6 β -hydroxylation showed high correlation ($R=0.90$, $p<0.001$) with the upper CYP3A Western blot band (table 7). It is possible that this testosterone activity is generally lower in male mice than in female mice. All strains had higher testosterone 6 β -hydroxylase activities in females than in corresponding males, with the exception of the C57bl/6 strain where the activities were approximately the same in both genders. However, the difference in activity between male and female NMRI mice was only 5%. Both genders of the CD1 strain showed lower expression of the upper CYP3A band as well as lower testosterone 6 β -hydroxylase activities than the other strains.

CYP3A11 is highly expressed in the liver, and is known to catalyse testosterone 6 β -hydroxylation (Yanagimoto *et al.* 1992). The upper band observed immunologically on the CYP3A blot in the present study might thus possibly be CYP3A11.

The bands on blots with rat CYP2D4 antibodies and the upper band on the CYP2D6 blots also showed high correlation (table 7) with testosterone 6 β -hydroxylation ($R=0.94$, $p<0.001$ and $R=0.71$, $p<0.05$, respectively) without any co-correlation at the protein level. This suggests that these proteins may also be capable of forming 6 β -hydroxytestosterone.

A correlation (table 7) was also found between the lower band on the rat CYP2B1 membranes and testosterone 6 β -hydroxylation ($R=0.64$, $p<0.05$). An involvement of this protein in testosterone 6 β -hydroxylation cannot be excluded.

Also, the bands on the blots with rat CYP2C6 antibodies showed correlation with testosterone 6 β -hydroxylation ($R=0.78$, $p<0.01$). However, this correlation may possibly be due to significant co-correlations with CYP2D4 ($R=0.67$, $p<0.05$) and CYP3A ($R=0.82$, $p<0.01$) at the protein level.

Lauric acid 12-hydroxylation

Two separate gels were run and blotted with antibodies against members of the CYP4A family (figure 2). One membrane was incubated with antibodies against rat CYP4A and the other with antibodies against human CYP4A11. Striking differences were detected between the two membranes. On the blot produced with rat CYP4A antibodies at least two bands were found, with the lowest band weaker than the others. Male and female samples generated upper bands with slightly different apparent molecular weights. On the blot produced with human CYP4A11 antibodies only one band was detected, and the males had markedly higher band intensities than the females. The NMRI strain showed lower band intensities (table 6) than the other strains. The male dominant band on the Western blot produced with rat CYP4A antibodies is probably the same band that was detected on the human CYP4A11 blot ($R=0.85$, $p<0.001$).

One human marker activity was used to detect CYP4A activity, namely lauric acid 12-hydroxylation. No systematic strain or gender differences were seen in this activity (table 5). The activity showed high correlation (figure 5) with the most intense band from each gender on the blot with the rat CYP4A antibody

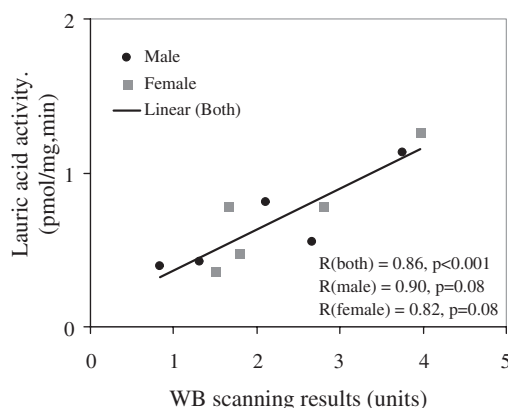


Figure 5. Bands detected by rat CYP4A antibodies in Western blots with microsomes from males and females had different apparent molecular weight (figure 2). However, if band intensities were plotted together regardless of apparent molecular weight, a good correlation to lauric acid activity was obtained. Western blots were performed and the scanning results (table 6) were obtained by densitometric measurements as described in Material and methods. Incubation conditions were as in table 3 and the activities are presented in table 7.

($R=0.86$, $p<0.001$), when disregarding the apparent difference in molecular weight (figure 2) between male and female bands. This indicates that both male and female proteins catalyse this reaction, and that there is possibly two related genes with gender-specific expression or that the expressed protein is processed differently in the two genders. When male and female pools were compared separately, the male and female correlation coefficients between upper CYP4A immunoreactivity and lauric acid 12-hydroxylation were $R=0.90$ ($p=0.08$) and $R=0.82$ ($p=0.08$), respectively (figure 5). These correlations were not found to be significant due to the low number of samples. Both genders of the 129/SvJ strain showed higher expression of the upper CYP4A band than the other strains. This was true also for the lauric acid activity. The difference in expression between male and female NMRI was more than twofold (table 6), with the males showing the highest expression.

High correlation (table 7) was also found between the CYP2E1 immunoreactivity and lauric acid 12-hydroxylation ($R=0.86$, $p<0.001$). It is known that human CYP2E1 hydroxylates lauric acid, although mostly in the 11-position (Castle *et al.* 1995). However there is also a high co-correlation at the protein level between CYP2E1 and the bands detected with rat CYP4A antibodies ($R=0.76$, $p<0.01$). Since the correlation between lauric acid hydroxylation and the CYP2E1 and CYP4A enzymes are equally high, none of them can be excluded as mouse enzymes involved in lauric acid metabolism.

A correlation was also found between lauric acid activity and the immunoreactivity in the Western blot with rat CYP2D1 antibodies ($R=0.69$, $p<0.05$). This is possibly due to high co-correlation between the rat CYP2D1 and the proteins detected by rat CYP2E1 ($R=0.64$, $p<0.05$) and human CYP4A11 ($R=0.56$ but $p=0.08$) antibodies.

The bands detected with human CYP2A6 antibodies showed correlations with lauric acid 12-hydroxylation ($R=0.71$, $p<0.05$) as well. However, the CYP2A6 immunoreactivity correlation with lauric acid hydroxylation is probably due to significant co-correlations between the bands detected by human CYP2A6

antibodies and bands detected by antibodies against CYP2E1 or CYP4A ($R=0.67$, $p<0.05$ and $R=0.65$, $p<0.05$, respectively) at the protein level.

Literature comparisons

All activities measured were compared with data obtained from other studies (Bogaards *et al.* 2000, Mankowski *et al.* 2000, Sapone *et al.* 2003) (table 8).

Several activities (diclofenac 4'-hydroxylation, bufuralol 1'-hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone 6-hydroxylation and *p*-nitrophenol *O*-hydroxylation) showed good agreement (differed less than twofold) with the data reported in the literature (table 8) when recalculated as in the Materials and methods. For testosterone 6 β -hydroxylation, the data reported in this study agreed with one of the previous studies (Mankowski *et al.* 2000), whereas another study reported higher data (Bogaards *et al.* 2000). The difference in metabolic rates between the studies might depend on mouse strain differences. For 7-EFC deethylation the data in this study was at the lower limit for good agreement compared with Bogaards *et al.* (2000). For coumarin 7-hydroxylation, the activities reported in this study were high compared with data reported in other studies (Bogaards *et al.* 2000), but this is also the one activity displaying the largest differences in activity between strains within this study. For example, the 129/SvJ strain had almost 10-fold higher metabolism than the other strains.

For phenacetin *O*-deethylation, the activities reported in this study are three-fold higher than the data reported in the literature (Mankowski *et al.* 2000). This could possibly be explained by the low K_m values reported in the literature (which were also used here to recalculate rates to be comparable), the large difference in concentrations used and the method used to recalculate rates for comparison. Amiodarone and paclitaxel activities were not possible to compare with literature data since only the disappearance of the parent compound was measured in this study, and not the amiodarone *N*-deethylation and paclitaxel 6 α -hydroxylation. Lauric acid 12-hydroxylation was not possible to compare with literature data since no K_m value was found for mice microsomes. For *S*-mephenytoin the data presented in this study is the disappearance of *S*-mephenytoin, but 4'-hydroxylation was also measured (data not shown) and these values were within the same range as reported (Bogaards *et al.* 2000, Mankowski *et al.* 2000).

Since human probe substrates were used, mouse activities found in the present study were also compared with human data reported in the literature (Rahman *et al.* 1994, Zerilli *et al.* 1997, Bogaards *et al.* 2000, Mankowski *et al.* 2000, Ohyama *et al.* 2000). Relative to literature data, the mouse liver microsomes had higher rates of metabolism than human liver microsomes for the following substrates: bufuralol 1'-hydroxylation (4.5–22.3-fold), and dextromethorphan *O*-demethylation (2.2-fold) while human liver microsomes had higher rates for the following substrates: coumarin 7-hydroxylation (2.7-fold), diclofenac 4'-hydroxylation (7.7–31.3-fold) and testosterone 6 β -hydroxylation (6.8–16.0-fold).

Conclusion

The present study indicates that there are gender differences in CYP-dependent metabolic rates and CYP expression in mice. The gender differences in some cases might be strain specific. In general, males had higher expression of the proteins detected by human CYP4A11 antibodies. They also had higher

Table 8. Activity measurements in mouse and human liver microsomes. Comparison between data from the present study and literature data.

Substrate ^a	Recalculated average rates ^b (pmol mg ⁻¹ min ⁻¹)	Concentration used (μM)	$V_{\max}/2$ mouse ^b (pmol mg ⁻¹ min ⁻¹)	K_m mouse ^b (μM)	$V_{\max}/2$ human ^b (pmol mg ⁻¹ min ⁻¹)	K_m human ^b (μM)
Phenacetin	431	1	141 ^c	4 ^c	480 ^c	30 ^c
Coumarin	152	4	25 ^d	9 ^d	408 ^d	3 ^d
7-EFC	142	10	294 ^d	4 ^d	112 ^d	3 ^d
Diclofenac	112	5	72 ^d	14 ^d	857 ^d , 3500 ^c	7 ^d , 10 ^c
Bufuralol	268 ^{e,f}	5	505 ^d , 517 ^c	1 ^d , 3 ^c	60 ^d , 12 ^c	13 ^d , 5 ^c
Dextromethorphan	481	5	447 ^c	6 ^c	220 ^c	4 ^c
Chlorzoxazone	1986	40	1032 ^d	73 ^d	1023 ^d	120 ^d
<i>p</i> -Nitrophenol	804 ^g	100	695 ^h		1190 ⁱ	40 ⁱ
Testosterone	528 ^e , 827 ^f	80	1513 ^d , 870 ^c	88 ^d , 137 ^c	3576 ^d , 13200 ^c	80 ^d , 65 ^c
Lauric acid	697 ^g	50	189 ^d		726 ^d	
Amiodarone	122 ^g	1			250 ^j	32 ^j
Paclitaxel	1350 ^g	10			435 ^k	4 ^k
<i>S</i> -mephenytoin	75 ^l	10	< 1 ^d , 21 ^c	103 ^c	7 ^d , 100 ^c	40 ^c

^aSubstrate used in activity measurements. For incubation conditions and activities measured, see table 3.

^bAverage rates from the present study were recalculated as described in the Statistical methods.

^cMankowski *et al.* (2000) (C57bl/SJL mice or human).

^dBogaards *et al.* (2000); averages for male and female B6C3F1 mice and averages for male and female human pools.

^eRecalculated value to be compared with Bogaards *et al.* (2000).

^fRecalculated value to be compared with Mankowski *et al.* (2000).

^gValues not recalculated due to lack of mouse K_m values.

^hSapone *et al.* (2003).

ⁱZerilli *et al.* (1997).

^jOhyama *et al.* (2000).

^kRahman *et al.* (1994).

^lValue not recalculated due to a lack of K_m values for the disappearance of *S*-mephenytoin. Reported K_m values refer to *S*-mephenytoin 4'-hydroxylation.

metabolism than corresponding females for phenacetin *O*-deethylation, which was used as CYP1A2 marker activity. Females generally had a higher expression of the proteins detected by human CYP2A6 antibodies and human CYP2B6 antibodies, as well as by rat CYP2D4 antibodies, and higher metabolism of bufuralol 1'-hydroxylation and dextromethorphan *O*-demethylation, both used as markers for human CYP2D activity. The protein detected by human CYP2B6/rat CYP2B1/2 antibodies was female specific and may be mouse CYP2B9. This protein was not detectable in liver microsomes from the 129/SvJ strain, even in females.

Under the conditions of this study, the CD1 strain showed low activity and protein expression for several CYPs relative to the other strains. The CD1 strain had lower than average values for the following parameters: CYP1A2 (activity measured as phenacetin *O*-deethylation and protein in Western blot as detected by rat CYP1A2 antibodies), CYP2A (protein in Western blot as detected by human CYP2A6 antibodies), CYP2C (protein in Western blot as detected by rat CYP2C13 antibodies), CYP2D (protein in Western blot as detected by rat CYP2D1 and CYP2D4 antibodies), CYP2E1 (protein in Western blot as detected by rat CYP2E1 antibodies) and CYP3A (activity measured as testosterone 6 β -hydroxylation and protein in Western blot as detected by rat CYP3A antibodies). However, these mice were older (24 weeks) than all the other mice (4–12 weeks).

Mouse CYP1A2 was identified since it metabolized phenacetin, showed cross-reactivity with antibodies against rat CYP1A2, and had high correlation between the activity and the protein expression.

Mouse CYP2A5 probably does not cross-react specifically with any of the antibodies used since no correlation was found between coumarin 7-hydroxylation, which is considered a marker substrate for mouse CYP2A5, and any of the Western blot band intensities measured in this study.

Several mouse CYP2C enzymes exist with similar molecular weights and are thus difficult to separate and detect on Western blots, even though several CYP2C antibodies were used (antibodies directed against rat CYP2C6, rat CYP2C11, rat CYP2C13 and human CYP2C8/9/19 also cross-reacting with rat CYP2C12). Bands detected in Western blots with CYP2C antibodies did not correlate with any CYP2C marker activities.

A female-dominant mouse CYP detected with rat CYP2D4 antibodies correlated well with bufuralol 1'-hydroxylation.

A protein was identified as mouse CYP2E1 since it cross-reacted specifically with rat CYP2E1 antibodies and metabolized both *p*-nitrophenol and chlorzoxazone. The protein identified on the CYP2E1 blot correlated better with *p*-nitrophenol metabolism than with chlorzoxazone 6-hydroxylation, indicating that *p*-nitrophenol is a more specific substrate for mouse CYP2E1 under the conditions used.

Testosterone 6 β -hydroxylation correlated well with the upper band on the blot with antibodies against CYP3A, suggesting that this band may be CYP3A11.

Lauric acid showed high correlation with the intense bands on the blot with the rat CYP4A antibody, when disregarding the apparent difference in molecular weight between male and female bands, indicating that both genders have proteins capable of catalysing this enzymatic reaction.

The activities measured were generally in good agreement with mouse activities reported in the literature. When comparing with human activities, mouse liver microsomes were faster than human liver microsomes when measuring bufuralol

1'-hydroxylation and dextromethorphan O-demethylation and slower metabolism when measuring coumarin 7-hydroxylation, diclofenac 4'-hydroxylation, testosterone 6 β -hydroxylation and lauric acid 12-hydroxylation.

In general, it was difficult to identify the proteins detected on Western blots and mouse CYP enzymes responsible for the activities measured. To characterize further CYP-dependent metabolism in mice, better tools are needed, such as mouse marker substrates, antibodies specific for mouse CYPs and purified or cDNA-expressed enzymes. Identification of strain and gender differences in expression of specific mouse CYPs and their substrate specificity is urgently needed to understand better and fully use mouse models in pharmacological and toxicological research for extrapolation to man. This study indicates that gender differences may in some cases be strain specific.

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Identification of the novel canine CYP1A2 1117 C > T SNP causing protein deletion

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1. The pharmacokinetics of YM-64227 (4-cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]-pyrimidine-2-(1*H*)-one), a novel and selective phosphodiesterase type 4 inhibitor, was characterized in beagle dogs. Based on the plasma parent drug to major hydroxylated metabolite ratio, 21 dogs were phenotyped as 16 extensive metabolizers (EM) and five poor metabolizers (PM).

2. Nucleotide sequences of CYPs 1A2, 2B11, 2C21, 2D15, 2E1 and 3A12 were investigated in the EM and PM dogs. A CYP1A2 1117 C > T single nucleotide polymorphism was found, which resulted in an amino acid change from an Arg codon to a stop codon at position 373. All dogs phenotyped as PM were T/T homozygous, whereas EMs were C/C homozygous and C/T heterozygous.

3. In Western blotting of liver microsomes, CYP1A protein expression was detected in the C/C and C/T types, but not in the T/T type.

4. Of 65 dogs genotyped using genome DNA, the frequencies of the C and T alleles were 0.61 and 0.39, respectively, suggesting approximately 15% of the dogs would not express the CYP1A2 protein.

5. The findings provide a coherent explanation for the inter-individual variability in the pharmacokinetics of CYP1A2 substrate drugs in dogs.

Introduction

Cytochrome P450 (CYP) is a superfamily of enzymes that play an important role in the oxidative metabolism of a wide variety of xenobiotics as well as endogenous compounds (Nelson *et al.* 1996). Metabolic activities of CYP are affected by several factors such as inhibition by concomitant drugs, induction and genetic polymorphism (Pelkonen *et al.* 1998). In clinical settings, CYP polymorphism has been widely investigated, and it is well known that mutated alleles of CYP2C9, CYP2C19 and CYP2D6 cause increases in plasma drug concentrations that might cause unforeseeable side-effects (Kagimoto *et al.* 1990, Sullivan-Klose *et al.* 1996, Furuta *et al.* 1999).

A large number of CYP cDNA clones have been isolated, sequenced and extensively studied in humans as well as in rodents and rabbits (<http://drnelson.utmem.edu/CytochromeP450.html>). In contrast, the number of studies on canine CYP cDNAs is small, even though the dog is extensively used in pharmacology research as well as in drug safety assessment studies. At present, the canine CYP enzymes involved in xenobiotic metabolism that have been cloned and sequenced

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include CYP1A1, CYP1A2 (Uchida *et al.* 1990), CYP2B11 (Graves *et al.* 1990), CYP2C21, CYP2C41 (Uchida *et al.* 1990, Blaisdell *et al.* 1998), CYP2D15 (Sakamoto *et al.* 1995), CYP2E1 (Lankford *et al.* 2000), CYP3A12 and CYP3A26 (Ciaccio *et al.* 1991, Fraser *et al.* 1997). Although gene analysis has revealed that some of these CYPs have variants, no relevant variation in plasma concentration of a given drug in dogs has been reported as the result of reduced metabolic activities. Moreover, only a limited number of pharmacokinetic reports have made reference to polymorphism in dogs.

The polymorphic disposition of a drug in dogs was first reported in the pharmacokinetics of celecoxib, a cyclooxygenase 2 inhibitor (Paulson *et al.* 1999). There are at least two populations of dogs distinguished by the elimination rate of celecoxib from plasma. Celecoxib is metabolized primarily by CYP2D15 in dogs. However, the metabolic activity of CYP2D did not correlate with celecoxib metabolism, and other CYP subfamilies, in addition to CYP2D, were suggested to contribute to this polymorphism. In a pharmacokinetic study of GTS-21, a selective α 7-nicotinic acetylcholine receptor partial agonist, the individual variability in plasma concentration was examined in dogs (Azuma *et al.* 2002). The contents of CYP1A isoform(s) in the liver microsomes from the animals were consistent with the varying metabolite formation, suggesting involvement of CYP1A in the variable pharmacokinetics of GTS-21. As a second example, in conjunction with CYP1A, remarkable inter-individual variations of plasma concentration of unchanged drug and its hydroxy metabolite were observed in dogs after oral administration of AC-3933, a novel cognitive enhancer (Mise *et al.* 2004). The poor metabolizer (PM) dogs defined by the plasma metabolite/AC-3933 ratio lacked CYP1A2 protein, and AC-3933 metabolism in extensive metabolizer (EM) dogs was inhibited by CYP1A2 inhibitors and anti-CYP1A2 antibodies. Mise *et al.* concluded that the expression of CYP1A2 in dogs is polymorphic and causes the large variation in AC-3933 pharmacokinetics.

Following the above investigations, canine polymorphism with YM-64227, 4-cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidin-2(1*H*)-one, a novel phosphodiesterase type 4 inhibitor (Iwata *et al.* 1998), was found. The plasma concentration of the main metabolite MM-2 was much smaller in the PM-type dogs than in the EM-type dogs, and vice versa with unchanged YM-64227. This phenomenon triggered the present study on canine CYP polymorphisms. Polymorphic disposition could complicate the assessment of pharmacological effects and toxicity of drugs. Thus, it is considered important to clarify the cause of the variability. Clarification would provide valuable information for the selection of appropriate animals to evaluate results more accurately. The purpose of the present study is to elucidate the polymorphism in the CYPs-dependent metabolism of xenobiotics in dogs.

Materials and methods

Chemicals

YM-64227, its hydroxylated metabolite (MM-2) (figure 1) and the internal standard 4-(3-chlorophenyl)-1-ethyl-7-(1-hydroxyethyl)pyrido[2,3-d]pyrimidin-2 (1*H*)-one were synthesized in the Chemistry Laboratories of Yamanouchi Pharmaceutical Co., Ltd. Their chemical purities were more than 99%. High-performance liquid chromatography-grade acetonitrile, acetic acid and *tert*-butyl methyl ether were purchased from Kanto Chemical Co. (Tokyo, Japan). Purified water from a Milli-Q system (Millipore Co., Bedford, MA, USA) was used throughout the study. All other chemicals used were commercially available and of the highest purity.

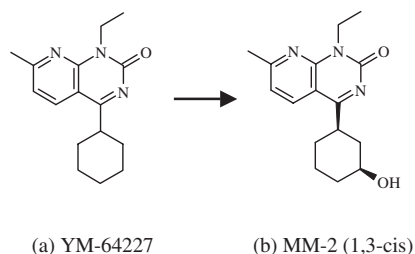


Figure 1. Chemical structures of (a) YM-64227 and (b) the major metabolite MM-2 (1,3-cis).

Animals

Male beagle dogs (body weight 8.9–13.0 kg) were supplied by Hazelton/LRE Research Products (Kalamazoo, MI, USA). They were kept in a climate-controlled room (temperature $23 \pm 1^\circ\text{C}$, relative humidity $55 \pm 1\%$) with a light/dark cycle of 13 h/11 h. They had free access to both pellet diet (MF; Oriental Yeast Co., Ltd, Tokyo, Japan) and water during the studies.

Phenotyping

YM-64227 suspended in 0.5% methylcellulose solution was administered orally at 0.3 mg kg^{-1} to dogs ($n = 21$) following an overnight fasting. Blood (about 5 ml) was collected from the forelimb vein using a heparinized syringe after 0.5 h. Concentrations of YM-64227 and MM-2 were quantified by high-performance liquid chromatography (Tenmizu *et al.* 2004). In brief, the plasma samples were extracted with *tert*-butyl methyl ether under alkali conditions. The analytes were separated on a phenyl ethyl column ($5 \mu\text{m}$, $250 \times 4.6 \text{ mm i.d.}$, Nacalai Tesque, Inc., Kyoto, Japan), operating at 40°C and using an acetonitrile–acetic acid gradient at a flow rate of 1.0 ml min^{-1} . The fluorescence signal was monitored at excitation and emission wavelengths of 330 and 400 nm, respectively. The validated quantitation range of the method was $0.4\text{--}200 \text{ ng ml}^{-1}$ for YM-64227 and MM-2 using 0.5 ml of the plasma sample. The EM and PM phenotypes were classified by use of metabolic ratio criteria of 0.0 (the $\log_{10}$ YM-64227/MM-2 concentrations ratio of the post-dose 0.5-h plasma). An individual with a metabolic ratio of < 0.0 was considered an EM-type dog and $0.0 \leq$ was a PM-type dog.

Canine CYP cDNA sequencing

Livers were obtained from dogs characterized as EM or PM in the phenotyping study. Total RNA for reverse transcriptase polymerase chain reaction (RT-PCR) was extracted from the liver by RNeasy Mini kit (Qiagen, Tokyo, Japan). A PCR-based strategy of CYPs1A2, 2B11, 2C21, 2D15, 2E1 and 3A12 was used as primer sets designed on the basis of reported sequence (Graves *et al.* 1990, Uchida *et al.* 1990, Ciaccio *et al.* 1991, Sakamoto *et al.* 1995, Lankford *et al.* 2000). The PCR reaction was performed using KOD-plus- (Toyobo, Tokyo, Japan). PCR conditions, performed on a GeneAmp PCR System 9700, consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles of: 94°C for 15 s, 55°C for 30 s and 68°C for 1.5 min. Sequencing was done on an ABI Prism 377 automated sequencer (Perkin-Elmer Corporation, Foster City, CA, USA).

Determination of the canine CYP1A2 SNP position number

Although canine CYP1A2 had been cloned by Uchida *et al.* (1990), the CYP1A2 cDNA reported was not complete and lacked the translational initiation codon. This missing region of the translational initiation codon was determined by 5'-rapid amplification of cDNA ends (5'RACE). The GeneRacer kit (Invitrogen, Tokyo, Japan) provides a method to obtain 5' ends of cDNA using known cDNA sequence from expressed sequence tags subtracted cDNA. The sequence primers for 5'RACE are shown in table 1. Sequencing was done on an ABI Prism 377 automated sequencer.

Genotyping

An allele-specific primer PCR (ASP-PCR) and a direct sequence analysis using genomic DNA were developed for single nucleotide polymorphism (SNP) analysis. Genomic DNA was extracted from 100 μl whole blood obtained from 65 dogs with a GenTLE Kun kit (TaKaRa) according to the manufacturer's directions.

The sequences of primers for ASP-PCR are shown in table 1. This analysis was applied for wild and mutant allele-specific primers, which were designed to distinguish the SNP position. PCR conditions consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles of: 94°C for 15 s, 59°C for 30 s and 68°C for 30 s. Other PCR conditions were the same as described above for canine CYP cDNA sequencing.

Table 1. Primer sets used to study the canine CYP1A2 cDNA and gene.

	Forward primer sequence	Position		Reverse primer sequence	Position
Gene specific primer sets for 5'RACE					
GeneRacer 5' primer	5'-CGACTGGAGCACGAGGACACTGA-3'	–	GSP 3' primer	5'-GGACTCTTCAGGCCTTTGGGAAGC-3'	–
RT-PCR primer sets for direct sequence of canine CYP1A2 cDNA					
CYP1A2-S01	5'-CAGCCTCCACAATCCTACTG-3'	–51–32	CYP1A2-A01	5'-AGGCAATGGAGAAGGTGTTG-3'	439–420
CYP1A2-S02	5'-CCTCCACCATCTTCTGCTTG-3'	38–57	CYP1A2-A02	5'-ATGTCCCTGGACACTGCGCTC-3'	836–817
CYP1A2-S03	5'-TCCCCCTCCTAATGAGCTCC-3'	638–657	CYP1A2-A03	5'-GAGGCCATGGGTGATCCCTC-3'	1589–1570
nested-PCR primer for direct sequence of canine CYP1A2 cDNA					
			CYP1A2-A04	5'-TGGACTCTTCAGGCCTTTGG-3'	116–97
			CYP1A2-A05	5'-CAATGACATTGGCCACTGAC-3'	589–570
CYP1A2-S04	5'-CCCATTCTCCAATATATGCC-3'	706–725			
PCR primer sets for ASP-PCR					
ASP-PCR_Wild	5'-TTCATCCTGGAGATCTTCCG-3'		ASP-PCR_reverse	5'-AATTGGAGGTTGACCGCATC-3'	
ASP-PCR_Mutant	5'-TTCATCCTGGAGATCTTCTG-3'				
PCR primer sets for direct sequence of canine CYP1A2 1117 C>T SNP					
CYP1A2DS-S01	5'-CTGGAGTCTTATGTACCT-3'		CYP1A2DS-A01	5'-AATTGGAGGTTGACCGCATC-3'	
nested PCR primer sets for direct sequence of canine CYP1A2 1117 C>T SNP					
CYP1A2DS-S02	5'-CCCGGTTTGGTCTTCTGTGT-3'		CYP1A2DS-A02	5'-TGACGTGGCCAGAAGTGAGA-3'	

The sequences of primers for direct sequence analysis are shown in table 1. This analysis was applied for direct observation of nucleotide sequence waveform. PCR conditions consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles of: 94°C for 15 s, 56°C for 30 s and 68°C for 30 s. Other PCR conditions were the same as described above. Sequencing was done on an ABI Prism 377 automated sequencer.

Western blotting

Liver tissue were minced and homogenized in 3 vols 1.15% KCl on ice. The homogenates were centrifuged at 9000 *g* for 20 min. The supernatants were transferred to a centrifuge tube and centrifuged at 105 000 *g* for 1 h. The pellet was resuspended in 100 mM Na,K-phosphate buffer and quickly frozen in liquid nitrogen. The canine liver microsomes were kept at -80°C until use. Protein concentrations in canine liver microsomes were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

For immunodetection of CYP1A2 enzyme in each individual canine liver sample, 1.0 µg microsomal protein was resolved by sodium dodecylsulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Clear Blot membrane-P: Atto, Tokyo, Japan). The membrane was incubated sequentially with an anti-rat CYP1A1 antibody (Daiichi Pure Chemicals, Tokyo, Japan), which has a cross-reactivity for canine CYP1A (Mise *et al.* 2004), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Cappel, OH, USA). CYP1A expression was detected by chemiluminescence using an ECL Advance Western Blotting Detection kit (Amersham Biosciences, Piscataway, NS, USA).

Accession numbers

The GeneBank accession number for the full-length canine CYP1A2 cDNA is AB159771; that for the part of canine CYP1A2 gene is AB159772.

Results

Phenotyping

After oral administration of YM-64227 at 0.3 mg kg⁻¹, beagle dogs were categorized as EM type with high plasma concentrations of metabolite MM-2 and as PM type with low plasma concentrations of MM-2. The metabolic ratios differentiated the EM type (*n* = 16) from the PM type (*n* = 5) (figure 2).

Canine CYP cDNA sequencing

The major canine CYP enzymes CYPs1A2, 2B11, 2C21, 2D15, 2E1 and 3A12 were cloned from canine liver cDNA by RT-PCR. In canine CYP1A2, 22 SNPs were determined. Among SNPs found in the present study, the most remarkable

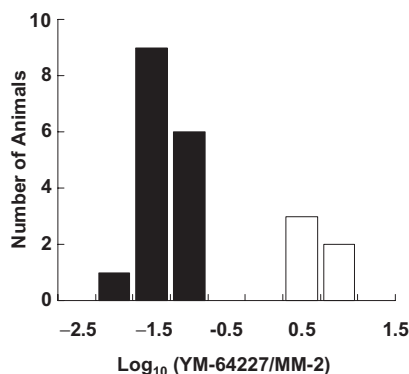


Figure 2. Histogram of post-dose 0.5-h plasma YM-64227/MM-2 concentration ratios after oral administration of YM-64227 at 0.3 mg kg⁻¹ to beagle dogs. Based on the plasma YM-64227/MM-2 concentration ratio, 21 dogs were phenotyped as EM (*n* = 16) or PM (*n* = 5).

was the change from an Arg codon to a stop codon, resulting in a premature protein. In other canine CYPs investigated, CYP2B11, 2C21, 2D15, 2E1 and 3A12, no remarkable SNPs that might cause the deficit of enzymatic functions, such as a premature stop codon, a whole gene deletion or a frame shift (data not shown).

Determination of the canine CYP1A2 SNP position number

The CYP1A2 SNP position number could not be assigned because the nucleotide sequence of canine CYP1A2 reported was not complete and lacked the translational initiation codon (Uchida *et al.* 1990). To identify the complete canine CYP1A2 cDNA nucleotide sequence, a part of the novel 5' region nucleotide was determined by the 5'RACE method. Based on the full length of the nucleotide sequence, the 22 SNPs of CYP1A2 were assigned as 58 G > A (Val₂₀ Ile), 171 C > T (Pro₅₇ Pro), 255 T > C (Ser₈₅ Ser), 330 C > G (Phe₁₁₀ Leu), 337 G > A, 339 G > T (Val₁₁₃ Ile), 355 C > A (Leu₁₁₉ Met), 358 A > T (Thr₁₂₀ Ser), 500 C > T (Ala₁₆₇ Val), 522 G > A (Glu₁₇₄ Glu), 568 A > C (Met₁₉₀ Leu), 682 A > T, 683 A > C (Asn₂₂₈ Ser), 694 G > T (Val₂₃₂ Leu), 764 C > T (Thr₂₅₅ Met), 768 C > T (Phe₂₅₆ Phe), 872 C > T (Ser₂₉₁ Phe), 1117 C > T (Arg₃₇₃ Stop), 1173 C > G (Thr₃₉₁ Thr), 1243 C > G (Gln₄₁₅ Glu), 1299 C > T (Gly₄₃₃ Gly) and 1303 G > A (Ala₄₃₅ Thr). Based on haplotype analysis, alleles were designated as CYP1A2*1A to *4B (table 2). Haplotypes could be defined when homozygous at a single polymorphic site or when multiple sites were identified (Tirona *et al.* 2001). Additionally, when homozygous for a SNP at one site were found to be heterozygous for another SNP at a different site, an allelic assignment was possible. Furthermore, the presence of SNPs in the liver-derived full-length cDNAs provided unequivocal verification of haplotype. Among them, CYPs1A2*3A, *4A, and *4B were the alleles that had 1117 C > T SNP. The originally deposited CYP1A2 sequence is designated CYP1A2*1A. It was suggested that SNP position numbers 58–768 in deduced exon 1 configure the haplotype block. Each CYP1A2 allele was largely categorized by the exon 1 haplotype block and 1117 C > T SNP.

Genotyping

Although mammalian cross-species genome structures are generally different, the length of the internal exon encoded proteins is the same (Salavaggione *et al.* 2002). Based on the similarity of the mammalian CYP1A subfamily, the 1117 C > T SNP is predicted to be located in exon 4 of canine CYP1A2. Accordingly, the most appropriate PCR forward primer was designed for exon 3 and reverse primer for deduced exon 5. An amplified PCR fragment nucleotide sequence was determined by direct sequence analysis. Splice junctions were dictated according to the cDNA nucleotide sequence and the GT-AG rule. Thus, the CYP1A2 genome nucleotide sequence was determined around the SNP of interest.

Allele-specific primer and direct sequence primer sets were designed such that individual dogs could be readily genotyped for the 1117 C > T SNP from genomic DNA samples. Figure 3(A) shows an agarose gel electrophoresis photograph of ASP-PCR; figure 3(B) shows electropherograms of direct sequence. Both methods clearly distinguished between 1117 C and 1117 T. Genotyping is summarized in table 3. C/C-type dogs comprised 25 animals, C/T-type dogs comprised 29 animals and T/T-type dogs comprised 11 animals. The allele frequency of the

Table 2. Schematic of canine CYP1A2 allele variants.

Allele designation	Deduced exon 1 region																2	4	Exon 5		Exon 6	
	58	171	255	330	337	339	355	358	500	522	568	682	683	694	764	768	872	<u>1117</u>	1173	1243	1299	1303
CYP1A2*1A	A	T	C	G	A	T	A	T	C	A	C	T	C	T	C	T	C	C	C	C	C	A
CYP1A2*2A	G	C	T	C	G	G	C	A	C	G	A	A	A	G	C	C	C	C	C	C	C	G
CYP1A2*2B	G	C	T	C	G	G	C	A	C	G	A	A	A	G	C	C	C	C	C	G	T	A
CYP1A2*2C	G	C	T	C	G	G	C	A	C	G	A	A	A	G	C	C	C	C	C	G	C	A
CYP1A2*2D	G	C	T	C	G	G	C	A	C	G	A	A	A	G	C	C	C	C	C	C	T	G
CYP1A2*2E	G	C	T	C	G	G	C	A	T	G	C	A	A	G	T	C	T	C	G	C	T	G
CYP1A2*3A	A	T	C	G	A	T	A	T	C	A	C	T	C	T	C	T	C	T	C	C	C	G
CYP1A2*4A	G	C	T	C	G	G	C	A	C	G	A	A	A	G	C	C	C	T	C	C	C	A
CYP1A2*4B	G	C	T	C	G	G	C	A	T	G	C	A	A	G	T	C	T	T	G	C	T	G

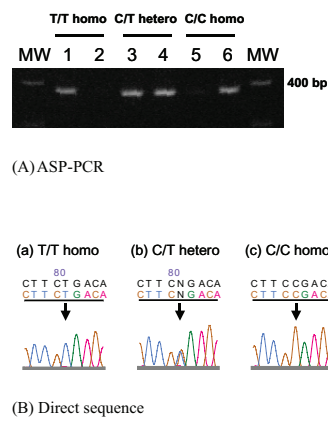


Figure 3. (A) Establishment of ASP-PCR. Lanes 2, 4 and 6 were used by the ASP-PCR_wild-type primer; lanes 1, 3 and 5 were used by the ASP-PCR_mutant-type primer. Lane MW expressed the DNA molecular weight size marker. (B) Electropherograms for the 1117 C>T SNP. Arrows indicate the SNP positions. In the electropherograms, A, C, G and T are coloured red, yellow, green and blue, respectively.

Table 3. Genotype frequency of canine CYP1A2 1117 C>T SNP for the 65 beagle dogs.

Genotyping (CYP1A2 1117 C>T SNP)			
Genotype	C/C	C/T	T/T
Frequency	0.38	0.45	0.17
Number of animals (<i>n</i> = 65)	(25)	(29)	(11)

wild- and mutant-types was 0.61 and 0.39, respectively, in 65 dogs. Of 21 dogs phenotyped, all PM-type dogs (*n* = 5) were genotyped as T/T type, and the EM-type dogs (*n* = 16) were genotyped as C/C (*n* = 10) and C/T (*n* = 6) type.

Western blotting

CYP1A expression in canine liver microsomes of each genotyped group was monitored by Western blot (figure 4). An approximately 50-kDa band was detected in C/C- and C/T-type groups. However, CYP1A proteins in the T/T-type microsomes were undetectable.

Discussion

The current study sequenced major canine CYP cDNAs from liver and 22 novel SNPs were discovered in CYP1A2. Canine CYP1A2 cDNA reported in the literature was not complete and lacked the translational initiation codon (Uchida *et al.* 1990). Hence, the full-length CYP1A2 cDNA nucleotide sequence was determined, and subsequently these SNP regions were numbered. Given the large number of polymorphisms observed in the current study and the likelihood that additional polymorphisms will be found, standardized names for CYP1A2 alleles are proposed based on the recommendations of Nebert (2002). Hence, the first published sequence for CYP1A2 is termed *1A (Uchida *et al.* 1990).

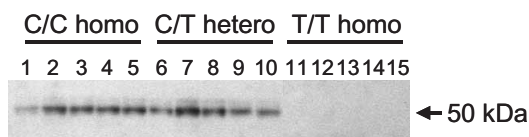


Figure 4. Western blot analysis of CYP1A. All the protein samples derived from each genotyped microsome were resolved by sodium dodecylsulphate-polyacrylamide gel electrophoresis. Equal amounts of protein (1.0 µg) were loaded in each lane. Immunoblots were performed with anti-rat CYP1A1 and developed with the ECL detection reagent. An approximately 50-kDa band was expressed as canine CYP1A protein, except for T/T-type microsomes.

The additional haplotypes were designated CYP1A2*2A to *4B (table 2). Among them, the CYPs1A2*3A, *4A and *4B alleles had the 1117 C>T SNP causing a stop codon, the most influential SNP.

In the Western blotting study, CYP1A protein expression was detected in C/C- and C/T-type dog liver microsomes. The antibody used in this study was for rat CYP1A1, and the specificity for canine CYP1A1 and CYP1A2 is unclear. However, the detected protein is considered as canine CYP1A2, since only CYP1A2 is constitutively expressed in dog liver (Uchida *et al.* 1990). In contrast, the liver microsomes of the T/T type did not yield any immunoreactive bands in Western blotting. Based on the predicted amino acid sequence, the premature protein is less than 75% of its normal size, and may not possess the recognition site for the CYP1A antibody. Meanwhile, the CYP family amino acid sequence, deduced from the full-length cDNA, has a hydrophobic amino terminus as well as the conserved putative haem-binding domain (Graham-Lorence and Peterson 1996) (amino acid position 430–443). As the 1117 C>T mutation changes to a stop codon, the premature CYP1A2 protein may not express this haem-binding domain. These assumptions were consistent with the facts that in all five PM-type dogs classified during the phenotyping with YM-64227, homozygous 1117 C>T SNP was found and CYP1A protein was not detected by Western blotting. In-house data show that the formation of MM-2, the major metabolite of YM-64227, is mediated by CYP1A2. Therefore, the 1117 C>T SNP has probably caused a lack of mature CYP1A2 expression, resulting in a significant decrease in the metabolism of YM-64227. Subsequently, blood samples were analysed from 65 beagle dogs to estimate the SNP frequency using the direct sequence method. The frequencies of the T alleles were 0.39, and the T/T-type frequency by the Hardy–Weinberg equilibrium was calculated as approximately 0.15. This value implies that about 15% of the beagle dogs would not express CYP1A2 protein. When the same blood samples were analysed by the newly developed ASP-PCR method, the same result was obtained. The ASP-PCR method is more timesaving and favourable for the routine analyses of a large number of samples.

Human CYP1A2 plays a major role in the metabolism of many commonly used drugs in clinical practice, such as clozapine, imipramine, phenacetin, theophylline and tacrine (Bertz and Granneman 1997), and of some neurotoxins (Coleman *et al.* 1996). Furthermore, CYP1A2 activates several aromatic amines and thus is a key enzyme in chemical carcinogenesis (Gooderham *et al.* 1996). A number of human CYP1A2 gene alleles have been identified (<http://www.imm.ki.se/CYPalleles/cyp1a2.htm>), among which CYP1A2*7 and CYP1A2*11 are known to cause a decrease in the metabolic clearance of CYP1A2. CYP1A2*7 contains a point mutation in the donor splice site of intron 6 (3534 G>A), which leads to

a truncated non-functional enzyme (Allorge *et al.* 2003). CYP1A2*11 consisted of an amino acid change from Phe to Leu at position 186, causing a significant reduction in metabolic activities of CYP1A2 (Murayama *et al.* 2004). However, these SNPs may not be clinically significant because their frequencies are very low: only one heterozygote was found in 100 (CYP1A2*7) and 206 (CYP1A2*11) subjects. Point mutations in the 5'-flanking region (CYP1A2*1C, Nakajima *et al.* 1999) and intron 1 (CYP1A2*1K, Aklillu *et al.* 2003) were identified to cause expression level and reduced inducibility of CYP1A2.

No remarkable SNP, which causes decreased metabolic activity, has been found in dogs until very recently. However, reduced metabolic activity for AC-3933 in a group of dogs lacking CYP1A2 protein (Mise *et al.* 2004) strongly suggested that the dog is polymorphic in the metabolic elimination of some drugs by CYP1A2, and the fairly good correlation between canine CYP1A contents and GTS-21 metabolic activities (Azuma *et al.* 2002) supported this assertion. The present paper described that the canine CYP1A2 1117 C>T SNP causes a truncated non-functional enzyme, and approximately 15% of the beagle dogs would not express CYP1A2 protein. In the study with AC-3933, two of 20 dogs were characterized as PM dogs (Mise *et al.* 2004). This ratio is not greatly different from the figure predicted from the C and T allele frequency, and five of 21 dogs were categorized as PM dogs in this study with YM-64227. Therefore, it is highly possible that canine CYP1A2 1117 C>T SNP is the cause of polymorphism of at least three in the four drugs described in the Introduction, i.e. GTS-21, AC-3933 and YM-64227. Theoretically, hepatic clearance-type drugs primarily eliminated by canine CYP1A2 can be susceptible to this polymorphism. Various drugs and environmental chemicals are substrates for human CYP1A2, as described above. Substrate specificities of canine CYP1A2 have not been studied extensively (Shou *et al.* 2003). However, it can be assumed that some of them would be metabolized by canine CYP1A2 as well, and a considerable number of chemicals being under development or synthesized as drug candidates may be metabolized by this CYP enzyme. Plasma concentrations reflect the pharmacological effects and govern toxicities of the drugs of interest. Therefore, genotyping of dogs for CYP1A2 polymorphism by a timesaving method provides a great advantage in evaluating a drug candidate in pharmacological research or safety assessment studies.

In conclusion, 22 novel SNPs were discovered in canine CYP1A2. Among them, CYP1A2 1117 C>T SNP results in an amino acid change from an Arg codon to a stop codon at position 373. Subsequent reduction in the metabolic activity was reflected in the pharmacokinetics of YM-64227. Based on allele frequency analysis, approximately 15% of the beagle dogs would not express CYP1A2 protein. The findings are valuable when considering the inter-individual variability in the pharmacokinetics of several CYP1A2 substrate drugs in dogs.

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Genetic polymorphisms of cytochrome P450 2C9 causing reduced phenprocoumon (*S*)-7-hydroxylation *in vitro* and *in vivo*

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1. The effect of cytochrome P450 (CYP) 2C9 polymorphisms on the stereoselective biotransformation of the oral anticoagulant phenprocoumon (PPC) to inactive, mono-hydroxylated metabolites was studied *in vitro* and *in vivo*.

2. In human liver microsomes, the (*S*)-7-hydroxylation — being the major metabolic pathway — was significantly compromised in a gene–dose-dependent manner in samples expressing the CYP2C9*2 or CYP2C9*3 allele. The CYP2C9*3/*3 genotype corresponded to an almost fourfold lower (*S*)-7-hydroxylation rate than CYP2C9*1/*1 (wild-type).

3. The intrinsic clearance of human recombinant CYP2C9*2 and CYP2C9*3 for the (*S*)-7-hydroxylation was 28.9 and 50.9% lower than of CYP2C9*1, respectively.

4. The area under the plasma concentration–time curve (AUC) of PPC metabolites after oral intake of 12 mg racemic PPC was significantly lower in volunteers expressing the CYP2C9*2 or CYP2C9*3 allele. Increasing plasma AUC metabolic ratios (parent compound/metabolite) in CYP2C9*2 and CYP2C9*3 variant allele carriers were found for each hydroxylation reaction and the CYP2C9*3/*3 genotype corresponded to an about 10-fold higher metabolic ratio of PPC (*S*)-7-hydroxylation relative to CYP2C9*1/*1.

5. CYP2C9 polymorphisms cause a markedly compromised PPC (*S*)-7-hydroxylation. However, PPC metabolism appears overall less influenced by CYP2C9 genotype compared with other oral anticoagulants and it may thus be a valuable alternative for therapeutic anticoagulation of patients expressing CYP2C9 variant alleles.

Introduction

The oral anticoagulant phenprocoumon (PPC) is extensively used for the prevention of thromboembolic disorders in many European countries (Hirsh *et al.* 1998). Chemically, it belongs to the group of 4-hydroxycoumarins (figure 1), including warfarin, acenocoumarol and dicoumarol that all elicit their anticoagulant effect by inhibition of vitamin K epoxide reductase (De Boer-van den Berg *et al.* 1986). Due to their narrow therapeutic index, the anticoagulant response needs to be closely monitored by regular determination of the international normalized ratio (INR) throughout treatment (Hirsh *et al.* 1998). Yet, large

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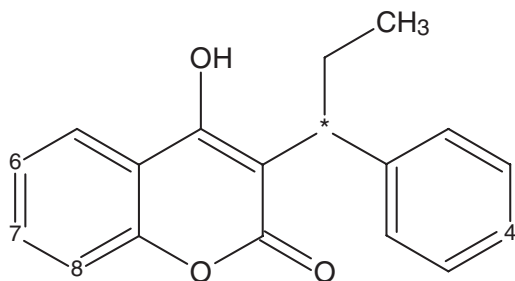


Figure 1. Chemical structure of phenprocoumon. The four different hydroxylation positions are indicated by their respective numbers and the chiral centre is also marked (*).

interindividual differences of the dose–response relationship often give rise to bleeding complications or insufficient anticoagulation (Levine *et al.* 2001). These differences are mainly based on variation in the activity and expression of drug-metabolizing cytochrome-P450 (CYP) enzymes in the liver (Hirsh *et al.* 1998). Thus, it is important to improve the knowledge about genetic factors influencing the biotransformation of anticoagulants to inactive metabolites.

Oral anticoagulants are administered as racemic mixtures of their (*S*)- and (*R*)-enantiomers that are almost completely absorbed from the gastrointestinal tract and highly protein-bound in plasma (De Vries 1990). The hydroxylation of warfarin is catalysed in a stereoselective manner by hepatic CYP enzymes. Its (*S*)-enantiomer is primarily hydroxylated in the 6- and 7-position by CYP2C9, whereas (*R*)-warfarin hydroxylation is catalysed by CYP1A2 (6- and 8-hydroxywarfarin), CYP2C19 (6- and 8-hydroxywarfarin), and CYP3A4 (10-hydroxywarfarin) (Wienkers *et al.* 1996, Zhang *et al.* 1997, Kaminsky and Zhang 1997). CYP2C9 is also the principal enzyme catalysing the hydroxylation of both acenocoumarol enantiomers mainly in the 6- and 7-position (Thijssen *et al.* 2000a). We have recently identified CYP2C9 and CYP3A4 as major enzymes catalysing the hydroxylation of (*S*)- and (*R*)-PPC *in vitro* (Ufer *et al.* 2004b).

Of the CYP2C9 alleles, CYP2C9*2 and CYP2C9*3 have been shown to cause a clinically relevant reduction in enzyme activity leading to a lower dose requirement and an increased risk of overanticoagulation by warfarin and acenocoumarol (Aithal *et al.* 1999, Thijssen *et al.* 2000b, 2001, Taube *et al.* 2000, Visser *et al.* 2004). The CYP2C9*2 variant differs from CYP2C9*1 (wild-type) by a C₄₃₀ → T substitution in exon 3 resulting in an amino acid exchange of arginine by cysteine at position 144 (Stubbins *et al.* 1996, Sullivan-Klose *et al.* 1996). CYP2C9*3 is characterized by a A₁₀₇₅ → C in exon 7 giving rise to the replacement of isoleucine by leucine at position 359 (Stubbins *et al.* 1996, Sullivan-Klose *et al.* 1996, Lee *et al.* 2002). In Caucasians, the variant allele frequency has been reported to range from 11 to 12.5% (CYP2C9*2) and 7.5 to 8.5% (CYP2C9*3), respectively (Stubbins *et al.* 1996, Yasar *et al.* 1999).

In the clinical situation, a higher risk of bleeding complications on PPC long-term treatment has been described for patients being heterozygous for the CYP2C9*3 allele (Hummers-Pradier *et al.* 2003). In contrast, no significant difference of (*S*)- and (*R*)-PPC pharmacokinetics in healthy volunteers was found when comparing CYP2C9*1/*1 wild-type subjects with hetero- or homozygous carriers of the CYP2C9*2 or CYP2C9*3 alleles (Kirchheiner *et al.* 2004). Consistent with these volunteer data, therapeutic anticoagulation was not

influenced by CYP2C9 genotype in incident users of PPC, whereas a significant effect of CYP2C9 genotype on dose requirement and anticoagulant response of acenocoumarol was found (Visser *et al.* 2004).

Given this controversial background, we have investigated the impact of CYP2C9 polymorphisms on the hydroxylation of (*S*)- and (*R*)-PPC *in vitro* and *in vivo*. For the *in vitro* studies, we used cDNA-expressed, human recombinant CYP2C9*1, CYP2C9*2 and CYP2C9*3 enzymes as well as pooled microsomes from human livers with different CYP2C9 genotypes. The *in vivo* study was performed in healthy volunteers expressing all six allele combinations of CYP2C9*1, CYP2C9*2 and CYP2C9*3 (Kirchheiner *et al.* 2004).

Materials and methods

(*S*)- and (*R*)-PPC (>99% chemical and enantiomer purity) were kindly provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland). Synthesized 4', 6- and 7-hydroxy-PPC were a generous gift of Professor W. F. Trager (Seattle, WA, USA) (Pohl *et al.* 1975). Deuterium-labelled 7-hydroxywarfarin used as internal standard was purchased from Ultrafine Chemicals (Manchester, UK) and dissolved in dimethylsulfoxide. All other stock solutions were prepared in pure methanol and stored at -20°C in the dark until use. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Sigma Chemicals (St Louis, MO, USA). Acetonitrile, methanol, dimethylsulfoxide, ammonia, ammonium acetate, glacial acetic acid, sodium acetate, formic acid, *ortho*-phosphoric acid and potassium phosphate were from Merck (Darmstadt, Germany). Chemicals were of the purest grade available and solvents of high-performance liquid chromatography (HPLC) grade. Purified water was prepared using a Millipore Milli-Q purification system (Bedford, MA, USA).

Human liver microsomes were isolated from organ donors (eight male, six female; age range 22–75 years) of a human liver bank (approved by the Ethics Committee at Karolinska Institutet) and stored in potassium phosphate buffer (50 mM, pH 7.4) at -80°C until use (Von Bahr *et al.* 1980). The concentrations of liver microsomal protein, cytochrome *b*₅, total CYP and NADPH-cytochrome c reductase as well as the NADPH oxidation activity were assessed by spectrophotometric measurements (Lowry *et al.* 1951, Omura and Sato 1964, Yasukochi and Masters 1976). Immunoblotting of CYP2C9 apoprotein levels was performed using CYP2C9 specific patient sera that were kindly provided by Professor P. Beaune (Paris, France) (Towbin *et al.* 1979, Yasar *et al.* 2001). DNA was extracted from human liver tissue using QIAamp Tissue DNA preparation kit (Quiagen, Hilden, Germany) and genotyped for the CYP2C9*1, CYP2C9*2 and CYP2C9*3 allele using endonucleases *Ava*II, *Nsi*I and *Kpn*I as described (Yasar *et al.* 1999). Human recombinant CYP2C9*1, CYP2C9*2 and CYP2C9*3 were isolated from microsomes of baculovirus-infected insect cells co-expressing NADPH-CYP reductase (Gentest, Woburn, MA, USA).

In vitro assay using human liver microsomes and recombinant enzymes

Kinetic parameters of (*S*)- and (*R*)-PPC hydroxylation in microsomes from human liver specimens genotyped CYP2C9*1/*1 have previously been reported and the substrate concentration of 5 μM used in the present study was selected accordingly (see the Results) (Ufer *et al.* 2004b). Pooled microsomes were prepared from individual human liver specimens with the following CYP2C9 genotypes: CYP2C9*1/*1 ($n=5$), CYP2C9*1/*2 ($n=2$), CYP2C9*2/*2 ($n=2$), CYP2C9*1/*3 ($n=2$), CYP2C9*2/*3 ($n=2$), and CYP2C9*3/*3 ($n=1$). (*S*)- and (*R*)-PPC dissolved in methanol was separately added to the incubation tubes and evaporated to dryness. Each 500- μl standard incubation mixture containing 50 mM potassium phosphate buffer (pH 7.4) and liver microsomes (0.5 mg protein mL^{-1}) was pre-incubated at 37°C for 5 min in a shaking water bath. Incubations were initiated by addition of NADPH (1 mM) and performed for 40 min. Each reaction was stopped by addition of 0.5 M phosphoric acid (20 μl) and immediate freezing on dry ice.

Kinetic experiments with human recombinant CYP2C9*1, CYP2C9*2 and CYP2C9*3 enzymes were essentially carried out as described for human liver microsomes. Incubations were performed for 30 min in a total volume of 250 μl using 5 pmol CYP2C9 enzyme per incubation. Kinetic parameters were determined from reactions at 9 different substrate concentrations ranging from 0.5 to 250 μM .

Linearity of metabolite formation with respect to time and protein concentration was confirmed as described (Ufer *et al.* 2004b). Incubations were performed in duplicate and PPC metabolites quantified by HPLC-mass spectrometry (MS) (Ufer *et al.* 2004a, b).

In vivo sample collection and genotyping of study subjects

Twenty-three healthy volunteers (20 male, three female; age range 20–66 years) gave their written informed consent and constituted six different CYP2C9 genotype groups: 2C9*1/*1 ($n=4$), 2C9*1/

*2 ($n=4$), 2C9*2/*2 ($n=3$), 2C9*1/*3 ($n=5$), 2C9*2/*3 ($n=4$), and 2C9*3/*3 ($n=3$) (Kirchheiner *et al.* 2004). DNA was extracted by using the MagNA Pure LCTM Total Nucleic Acid Isolation kit (Roche Diagnostics, Mannheim, Germany) (Kessler *et al.* 2001) and CYP2C9 genotyping was performed as described (Aynacioglu *et al.* 1999, Kirchheiner *et al.* 2004). Each volunteer received a single, oral dose of 12 mg racemic PPC (Marcumar[®], Roche, Grenzach-Wyhlen, Germany). Plasma concentrations of PPC metabolites were determined at 0, 4, 9, 24, 48, 96 and 144 h after drug intake.

Quantification of racemic PPC metabolites in human plasma by HPLC-MS

The quantification of 4'-, 6- and 7-hydroxy-PPC has been subject to a separate communication (Ufer *et al.* 2004a). Briefly, each plasma sample was centrifuged (17 500 *g*, 10 min) and the supernatant purified by solid-phase extraction (SPE) using Sep Pak C18 light cartridges supplied by Waters (Eschborn, Germany). After conditioning (1 ml methanol) and equilibration (1 ml water), 1 ml of the supernatant containing deuterium-labelled 7-hydroxy-warfarin as internal standard was passed through the cartridges followed by washing with 500 μ l acetonitrile (20%, pH 3.0). Samples were eluted with acetonitrile (40%, pH 10.0), vacuum centrifuged and 25 μ l of the eluate were injected into the HPLC system.

The HPLC-MS system was equipped with Chemstation software consisting of a reversed-phase Luna C18 100 $\times$ 2.0 mm i.d. column with 3 μ m particle size (Phenomenex, Torrance, CA, USA) and a single-quadrupole mass spectrometer (Hewlett Packard 1100, Agilent Technologies, Avondale, PA, USA). This was equipped with an atmospheric pressure-electrospray ionization (AP-ESI) source used in the negative ionization mode with the following spray chamber settings: nebulizer pressure, 20 psi; capillary voltage, 2000 V; fragmentor voltage, 70 V; drying gas temperature, 350°C; drying gas flow rate, 101 min⁻¹. Selected ion monitoring (SIM) was chosen and deprotonated molecular ions were monitored at m/z 295 (PPC metabolites) and m/z 328 (internal standard). Chromatographic separation was achieved with an eluent A (2% acetonitrile) and B (70% acetonitrile) in 25 mM formic acid. A step gradient was run with 55% (0–5 min), 100% (5–7 min) and 55% (7–11 min) of eluent B. Intra- and interday coefficients of variation were <10% and the limit of detection was 1 nM.

Stereoselective quantification of (S)-/(R)-PPC and its monohydroxylated metabolites in human plasma by HPLC-MS/MS

(S)- and (R)-PPC were quantified by HPLC-MS/MS as described (Kammerer *et al.* 2004). The stereoselective quantification 4'-, 6- and 7-hydroxy-PPC has been subject to a separate communication (Kammerer *et al.*, unpublished data). Briefly, each plasma sample was adjusted to pH 3.0 and centrifuged (19 800 *g*, 5 min). The supernatant was purified by SPE using Sep Pak C18 cartridges supplied by Waters. After conditioning (2 ml methanol) and equilibration (2 ml 1% methanol, pH 2.8), 1 ml of the supernatant was passed through the cartridges followed by washing with 3 ml methanol (1%, pH 2.8). After drying under full vacuum for 10 min, the analytes were eluted with 1 ml methanol and the eluate was evaporated to dryness at 40°C for 2 h in a SpeedVac vacuum concentrator (Thermo Life Sciences, Holbrook, NJ, USA). The residue was reconstituted in 5 mM ammonium acetate buffer/methanol (pH 3.9) and 20 μ l were injected into the HPLC system.

For the stereoselective determination of PPC metabolites, a two-step achiral–chiral analytical procedure was used (Kammerer *et al.*, unpublished data). The initial achiral separation of PPC metabolites was achieved on a Surveyor HPLC-system consisting of a vacuum-degasser, a quaternary pump, a thermostated autosampler and a C-18 column (Grom Sil 120ODS-3CP, 150 $\times$ 2 mm, 5 μ m, 10 $\times$ 1 mm precolumn). The achirally separated metabolites were parked on Chira Grom 2 columns. Each of these racemic metabolites was individually transferred on a chiral Chira-Grom-2 column (250 $\times$ 2 mm, 8 μ m) connected to a Spectra Series P100 isocratic pump. This was achieved by using double-headed Rheodyne motor switch valves (Besta Technik, Wilhelmsfeld, Germany) controlled by the autosampler. The chirally discriminated analytes were detected by a TSQ Quantum triple-stage-quadrupole MS equipped with an ESI source and controlled by the Xcalibur software 1.3. Stereo-specific identification was achieved by comparison to liver microsomal samples of separately incubated and stereochemically pure (S)- and (R)-PPC. The MS settings were as follows: The sprayer needle was adjusted in the 90° position towards the orifice, spray voltage 4000 V in positive ionization mode, capillary temperature: 320°C, sheath gas: 40 units, auxiliary gas: off, collision gas pressure (Argon): 1.0 mTorr. HPLC columns were from Grom Analytik (Herrenberg, Germany) and the HPLC-MS system from Thermo Finnigan (San Jose, CA, USA).

Data analysis and statistics

Data analysis was performed using STATISTICA 6.1 (StatSoft, Tulsa, OK, USA) and StatXact 6.0 (Cytel, Cambridge, MA, USA). Recombinant data were compared by one-way analysis of variance and post-hoc comparisons with the Student Newman–Keuls test. The six genotype groups were compared using the non-parametric Jonckheere–Terpstra trend test. The following order of CYP2C9 genotypes was set a priori: *1/*1, *1/*2, *2/*2, *1/*3, *2/*3 and *3/*3. Kinetic parameters were determined by non-linear regression analysis using the curve-fitting software GraFit 4.03 (Erithacus

Software Ltd, Staines, UK). Pharmacokinetic modelling of *in vivo* data was done by using WinNonlin 4.0.1 (Pharsight, Mountain View, CA, USA).

Results

(S)- and (R)-PPC-hydroxylation in human liver microsomes

Previous kinetic results obtained in CYP2C9*1/*1 wild-type livers indicated two catalytic components operating with low (K_m about 150 μM) or high (K_m about 10 μM) affinity (Ufer *et al.* 2004b). Thus, the selected concentration of 5 μM was clearly in the low- K_m range corresponding to the high-affinity catalysts of *(S)*- and *(R)*-PPC hydroxylation, namely CYP2C8 and CYP2C9 (Ufer *et al.* 2004b). Moreover, this concentration corresponded to plasma concentrations obtained *in vivo* ranging from about 2 to 15 μM during long-term PPC treatment (Petersen *et al.* 1993) or after single, oral dose administration of 12 mg racemic PPC (Kirchheiner *et al.* 2004). Concentrations of liver microsomal protein, cytochrome b_5 , total CYP and NADPH-cytochrome c reductase as well as NADPH oxidation activity varied in a previously reported range (Guengerich 1995). CYP2C9 apoprotein levels of the individual liver specimens varied about threefold and were not significantly influenced by CYP2C9 genotype (data not shown).

The *(S)*-7-hydroxylation reaction was quantitatively most important in CYP2C9*1/*1 wild-type liver specimens and accounted for almost 50% of total metabolite formation from *(S)*- and *(R)*-PPC (figure 2). The rate of *(S)*- and *(R)*-PPC-7-hydroxylation significantly decreased with increasing number of CYP2C9 variant alleles indicating a gene-dose effect (Jonckheere-Terpstra trend test $p=0.0002$ and 0.0082) and CYP2C9*3 corresponded to a greater decrease than the CYP2C9*2 allele (figure 3). In liver specimens of the CYP2C9*3/*3 genotype, the *(S)*-7-hydroxylation rate was almost fourfold lower relative to CYP2C9*1/*1 (figure 3). At the same time, the 4'-hydroxylation rate of either enantiomer was not influenced by CYP2C9 genotype and this pathway became most important

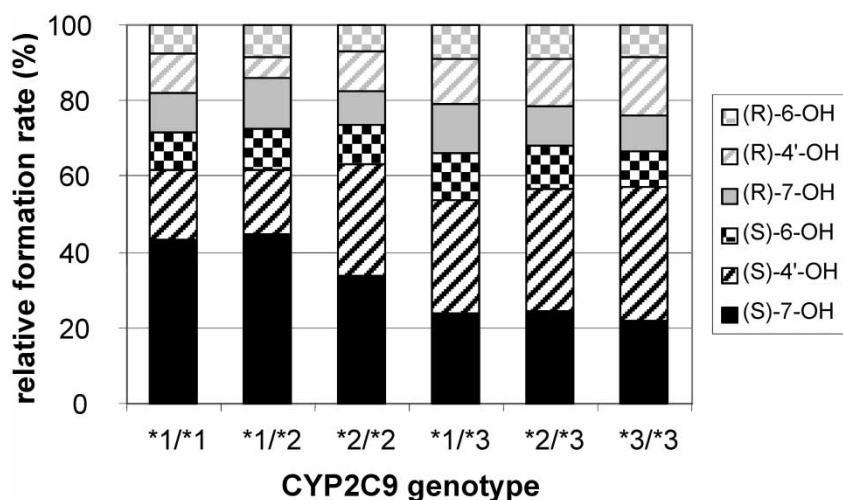


Figure 2. Mean relative hydroxylation rates of PPC in human liver microsomes with different CYP2C9 genotypes at a substrate concentration of 5 μM . The mean standard deviation of each pathway ranged from 0.3 to 2.1%. Data are derived from triplicate determinations.

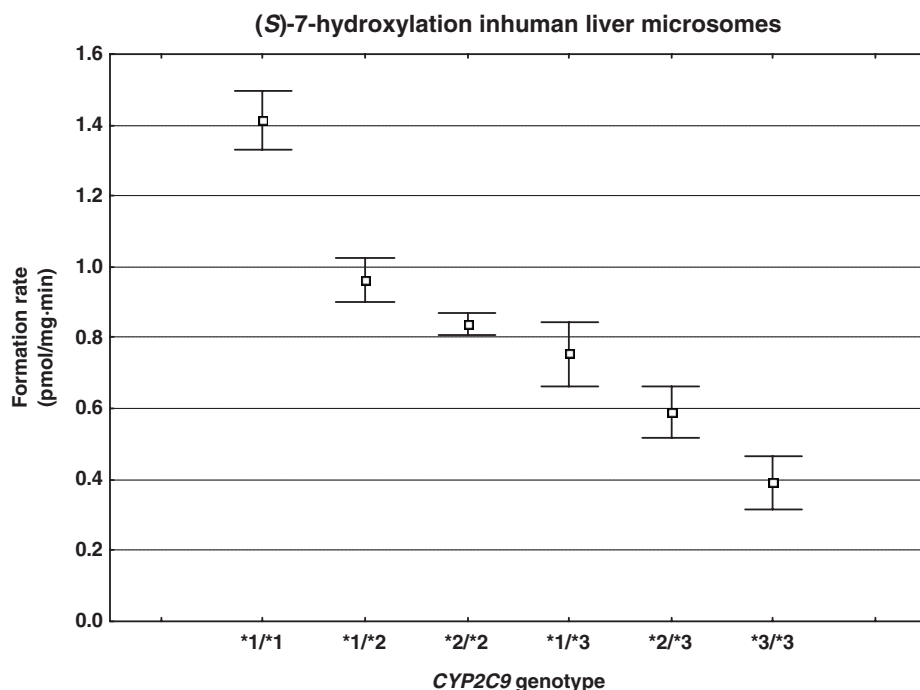


Figure 3. (S)-7-Hydroxylation of PPC in human liver microsomes with different CYP2C9 genotypes at a substrate concentration of 5 μ M. Results are the mean $\pm$ standard deviation calculated from three independent experiments.

in livers being hetero- or homozygous for CYP2C9*3 (figure 2). The (S)- and (R)-PPC-6-hydroxylation rate was about two-fold lower in CYP2C9*3/*3 livers, but no significant trend was found.

*(S)- and (R)-PPC-hydroxylation by human cDNA-expressed CYP2C9*1, *2, *3*

Each of the three CYP2C9 enzymes showed a significant (S)- and (R)-PPC hydroxylation activity. The (S)-7-hydroxylation was identified as major pathway with the highest intrinsic clearance ($V_{\max}/K_m$). This pathway corresponded to 34.0% (CYP2C9*1), 29.9% (CYP2C9*2) and 22.9% (CYP2C9*3) of total intrinsic clearance calculated as the sum of all six hydroxylation reactions (tables 1 and 2).

CYP2C9*2 showed a 28.9% lower clearance than CYP2C9*1 in the formation of (S)-7-hydroxy-PPC. A greater reduction of (S)-7-hydroxylation clearance by 50.9% was observed when using the CYP2C9*3 enzyme. These differences were primarily due to a significantly lower $V_{\max}$ (table 1).

However, the *in vitro* intrinsic clearance of the other five hydroxylation reactions was not significantly reduced when catalysed by CYP2C9*2 or CYP2C9*3 relative to the CYP2C9*1 enzyme (tables 1 and 2).

PPC hydroxylation in healthy volunteers with different CYP2C9 genotypes

Plasma concentration–time curves of PPC metabolites in healthy volunteers of six different CYP2C9 genotypes are shown in figure 4a–c. The determined areas under the plasma concentration time curve (AUC) resulted in a significantly decreasing trend with increasing number of CYP2C9 variant alleles. This effect

Table 1. Apparent kinetic parameters of (*S*)-phenprocoumon hydroxylation using human recombinant enzymes CYP2C9*1, CYP2C9*2 and CYP2C9*3.

	$V_{\max}$ (pmol nmol ⁻¹ CYP min)	Apparent K_m (μM)	$V_{\max}/K_m$ (μl nmol ⁻¹ CYP min)
4'-Hydroxylation			
CYP2C9*1	15.5 (±0.41)	1.63 (±0.11)	9.48 (±0.38)
CYP2C9*2	11.1 (±0.75) ^a	1.71 (±0.39)	6.51 (±1.10)
CYP2C9*3	14.9 (±0.63) ^b	1.54 (±0.27)	9.67 (±1.32)
6-Hydroxylation			
CYP2C9*1	9.56 (±0.34)	1.40 (±0.17)	6.83 (±0.61)
CYP2C9*2	7.37 (±0.17) ^a	1.53 (±0.11)	4.83 (±0.23)
CYP2C9*3	7.81 (±0.09) ^a	1.68 (±0.18)	4.64 (±0.43)
7-Hydroxylation			
CYP2C9*1	29.2 (±1.42)	1.48 (±0.10)	19.7 (±0.31)
CYP2C9*2	23.2 (±1.43) ^a	1.65 (±0.14)	14.0 (±0.30) ^a
CYP2C9*3	17.3 (±0.88) ^{a,b}	1.79 (±0.38)	9.67 (±1.61) ^a

Data were calculated from duplicate incubations at nine different substrate concentrations. The standard error for the determination of the maximum reaction velocity $V_{\max}$ and apparent Michaelis–Menten constant K_m by non-linear regression analysis was <20% (K_m) and <5% ($V_{\max}$), respectively.

^aSignificantly different from CYP2C9*1, $p < 0.05$; ^bsignificantly different from CYP2C9*2, $p < 0.05$.

Table 2. Apparent kinetic parameters of (*R*)-phenprocoumon hydroxylation using human recombinant enzymes CYP2C9*1, CYP2C9*2 and CYP2C9*3.

	$V_{\max}$ (pmol nmol ⁻¹ CYP min)	Apparent K_m (μM)	$V_{\max}/K_m$ (μl nmol ⁻¹ CYP min)
4'-Hydroxylation			
CYP2C9*1	7.75 (±0.02)	0.80 (±0.03)	9.74 (±0.45)
CYP2C9*2	5.92 (±0.01) ^a	0.72 (±0.00)	8.21 (±0.00)
CYP2C9*3	6.00 (±0.07) ^a	0.86 (±0.11)	6.95 (±0.78)
6-Hydroxylation			
CYP2C9*1	7.14 (±0.04)	0.87 (±0.11)	8.23 (±1.13)
CYP2C9*2	6.82 (±0.01) ^a	0.80 (±0.00)	8.50 (±0.00)
CYP2C9*3	5.92 (±0.02) ^{a,b}	0.79 (±0.02)	7.46 (±0.19)
7-Hydroxylation			
CYP2C9*1	8.72 (±0.11)	2.16 (±0.29)	4.03 (±0.50)
CYP2C9*2	7.22 (±0.27) ^a	1.51 (±0.02)	4.80 (±0.10)
CYP2C9*3	7.43 (±0.19) ^a	1.93 (±0.64)	3.86 (±1.33)

Data were calculated from duplicate incubations at nine different substrate concentrations. The standard error for the determination of the maximum reaction velocity $V_{\max}$ and apparent Michaelis–Menten constant K_m by non-linear regression analysis was <20% (K_m) and <5% ($V_{\max}$), respectively.

^aSignificantly different from CYP2C9*1, $p < 0.05$; ^bsignificantly different from CYP2C9*2, $p < 0.05$.

of CYP2C9 genotype was more pronounced with respect to the formation of 6- and 7-hydroxy-PPC than 4'-hydroxy-PPC (figure 4a–c). Irrespective of CYP2C9 genotype, the $C_{\max}$ of each metabolite was barely reached at the last sampling time point of 144 h after oral administration of 12 mg racemic PPC indicating a very slow formation of PPC metabolites (figure 4a–c).

Plasma AUC ratios of (*S*)- and (*R*)-PPC versus (*S*)- and (*R*)-hydroxy-PPC

The plasma concentration–time curves of (*S*)- and (*R*)-PPC and its metabolites were stereoselectively assessed in single study subjects of each CYP2C9 genotype.

Table 3. Plasma AUC ratios of (S)-PPC/(S)-4'-, 6- and 7-hydroxy-PPC shown for each CYP2C9 genotype.

	(S)-PPC/(S)-4'-OH-PPC*	(S)-PPC/(S)-6-OH-PPC*	(S)-PPC/(S)-7-OH-PPC*
2C9*1/*1	147.3	515.4	40.61
2C9*1/*2	140.2	467.9	40.99
2C9*2/*2	159.1	1372.3	100.8
2C9*1/*3	218.0	2020.5	97.0
2C9*2/*3	295.8	2133.3	147.8
2C9*3/*3	410.7	2468.3	422.8

Each metabolite was stereoselectively analysed in duplicate and AUC ratios were calculated from mean plasma concentration–time curves of six study subjects with different CYP2C9 genotypes. Trend analysis was done by means of the non-parametric Jonckheere–Terpstra test with a significance level of $p < 0.05$ (*).

Table 4. Plasma AUC ratios of (R)-PPC/(R)-4'-, 6- and 7-hydroxy-PPC shown for each CYP2C9 genotype.

	(R)-PPC/(R)-4'-OH-PPC	(R)-PPC/(R)-6-OH-PPC	(R)-PPC/(R)-7-OH-PPC*
2C9*1/*1	107.8	103.8	20.62
2C9*1/*2	154.3	134.7	31.42
2C9*2/*2	102.0	221.9	42.41
2C9*1/*3	160.4	339.7	42.08
2C9*2/*3	152.1	257.0	46.22
2C9*3/*3	175.9	280.7	91.66

Each metabolite was stereoselectively analysed in duplicate and AUC ratios were calculated from mean plasma concentration–time curves of six study subjects with different CYP2C9 genotypes. Trend analysis was done by means of the non-parametric Jonckheere–Terpstra test with a significance level of $p < 0.05$ (*).

The determined plasma AUC metabolic ratios (parent compound/metabolite) are reported for each pathway and CYP2C9 genotype (tables 3 and 4). There was a significant trend towards higher ratios of each (S)-PPC hydroxylation reaction with increasing number of CYP2C9 variant alleles. Compared with the CYP2C9*1/*1 ratio, the CYP2C9*3/*3 genotype corresponded to an about 2.5-fold (4'-hydroxylation), 5-fold (6-hydroxylation) and 10-fold (7-hydroxylation) higher metabolic ratio, respectively (table 3). These differences were less pronounced with respect to the (R)-enantiomer and only significant for the 7-hydroxylation reaction (table 4).

Discussion

Genetic polymorphisms of the CYP2C9 gene have clearly been shown to be of clinical relevance for xenobiotic metabolism, particularly with respect to anti-coagulants and associated bleeding complications (Aithal *et al.* 1999, Taube *et al.* 2000, Thijssen *et al.* 2001, 2000b, Scordo *et al.* 2002). Recently, we have identified CYP2C9 as a major high-affinity catalyst of PPC hydroxylation *in vitro* (Ufer *et al.* 2004b). This is the first study to report the effect of CYP2C9*2 and CYP2C9*3 alleles on the stereoselective formation of PPC metabolites *in vitro* and *in vivo*.

In human liver microsomes, the CYP2C9*2 and CYP2C9*3 alleles were associated with a reduced (S)-7-hydroxylation rate being the major pathway of PPC phase I metabolism (figure 3). The magnitude of this effect was greater for

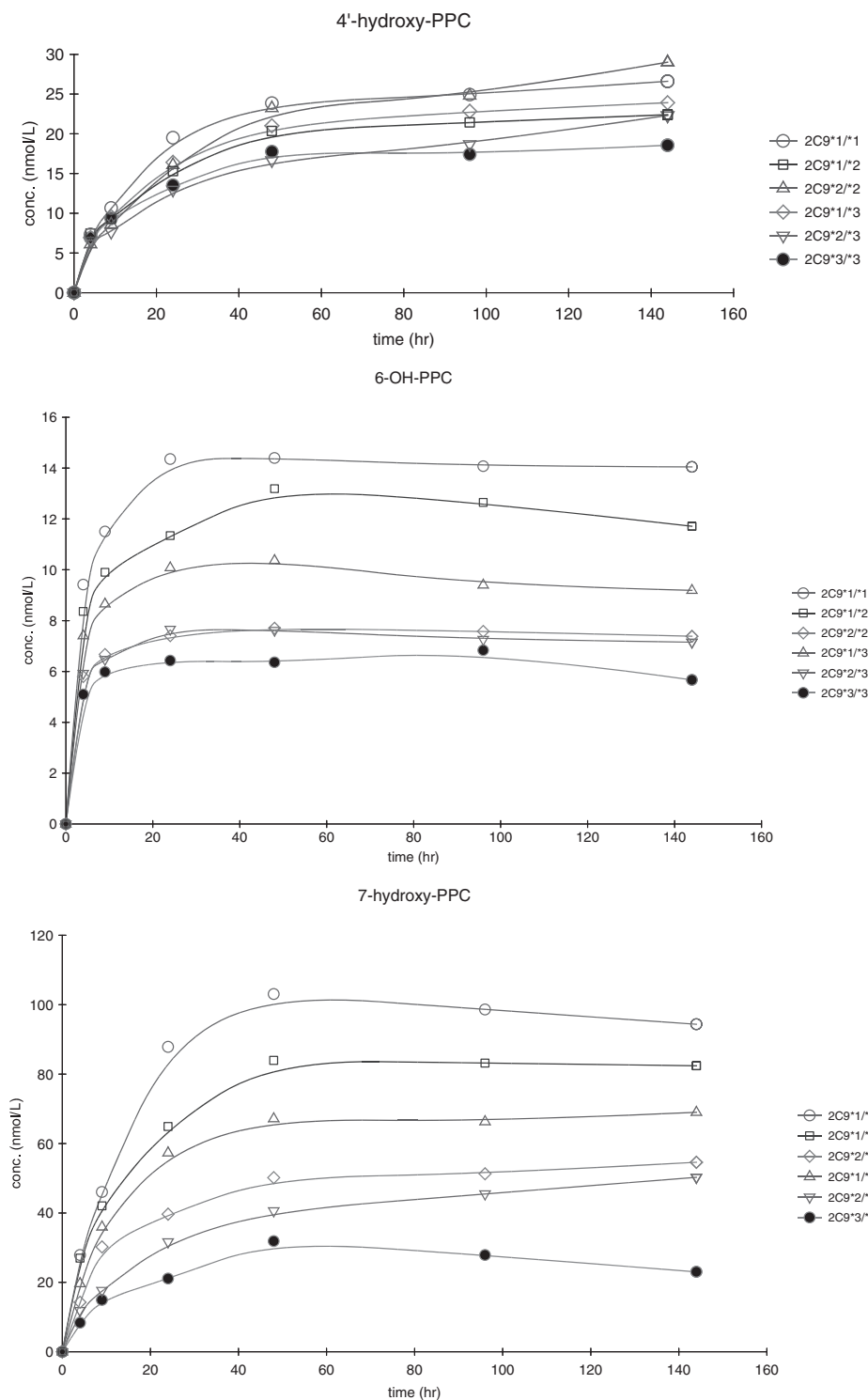


Figure 4. (a-c) Plasma concentration-time curves of 4'-, 6- and 7-hydroxy-PPC after oral administration of 12 mg racemic PPC shown for each CYP2C9 genotype. The corresponding AUC data have previously been reported by Kirchheiner *et al.* (2004).

the CYP2C9*3 than for the CYP2C9*2 allele and also for homozygous relative to heterozygous samples indicating a gene-dose effect. Such a graded reduction of hydroxylation activity is well in line with previous results of other CYP2C9-catalysed reactions (Yamazaki *et al.* 1998a, b). It is also conceivable on the basis of our data obtained with cDNA-expressed recombinant enzymes that the *in vitro* intrinsic clearance of the (*S*)-7-hydroxylation was about 30 or 50% lower than the corresponding wild-type clearance when using CYP2C9*2 or CYP2C9*3, respectively (tables 1 and 2).

Moreover, we have found a regioselective impact of CYP2C9 polymorphisms on PPC biotransformation, as the 4'-hydroxylation rate of (*S*)- and (*R*)-PPC was not significantly influenced by CYP2C9 genotype using human liver microsomes or recombinant enzymes (tables 1 and 2, and figure 2). Thus, regioselectivity of PPC hydroxylation shifts from the 7- to the 4'-position when catalysed by CYP2C9 variant alleles. Consequently, the (*S*)-4'-hydroxylation reaction was found to be the quantitatively most important pathway in microsomes from liver specimens that were hetero- or homozygous for the CYP2C9*3 allele (figure 2). These results are consistent with a previous *in vitro* study showing a more pronounced inhibition of (*S*)-PPC hydroxylation in the 7-position as compared with the 4'-position by the CYP2C9-specific chemical inhibitor sulfaphenazole in human liver microsomes (He *et al.* 1999). This suggested another catalyst of the 4'-hydroxylation reaction in addition to CYP2C9. We have recently identified CYP2C8 as an alternative high-affinity catalyst of the (*S*)-PPC 4'-hydroxylation that may render this pathway less dependent on variations of CYP2C9 activity (Ufer *et al.* 2004b).

These *in vitro* findings corroborated well with *in vivo* data obtained from healthy volunteers after oral intake of a single dose of 12 mg racemic PPC (Kirchheiner *et al.* 2004). A similar trend towards a lower plasma AUC of each PPC metabolite in hetero- or homozygous carriers of the CYP2C9*2 or CYP2C9*3 variant alleles was observed (figure 4a-c). Consistent with the *in vitro* data, this effect was also regioselective for the 6- and 7-position, whereas the 4'-hydroxylation reaction was less influenced by CYP2C9 genotype. Moreover, differences in plasma-AUC metabolic ratios in variant allele carriers of CYP2C9 were also greatest for the 7-hydroxylation reaction followed by 6- and 4'-hydroxylation (tables 3 and 4). Comparing these fold differences of metabolic ratios between the (*S*)- and (*R*)-enantiomer, a consistently greater impact of CYP2C9 polymorphisms on the hydroxylation of (*S*)-PPC was observed with respect to each hydroxylation reaction. This is in line with our previous *in vivo* findings for the parent compound also resulting in a greater reduction of (*S*)-PPC clearance relative to (*R*)-PPC in variant allele carriers of CYP2C9 (Kirchheiner *et al.* 2004).

Though the role of CYP2C9 polymorphisms as a cause of reduced PPC (*S*)-7-hydroxylation appears indisputable in view of our results, this effect is, however, much less pronounced compared with warfarin (*S*)-7-hydroxylation being the major clearance mechanism of this latter anticoagulant. Rettie and co-workers have reported on an about 5.5- and 27-fold reduction of warfarin intrinsic clearance for this pathway when using recombinant CYP2C9*2 or CYP2C9*3, respectively (Rettie *et al.* 1994, Haining *et al.* 1996). These data and their usefulness in predicting the *in vivo* clearance of warfarin have essentially been confirmed by others (Takahashi *et al.* 1998). Thus, the proposition of PPC as a safer anticoagulant than warfarin in carriers of CYP2C9 variant alleles is further

supported by our findings, as the (S)-7-hydroxylation reaction is compromised by only about 50% when catalysed by recombinant CYP2C9*3. *In vivo*, (S)-warfarin clearance was reduced by about 60 and 90% in heterozygous or homozygous carriers of the CYP2C9*3 allele, respectively (Takahashi and Echizen 2001). In terms of PPC, carriers of the CYP2C9*3/*3 genotype showed an oral clearance that was reduced by only about 35 and 25% regarding (S)- and (R)-PPC, respectively (Kirchheiner *et al.* 2004). It should, however, be noted in this regard that the differential impact of CYP2C9 genotype on the oral clearance of both anticoagulants may, however, also stem from a significant excretion of conjugated PPC accounting for almost 40% of an oral dose (Toon *et al.* 1985). In contrast, warfarin and acenocoumarol are almost exclusively eliminated after hepatic biotransformation as reduced or hydroxylated metabolites (Lewis and Trager 1970, Dieterle *et al.* 1977) and CYP2C9-catalysed (S)-6 and 7-hydroxylation has been shown to be most important (Hermans and Thijssen 1993).

To conclude, CYP2C9*2 and *3 polymorphisms are associated with a markedly compromised (S)-7-hydroxylation of PPC *in vitro* and *in vivo*. However, other pathways, such as the (S)-4'-hydroxylation, remain virtually unaffected by CYP2C9 genotype and may serve as alternative routes of metabolism in individuals with low CYP2C9 activity. Thus, PPC may be a valuable alternative for therapeutic anticoagulation of individuals carrying any CYP2C9 variant allele, as its metabolism appears to be less dependent on CYP2C9 activity compared with other commonly used anticoagulants, such as warfarin or acenocoumarol.

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Expression of basolateral and canalicular transporters in rat liver and cultures of primary hepatocytes

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1. Basolateral and canalicular proteins are expressed in the liver and besides their role in the transport of bilirubin, glutathione, hormones and various glucuronides, they also function as transporters of a wide range of drugs.
2. Despite their frequent use in drug research, little is known about the expression of genes coding for transporters in cultures of primary sandwiched hepatocytes.
3. The kinetics of gene expression of canalicular and basolateral membrane transporters in cultures of primary rat sandwiched hepatocytes were investigated, and the expression of cMOAT, spgp, mdr1a and mdr2 were shown to be comparable with transcript levels observed *in vivo*.
4. Strikingly, expression of the basolateral membrane transporter ntcp and oatp1 and 2 were dramatically reduced, the level being < 10 and < 5%, respectively, of those found *in vivo* in rat liver tissue.
5. Notably, mRNA expression of the canalicular membrane transporter mdr1b was increased up to 13-fold.
6. The findings point to a dramatic change in the expression of basolateral and canalicular transporters in cultured hepatocytes, and this should be considered when hepatocytes are used for drug profiling studies.

Introduction

Primary hepatocyte cultures are frequently used in experimental pharmacology and toxicology, and were shown to mimic many of the liver function observed *in vivo* (Seglen 1976, LeCluyse *et al.* 1996). Though hepatocyte cultures are considered reliable models for drug metabolism, drug interactions and enzyme induction and/or inhibition studies, they still have limitations.

In terms of cell isolation, a major step forward was the two-step collagenase perfusion method of Seglen (1976), but cultured primary hepatocytes progressively lose liver specific functions and a highly differentiated cellular phenotype (Gomez-Lechon *et al.* 1998). These limitations were partially overcome through addition of chemicals (e.g. 2% DMSO) (Cable and Isom 1997), co-culture with epithelial or mesenchymal cells (Guguen-Guillouzo *et al.* 1983), cultivation with extracellular matrix substrata (Gomez-Lechon *et al.* 1998), and other improved culture conditions to mimic the native extracellular environment (LeCluyse *et al.* 1994) of hepatocytes, all of which facilitated the culture of highly differentiated cells. Further, primary hepatocytes cultivated in a collagen sandwich maintain

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expression of drug-metabolizing enzymes and show high level of morphological differentiation for prolonged periods, as evidenced by the formation of bile duct canaliculi (LeCluyse *et al.* 1994). Nonetheless, there is still no culture system as yet available that completely resembles the macro- and micro-environment of the liver. Presumably this is one of the major reasons why hepatocytes in cultures may behave differently to those seen *in vivo*. Taking these limitations into account, primary hepatocytes are still of great value for biomedical research and can successfully be applied to particular investigations such as the aforementioned enzyme induction/inhibition studies (Sidhu *et al.* 1998, Sidhu and Omieeinski 1999) and for studying drug-induced hepatotoxicity including intrahepatic cholestasis (Trauner *et al.* 1997).

Because of their importance in normal liver function and their specific role in multidrug resistance, we wished to investigate the gene expression of canalicular and basolateral transporters in cultures of primary hepatocytes over time and compare these findings with an expression profile found *in vivo* in native rat liver tissue. Overall, the study aimed to clarify whether gene expression of hepatic transporters can be studied reliably in cultures of primary sandwiched hepatocytes, particularly when effects on drug transporters are being investigated.

Materials and methods

Animals

Male Sprague–Dawley rats weighing (150 ± 25 g; Charles River Laboratories, Sulzfeld, Germany) were housed under standard laboratory conditions. The animals received a commercial pelleted chow identified as 'altromin 1324N' (Altromin International, Lage, Germany) and water was given *ad libitum*. Before surgery, animals were anaesthetized with 10 mg ketamin and 1 mg xylazin/100 g body weight by intraperitoneal injection.

Hepatocyte isolation and cell culture

Isolation and cultivation of hepatocytes was done essentially as described (Borlak *et al.* 2002, 2003). Plastic culture dishes (60 mm; Falcon, Beeton Dickinson GmbH, Germany) were precoated with rat-tail collagen 1 day before cell isolation and stored at 37°C in 5% CO₂. Further, before cell isolation, a slice of liver was shock-frozen in liquid nitrogen to await mRNA isolation.

Hepatocytes were isolated according to the method of Seglen (1976), with modifications: Briefly, after laparotomy, the vena porta was prepared, cannulated and perfused with calcium-free Krebs–Ringer buffer (KRB). Once the organ was visibly lightened, perfusion was continued with 100 ml 1 mM EDTA buffer followed by perfusion with 0.5% collagenase-KRB solution. Collagenase perfusion was continued until softening of the liver tissue was apparent. The organ was then transferred to ice-cold wash-buffer, and hepatocytes were isolated by gently shaking of the organ. The resultant hepatocyte suspension was filtered through nylonmesh (100 µm) to remove debris and centrifuged twice at 500 rpm for 5 min at 4°C. The resulting pellet was resuspended in William's E medium containing 5% foetal calf serum (FCS) and the cell suspension was adjusted to 1 million cells ml⁻¹. A total of 3 ml of this suspension were used for cell seeding on collagen-coated culture dishes (Falcon). Before seeding, the viability of cells was determined by the trypan blue exclusion test. On average, viability was > 80%, and the

overall yield was about $100\text{--}300 \times 10^6$ cells/liver. Seeded cells were allowed to attach and non-attached cells were removed after 4 h. Twenty-four hours after seeding, cultures were overlaid with a second collagen layer. Medium was changed once daily using William's E medium containing 5% FCS. Cells were cultured in a Sanyo Incubator at 37°C and 5% CO₂.

Cells were inspected daily by light microscopy and photographed using a Zeiss Axiovert 25 Microscope.

Cells were harvested on the day of isolation, e.g. after 6 h of seeding, and subsequently once daily for 8 consecutive days. Cells were scraped from the culture dishes, suspended in wash buffer and centrifuged at 2000 rpm for 10 min, and the resulting pellet was resuspended in lysis buffer as recommended by the manufacturer (Macherey & Nagel, Düren, Germany), flash-frozen and stored at -80°C to await RNA isolation.

RNA isolation

Slices of flash-frozen liver and cell pellets from culture experiments were used for RNA isolation. Total RNA was isolated with the Macherey & Nagel Nucleo-Spin RNAII Kit according to the manufacturer's protocol. On average, 45 µg total RNA/6 million cells were yielded. The integrity of RNA was studied on a 1% agarose gel and 18 and 28 S ribosomal bands were visible without any signs of RNA degradation/fragmentation.

cDNA synthesis

Reverse transcription was done with 2 µg total RNA and 20 units AMV-Reverse Transkriptase (Promega, Mannheim, Germany) in a total reaction volume of 20 µl as described (Borlak and Thum 2001, Thum and Borlak 2002). The reaction was initiated at 70°C for 5 min followed by a cooling step on ice for 5 min and thereafter incubated at 42°C for 1 h. Reverse transcription was stopped by heating the reaction mixture to 95°C for 5 min and the resulting cDNA was stored at -20°C until further analysis.

RT-PCR

The PCR cocktail consisted of 10 ng cDNA, 10 µl Qiagen Hotstar Taq Mastermix and 10 µM oligonucleotides in a total reaction mix of 20 µl. Amplification products were studied on a 1.5% agarose-gel and stained with ethidium bromide. The cycling program for GAPDH, mrp2, mdr1a, mdr2, spgp, ntcp, oatp1 and oatp2 was: 15 min initial denaturing at 95°C and 30 s denaturation between each cycle at 94°C, 1 min annealing at 52–57°C, 1 min elongation at 72°C and final elongation at 72°C for 10 min. For mrp1b gene amplification, conditions were as follows: 95°C initial denaturation for 15 min, 30 s denaturation at 94°C between each cycle, annealing starting at 77°C for 1 min with 4°C lower temperature for each of the following consecutive cycle till 54°C was reached and repeated for 34 times. The number of PCR cycles were 30, 27, 28, 29, 33, 34, 35, 35 for GAPDH, spgp, mrp2, ntcp, oatp1, oatp2, mdr1a and mdr2, respectively (table 1).

Albumin synthesis

Details of the albumin assay are given by Borlak *et al.* (2002). Briefly, the amount of rat albumin secreted into the culture medium was measured by ELISA. Microtitre plates (Nunc, Maxisorb Wiesbaden, Germany) were incubated with the

Table 1. Primer sequences for RT-PCR experiments.

Gene	Forward primer	Reverse primer	Number of cycles	Reference
GAPDH	GGCCAAGGTCATCCATGA	TCAGTGTAGCCCAGGATG	30	Altschul <i>et al.</i> (1997)
mdr1a	GATGGAATTGATAATGTGGAC	AAGGATCAGGAACAATAAA	35	Vos <i>et al.</i> (1998)
mdr1b	GAAATAATGCTTATGAATCCCAAAG	GGTTTCATGGTTCGTCTCTTTGA	34	Vos <i>et al.</i> (1998)
mdr2	AAGAATTTGAAGTTGAGCTAAGTGA	TGGTTTCCACATCCAGCCTAT	35	Vos <i>et al.</i> (1998)
mrp2	GGCTGAGTGCTTGGAC	CTTCTGACGTCATCCTCAC	28	Paulusma <i>et al.</i> (1997)
ntcp	ATGCCCTTCTCTGGCTTTCT	GCTCCATGGTTCTGATGGTT	29	Vos <i>et al.</i> (1999)
oatp1	AAGCGAAGAAGCTGGAAACA	CACCACAGGTCTGTGCAGTT	33	Vos <i>et al.</i> (1999)
oatp2	TGCACACTTAGCATTCTGGC	TGCATGTAACCCAACTCCAA	34	Vos <i>et al.</i> (1999)
spgp	GAGGTTACTTAATAGCCTACG	CATCTATCATCACAGTTCCC	27	Vos <i>et al.</i> (1998)

cell culture supernatant. Albumin was detected with peroxidase-conjugated antibodies against rat albumin (Chappel Laboratories, USA). Quantification was done by assaying colour formation of the chromophore tetramethylbenzidine (TMB) at 450 nm with a Dynatech (Germany) ELISA reader. Rat albumin and anti rat-albumin antibody were purchased from Chappel Laboratories. As culture media contained FCS, cross-reactivity of the rat albumin antibody against bovine serum albumin was studied and found to be negative. Results are expressed in μg albumin per 3 million cells per 1 h.

Statistics

We employed an unpaired Student's *t*-test. Results were considered to be statistically significant at $p < 0.05$.

Results

Hepatocyte culture

Hepatocyte morphology was assessed qualitatively by phase-contrast light microscopy: essentially, no signs of gross morphological alterations were visible. Only cell cultures grown to near confluence (typically more than 90% of culture dish) and visible bile canaliculi were used for the study (figure 1).

The quality of hepatocyte culture was also assessed quantitatively by measuring albumin production. As expected, albumin production was less post-isolation, but recovered during cultivation (figure 2).

Gene expression of uptake and export transporters

Gene expression of hepatic transporters was quantified relative to expression of GAPDH, a house keeping gene. It should be noted that the SDs of some of the measurements were quite large, presumably reflecting more marked changes in individual experiments. Notably, the expression profiles of apical and basolateral

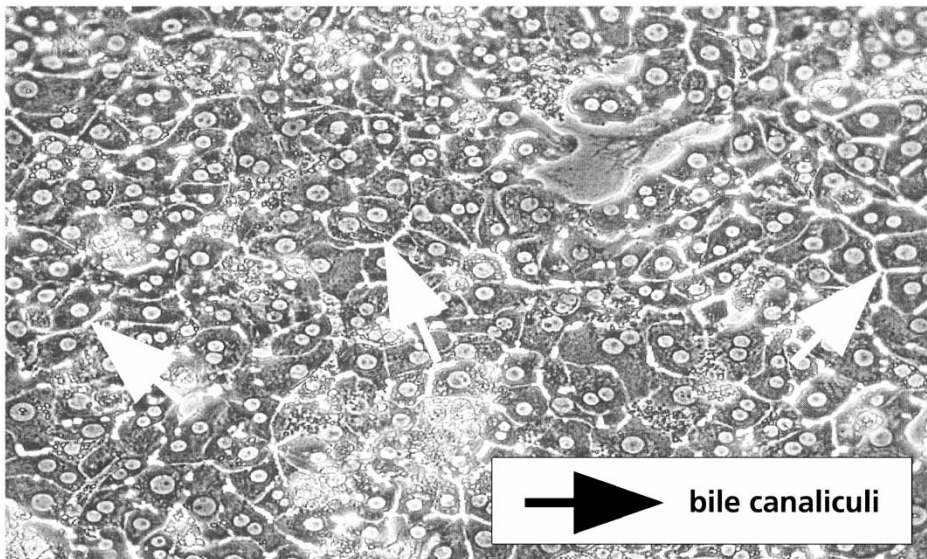


Figure 1. Primary sandwiched rat hepatocytes on day 2 in culture.

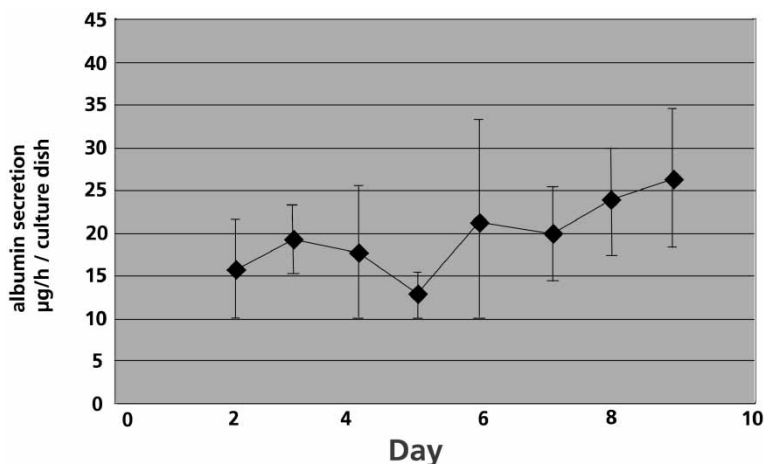


Figure 2. Rates of albumin secretion in primary sandwiched rat hepatocyte cultures ($n=5$).

Table 2. mRNA expression of various transporters relative to GAPDH expression (percentage change).

	Liver	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
mdr1a	100 ± 26	96 ± 15	97 ± 15	78 ± 16	69 ± 7*	75 ± 11*	23 ± 147	43 ± 36*	77 ± 70	78 ± 44
mdr2	100 ± 22	109 ± 20	86 ± 26	68 ± 54	66 ± 66	91 ± 26	89 ± 35	93 ± 16	126 ± 62	105 ± 53
mrp2	100 ± 64	68 ± 55	55 ± 57	65 ± 58	58 ± 90	98 ± 53	55 ± 70	47 ± 56	54 ± 63	55 ± 66
Spgp	100 ± 57	94 ± 77	94 ± 77	100 ± 25	95 ± 70	155 ± 125	87 ± 84	94 ± 180	149 ± 58	129 ± 134

Controls were set to 100%.

Data are percentage change and are the mean ± SD ($n=5$ independent experiments). Unpaired Student's t -test was performed: * $p < 0.05$ when compared with control.

transporters fall into three distinct groups. The first consists of transporters, where gene expression was basically unchanged and thus was viewed comparable with *in vivo* liver tissue expression levels. Consequently, mRNA transcripts of the apical export transporters mdr2 and spgp (table 2) remained similar during the entire cultivation period. There is not much of a trend in mdr1a that might have dipped on days 6 and 7, but was still at 80% of controls by the end of the experiment. Further, mrp2 declined with culture time, but did not reach statistical significance, due to variability amongst individual donors/cell culture experiments (table 2).

The second group consists of hepatic transporters with a substantially reduced transcript level during the entire cultivation period and included the basolateral uptake transporters oatp1, oatp2 and ntcp (figure 3a, b). Specifically, oatp1- and oatp2-transcript levels were reduced to about 5% of liver *in vivo* expression levels, whereas transcripts for ntcp decreased to approximately 5–10% of the expression values determined for whole liver extracts.

The third group consisted of the apical export transporter mdr1b, where a substantial induction of mRNA was observed, the levels being up to 13-fold above those found *in vivo* (figure 4a, b).

Discussion

As shown in figure 1, hepatocytes expressed a polyhedral morphology with a central spherical nucleus. *In vivo*, hepatocytes are arranged in plates with

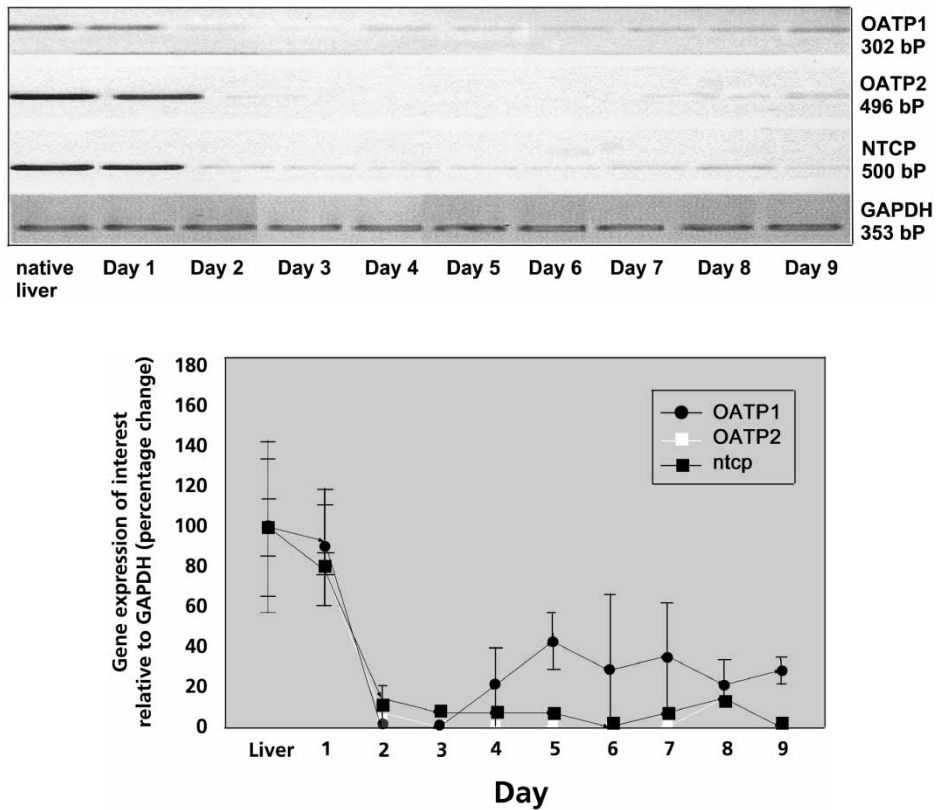


Figure 3. (a) Representative ethidium bromide-stained PCR amplification products of basolateral uptake transporters (25 ng/lane). (b) Kinetics of gene expression of basolateral uptake transporters in cultured primary sandwiched rat hepatocytes ($n=5$).

blood-filled sinusoids on each side of the plates. The cytoplasmic membrane has specialized domains providing a canalicular region on the lateral walls and numerous microvilli on the sinusoidal (basolateral) surfaces. Transport systems at the basolateral domain of the hepatocyte facilitate selective uptake of albumin bound organic anions, cations and neutral compounds that are excreted into bile. Therefore, the sodium-independent organic anion transporters *oatp1* and *oatp2* and the sodium-taurocholate co-transporter *ntcp* functions selectively to remove conjugated bile salts and estrone sulfate from the hepatic sinusoid.

As shown in the present study, cultures of primary sandwiched hepatocytes differed in the expression of genes coding for transporters and multidrug resistance associated proteins. We observed a substantial reduction in transcript level of basolateral uptake transporters and a large induction of the apical export transporter. Thus, upon hepatocyte isolation repression of basolateral uptake transporters was obvious for *oatp1*, *oatp2* and *ntcp* remaining suppressed during 8 days of culture, whereas the apical export transporter *mdr1b* was strikingly induced and remained approximately 10-fold elevated during the entire culture period. Consequently, morphological disruption of liver tissue, as achieved during cell isolation and subsequent culture, does greatly influence the transcriptional regulation of hepatic uptake and export transporters. This needs to be considered

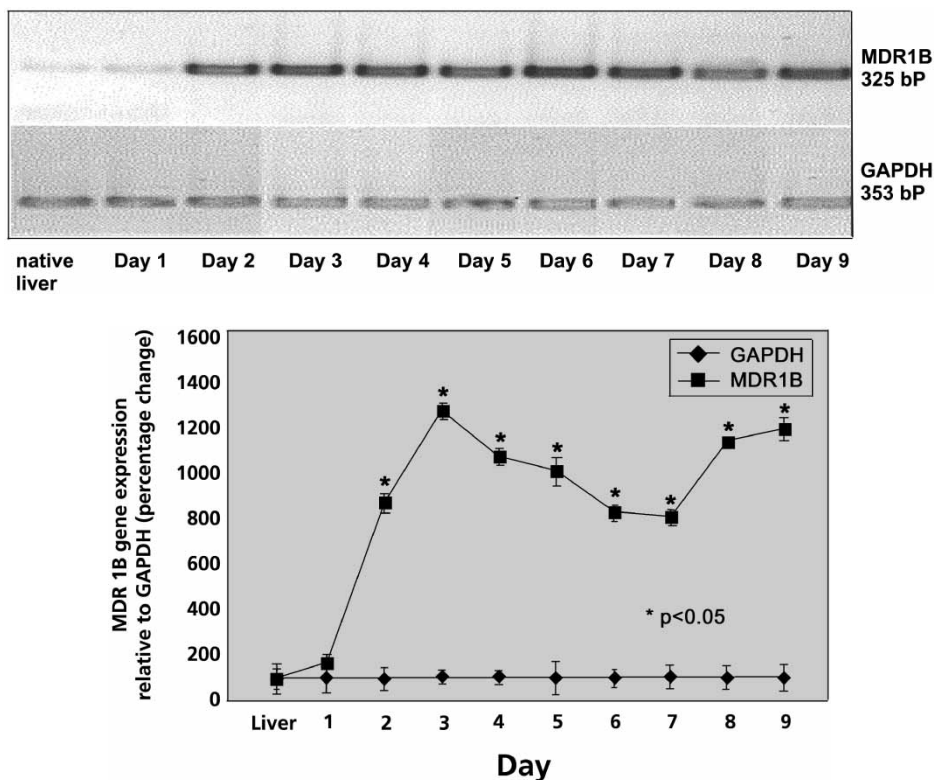


Figure 4. (a) Representative ethidium bromide-stained PCR amplification products of *mdr1b* in cultured primary sandwiched rat hepatocytes (25 ng/lane). (b) Kinetics of MDR1B gene expression in cultures of primary sandwiched rat hepatocytes ($n = 5$).

when hepatocyte cultures are used for drug transport and multidrug resistance studies.

There is limited information on the gene expression of various transporters in sandwiched rat hepatocyte cultures. Specifically, Luttringer *et al.* (2002) investigated the influence of the isolation procedure and cell culture condition on expression of transporters. These investigators suggest extracellular matrix (ECM) and dexamethasone (DEX) supplementation to be of importance in the formation of bile canaliculi networks, as evidenced by regulation of membrane transporters in cultured rat hepatocytes. Ziemann *et al.* (1999) reported *mdr1b* gene expression to be inducible by ROS in cultured rat hepatocyte monolayers, and Schrenk *et al.* (1996) studied *mdr1b* gene expression in rodent primary hepatocyte cultures as well as in a rat hepatoma cell line. Regulation of transporters differed upon drug treatment and during liver disease but little is known about their regulation *in vitro* in cultures of sandwiched rat hepatocytes.

It is well established that the canalicular membrane transporter functions export a variety of compounds including hydrophobic metabolites, peptides, hormones and toxic compounds out of hepatocytes into bile canaliculi. A summary of some major functions of hepatic transporters is given in table 3.

The substantial induction of the *mdr1b* gene is intriguing and points to a significant shift in transcriptional regulation. With the exception of *mdr1b*, expression of the canalicular membrane transporters studied herein was unaffected

Table 3. Examples for drug transport via various hepatic transport proteins.

Transporter	Physiological substrate	Pharmaceutical substrate	Reference
mrp2 (cMOAT)	bilirubin glucuronides, oxidized glutathione, glutathione conjugates	cytostatica: methotrexate	Roelofsen <i>et al.</i> (1995), Masuda <i>et al.</i> (1997)
spgp	Unknown	cytostatica: taxol	Childs <i>et al.</i> (1998)
mdr2	phosphatidylcholin, cholesterol	antifungal antibiotic: aureobasidin A	Childs <i>et al.</i> (1998), Kino <i>et al.</i> (1996)
mdr1a	hydrophobic metabolites, peptides and steroid hormones	cytostatica: doxorubicin; antibiotic: rifampicin; cardiotherapeutic: digoxin; muscle relaxants: vecuronium	Schinkel <i>et al.</i> (1997), Schuetz <i>et al.</i> (1996), Fromm <i>et al.</i> (1999), Smit <i>et al.</i> (1998)
mdr1b	hydrophobic metabolites, peptides and steroid hormones	cytostatica: doxorubicin; muscle relaxants: vecuronium	Schinkel <i>et al.</i> (1997), Smit <i>et al.</i> (1998)
ntcp	conjugated bile acids, estrone sulfate, thyroid hormones	bile acid-coupled cytostatica: chlorambucil-taurocholate	Hagenbuch and Meier (1994), Kullak-Ublick <i>et al.</i> (1997), Friesema <i>et al.</i> (1999)
oatp	conjugated and unconjugated bile acids, thyroid hormones	antihistamine: fexofenadine; thrombin inhibitor: CRC 220	Kullak-Ublick <i>et al.</i> (1995), Cvetkovic <i>et al.</i> (1999), Friesema <i>et al.</i> (1999), Eckhardt <i>et al.</i> (1996)
mrp1	glutathione disulfide, glucuronides	(HIV-1) protease inhibitors: ritonavir, nelfinavir, indinavir, (PIs)-saquinavir, cytostatica: chlorambucil	Srinivas <i>et al.</i> (1998), Keppler <i>et al.</i> (1998), Barnouin <i>et al.</i> (1998)

and as shown in figure 1, cultured hepatocyte formed bile canaliculi. The highly significant induction of *mdr1b* may be viewed as an adaptive response to the developing bile canaliculi. Presumably, the newly formed canaliculi may have not been sufficiently developed for the *mdr1b* gene to return to normal.

Further, there is evidence for *mdr1b* to be regulated by hepatocarcinogens including acetylaminofluorene (Deng *et al.* 2001), by intracellular ROS (Ziemann *et al.* 1999) and, as shown recently, by lipopolysaccharide treatment of SD rats (Cherrington *et al.* 2004). Interestingly, pretreatment of rats with dexamethasone prevented LPS-mediated increase in *mdr1b* gene expression (Cherrington *et al.* 2004). Moreover, there is evidence for *mdr1b* to be regulated by TNF α as a result of NF-kappaB signalling, but this was independent of p53 action (Ros *et al.* 2001). It is also of considerable interest that nuclear run-on assays demonstrated increased *mdr1b* mRNA levels to be the result of increased gene transcription (Hill *et al.* 1996). It remains to be determined whether processes linked to cellular dedifferentiation triggers enhanced *mdr1b* expression.

As mentioned above, the gene expression of the basolateral transporters *oatp1*, *oatp2* and *ntcp* was substantially reduced and it is tempting to speculate that this reduction is in response to the new level of natural and physiological substrates in the culture media. We additionally investigated bile secretion into culture media using the classic NAD/NBT enzymatic colorimetric bile acids method of Mashige (1981), but did not find changes (data not shown). Notably, we did not observe signs of cholestasis and/or steatosis during the entire culture period and thus, the gene repression of these transporters did not result in gross morphological alterations or changes in clinical chemistry parameters (data not shown).

Liang *et al.* (1993) reported the gene expression of the basolateral transporter *ntcp* to be dramatically reduced in cultures of primary hepatocytes. We confirm the findings of Liang *et al.* and add to the list of highly significantly repressed transporter *oatp1* and *oatp2*. Although the gene expression of the canalicular membrane transporters *spgp*, *mrp2*, *mdr1a* was similar to normal liver tissue, expression levels of *mdr1b* was extensively increased.

The gene regulation of hepatic transporters can be modulated by drugs and other xenobiotics and induction of multidrug resistance associated proteins by anticancerous drugs in tumour cells is well known. For instance, expression of the canalicular transporter cMRP is inducible by the antineoplastic drug cisplatin and multidrug resistance associated proteins are involved in the pathogenesis of drug induced intrahepatic cholestasis (Kauffmann *et al.* 1997). The basolateral transporter *ntcp* and the canalicular transporter *spgp* are down-regulated in sepsis and endotoxemic cholestasis (Green *et al.* 1996, Vos *et al.* 1998). Most of these observations are based on *in vivo* studies and in the case of the canalicular transporter cMOAT, its general inducibility has been demonstrated in cultures of primary hepatocytes.

In conclusion, we report expression of basolateral and canalicular transporters to be altered in cultures of primary hepatocytes. Presumably, this is an adaptive response to cell isolation and cultivation. Indeed, the mixture of endogenous substances and xenobiotics entering the hepatocyte *in vivo* via the sinusoids at the basolateral side is now replaced by a formulated medium. There is a need to continuously improve the experimental conditions for hepatocyte cultures to mimic more closely the *in vivo* situation so that expression of the basolateral transporters return to normal. This includes the development of newly defined

culture media containing a well balanced composition of conjugated bile salts and other physiological substrata for the normal regulation of transporters. As the canalicular transporters mrp2, spgp, mdr1a, mdr2 and mrp1 are expressed at levels similar to those found *in vivo*, primary hepatocyte cultures may be used to study their expression and regulation, as part of drug profiling and hepatotoxicity studies.

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Use of a cocktail of probe substrates for drug-metabolizing enzymes for the assessment of the metabolic capacity of hepatocyte preparations

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1. A cocktail of the following probe substrates for human drug-metabolizing enzymes was used to characterize hepatocyte preparations: phenacetin (for CYP1A2), diclofenac (CYP2C9), diazepam (CYP2C19), bufuralol (CYP2D6), midazolam (CYP3A4/5) and 7-hydroxycoumarin (for glucuronidation and sulphation).

2. The cocktail was incubated with cryopreserved human, dog or minipig hepatocytes or with freshly prepared rat hepatocytes. Sample analysis was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in an Open Access environment that allowed less experienced MS operators to login, submit and analyse sample sets using predefined settings without the immediate attendance of an experienced analyst. Intrinsic clearances (CL_{int}) were calculated from the disappearance of the compounds from the incubations.

3. Initially, the cocktail used for human, rat and dog hepatocyte incubations contained 7-ethoxycoumarin instead of 7-hydroxycoumarin. However, 7-ethoxycoumarin had an inhibitory effect on the metabolism of phenacetin.

4. The highest CL_{int} estimated with human and dog hepatocytes was observed for 7-hydroxycoumarin. For rat and minipig hepatocytes, the highest CL_{int} was observed for bufuralol. In incubations with dog and minipig hepatocytes, the lowest CL_{int} was seen with diclofenac, whereas for human and rat hepatocytes, the lowest value was observed with diazepam and phenacetin, respectively.

5. When the cocktail was incubated together with human hepatocytes and 1 μ M ketoconazole, the CL_{int} of midazolam was decreased to about 7.5% of the control value, whereas the metabolism of the other cocktail compounds was virtually unaffected by this CYP3A inhibitor.

6. It is suggested that a cocktail of specific human probe substrates for drug-metabolizing enzymes can be used routinely for the determination of the metabolic capacity of hepatocyte preparations in order to ensure the quality and reproducibility of experiments. Moreover, a cocktail of specific probe substrates can also be a useful tool for studies on enzyme inhibition.

Introduction

Hepatocytes provide an *in vitro* system with cellular integrity with respect to enzyme systems, co-factors and membrane transport proteins (Cross and Bayliss 2000, Brandon *et al.* 2003, Donato and Castell 2003). The use of hepatocytes for *in vitro* studies of drug metabolism has increased over the past years, which can be explained by the availability of cryopreserved hepatocytes with high viability and preserved enzyme activities (Li *et al.* 1999, Hewitt *et al.* 2000, Lau *et al.* 2002, Bachman *et al.* 2003). Cryopreserved human hepatocytes have the practical advan-

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tage of allowing a routine use of human liver material during drug development. In addition, hepatocytes from different human donors can be pooled to resemble an average human population in hepatic enzyme expression.

Previous studies have demonstrated that cytochrome P450-dependent activities and Phase II conjugations remain stable in these cryopreserved hepatocytes (Li *et al.* 1999). In Lau *et al.* (2002) and Bachmann *et al.* (2003), satisfactory correlations between *in vitro* intrinsic clearances obtained with human cryopreserved hepatocytes and *in vivo* clearances were demonstrated. In addition, Hewitt *et al.* (2000) have shown that the metabolic activities of monkey hepatocytes are well preserved during cryopreservation.

The activities of drug-metabolizing enzymes can be assayed by the use of specific probe substrates. The simultaneous use of several selective probes for individual enzymes could allow the assessment of many enzymes in the same experiment. Such drug cocktail methods have been employed *in vivo* in humans for phenotyping as well as for studies on drug-drug interactions (Chainuvati *et al.* 2003, Christensen *et al.* 2003, Blakey *et al.* 2004). Dierks *et al.* (2001) developed a cocktail for the evaluation of the activities of seven major human drug-metabolizing enzymes in microsomes. However, apart from the latter study, reports on the utilization of drug cocktails for *in vitro* experiments are scarce. This is somewhat surprising since a cocktail with specific probe substrates for drug-metabolizing enzymes could offer a time-saving tool for the characterization of *in vitro* systems.

The present study has developed a method for the simultaneous determination of the metabolic capacities of major drug-metabolizing enzymes in human hepatocytes by using a cocktail with specific human probe substrates. This cocktail was also used for the assessment of the metabolic capacity of rat, dog and minipig hepatocytes. The rat hepatocytes employed for this investigation were freshly prepared, whereas the human, dog and minipig hepatocytes were cryopreserved and commercially obtained. Probe substrates for human drug-metabolizing enzymes may not necessarily be probes for orthologues in other species (Guengerich 1997, Bogaards *et al.* 2000). However, the objective was to ensure the quality and reproducibility of hepatocyte experiments, and we have not focused on any substrate specificity comparison between species.

The compounds to be included in a cocktail have to be selected carefully so that no metabolic interactions occur in the incubations. Moreover, the compounds must be chosen so that they can be individually detected in the same assay. Our cocktail contained the following human cytochrome P450 probe substrates: phenacetin (for CYP1A2), diclofenac (CYP2C9), diazepam (CYP2C19), bufuralol (CYP2D6) and midazolam (CYP3A4/5). For reviews of the metabolism of these substrates, see Parkinson (1996) and Rendic and Di Carlo (1997). In addition to the CYP probe substrates, 7-hydroxycoumarin was included in the cocktail as a substrate for the phase II-conjugating enzymes UDP-glucuronosyltransferase (UGT) and sulphotransferase (SULT). Initially, the cocktail contained 7-ethoxycoumarin, which undergoes *O*-deethylation before glucuronidation and sulphation (Walsh *et al.* 1995), instead of 7-hydroxycoumarin.

Sample analysis was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in an Open Access environment that allows less experienced MS operators to login, submit and analyse sample sets using predefined settings without immediate attendance of an experienced analyst.

Intrinsic clearances (CL_{int}) were calculated from the disappearance of the compounds from the incubations. This approach requires that the K_m for a compound is higher than its initial concentration in the incubation. An advantage of using low substrate concentrations is that the risk of inter-substrate interactions is minimized. At the same time, the compound concentration has to be high enough to allow analysis. As a compromise, a substrate concentration of 2 μ M was selected.

It has been demonstrated that the activity of CYP3A4 in human hepatocytes is inhibited specifically by 1 μ M ketoconazole (Li *et al.* 1999). In order to investigate the usefulness of our cocktail for drug–drug interactions *in vitro*, human hepatocytes were incubated with the cocktail together with 1 μ M ketoconazole, and the CL_{int} values of the cocktail compounds were compared with the corresponding values obtained without the inhibitor.

Materials and methods

Chemicals

Forene® (isofluran) for anaesthesia came from Abbott Scandinavia AB (Kista, Sweden); bufuralol was from Ultrafine Chemicals (Manchester, UK); diazepam, diclofenac, 7-ethoxycoumarin, 7-hydroxycoumarin, midazolam, phenacetin, Krebs–Henseleit buffer, Williams' E medium, collagenase type XI, trypan blue, EGTA and trisma base were obtained from Sigma Chemical Co. (St Louis, MO, USA); ketoconazole was purchased from Janssen, Chimica (Geel, Belgium). All other chemicals were of analytical grade and obtained from common commercial suppliers.

Animals

Male Sprague–Dawley rats (8–9 weeks old; B&K Universal AB, Sollentuna, Sweden) were maintained under a 12-h light/dark cycle in steel cages with free access to commercial food pellets (Lactamin, Stockholm, Sweden) and tap water.

Preparation of rat hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats using a collagenase perfusion technique, modified from the procedure described by Bissell and Guzelian (1980). After anaesthetizing the rat with Forene (isofluran), the liver was perfused *in situ* at 37°C with a calcium-free buffer (pH 7.2) containing 0.5 mM EGTA, followed by perfusion with Williams' E medium containing collagenase type XI (0.16 mg ml⁻¹). After release from the liver, the hepatocytes were suspended in Williams' E medium, filtered, washed twice in Krebs–Henseleit buffer by centrifugation and resuspended in Krebs–Henseleit buffer. The viability (as determined by trypan blue exclusion) was routinely 85–95%. Fresh rat hepatocytes were prepared before each experiment. In total, 14 rat hepatocyte preparations were used for this investigation.

Cryopreserved hepatocytes

Cryopreserved hepatocytes prepared from human, male Beagle dog and male Gottingen minipig livers were purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA). Li *et al.* (1999) have described the procedure for cryopreservation of hepatocytes. Each purchased vial contained at least 5×10^6 viable hepatocytes, which were thawed as recommended by In Vitro Technologies. The viability (as determined by trypan blue exclusion) was routinely 70–80%. The incubation of the cocktail with human hepatocytes was repeated 13 times and hepatocytes from 10 different individuals were used. For a summary of the characteristics of the human samples, see table 1. For additional information on the levels of drug-metabolizing enzymes in these samples, see www.invitrotech.com. Each experiment was performed with pools of hepatocytes from two to four human individuals. The hepatocytes of the donors included in each experiment were chosen so as to resemble the average hepatic enzyme expression of a human population. The experiment with dog hepatocytes was repeated eight times with hepatocytes from three different male Beagle individuals (Lots ZRA, LPB and TXJ). These dog hepatocytes were in most cases tested as pools of hepatocytes from two of the individuals. In the case of minipig, hepatocytes from one male Gottingen individual (Lot AWR) were used in four experiments.

Table 1. Characteristics of the human liver samples (obtained from In Vitro Technologies).

Lot	Sex of donor	Age of donor (years)	Medical history
MQF	F	49	hypertension
DRL	M	44	none
117	F	47	none
NQT	M	53	colon polyps
OCF	F	36	fibromyalgia
OQD	M	51	brain tumour
RNG	F	67	myocardial infarction (3), coronary artery
CYE	M	52	none
HRK	F	66	hypertension
ENR	M	72	kidney stone

Incubations of hepatocytes with the cocktail

The substrate stock solution consisted of 20 μM each of phenacetin, diclofenac, diazepam, bufuralol, midazolam and 7-hydroxycoumarin in 1% dimethyl sulphoxide. Initially 7-ethoxycoumarin was used instead of 7-hydroxycoumarin in incubations with rat, human and dog hepatocytes. The stock solution was stored in aliquots at -20°C until incubation. All incubations were performed as duplicates in 96-well deep microplates (Treff Ag, Degersheim, Switzerland). The hepatocytes were diluted in Krebs–Henseleit buffer and 90 μl of the cell suspension were added to 10 μl of the cocktail stock solution so that the final hepatocyte concentration was 10^6 cells ml^{-1} , and the concentration of each enzyme substrate was 2 μM . Incubations were performed at 37°C under an atmosphere of 5% CO_2 /95% air and the plates were shaken gently.

The human, dog and minipig hepatocytes were incubated with the cocktail for 0, 15, 30, 45, 60 and 90 min, while the rat hepatocytes were incubated with the cocktail for 0, 5, 15, 30, 45 and 60 min. The reactions were stopped by the addition of 100 μl ice-cold acetonitrile. The plates were centrifuged (1900g, 5 min) and aliquots of the supernatant analysed by LC-MS/MS.

The cocktail compounds were screened for possible inter-substrate interactions in human hepatocytes by also incubating each compound individually. The results thus obtained were compared with the corresponding results obtained from incubations with the cocktail.

Inhibition with ketoconazole

Human hepatocytes were incubated with the cocktail, as described above, together with 1 μM ketoconazole. The experiment was performed four times with hepatocytes from one or two individuals in each experiment, with a total number of five individuals (Lots CYE, ENR, HRK, OCF and OQD). All samples were treated in exactly the same manner as described for the hepatocytes incubated without the inhibitor.

Analysis

LC-MS/MS analyses were performed with a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, UK) coupled to an Alliance 2790 binary pump and an autosampler (Waters Corp., Milford MA). An Open Access system was made possible by the software MassLynx (controls the LC-system and the mass spectrometer), QuanLynx (controls quantification and MS optimization) and OpenLynx (an application manager). Open Access enables less experienced MS operators to login samples and to access instrument functions with a minimum of interaction with the software. An Atlantis C-18, 3 μm , 30×2.1 mm analytical column was used. A six-port switching valve (Waters) was used to divert the mobile phase to waste the first 2 min after injection. Chromatography was performed using a generic gradient at a flow rate of 0.4 ml min^{-1} . The mobile phase consisted of solvent A: acetonitrile in 0.1% acetic acid (2:98 v/v), and solvent B: acetonitrile in 0.1% acetic acid (80:20 v/v). The gradient conditions were as follows: 0–0.5 min 2% B, 0.5–1.0 min 2–90% B, and 1.0–3.5 min 90% B.

Electrospray ionization in both positive and negative mode (ESI+/ESI–) with multiple reaction monitoring (MRM) was used. The capillary voltage was set to 3.2 kV and the source block and desolvation temperatures were set at 130 and 300°C , respectively. The nebulizer gas was set to maximum, the cone and desolvation gas were set to 70 and 680 lh^{-1} , respectively (high purity nitrogen). The analysers were set as follows: the low- and high-mass resolutions on both quadrupoles were set at 13.0 (approximately 0.9 amu FWHM) with ion energy of 1 V. The collision entrance and exit lenses were –2 and 1 V, respectively. High-purity argon was used as a collision induced dissociation (CID) gas. The CID gas was adjusted to give a collision cell pressure of approximately 2.1 e^{-3} mBar.

MRM transitions were monitored as follows: diclofenac m/z 293.8 $\rightarrow$ 250.1, ESI–, cone voltage 19 V, collision energy 12 eV; 7-hydroxycoumarin m/z 160.9 $\rightarrow$ 133.1, ESI–, cone voltage 37 V, collision

energy 19 eV; midazolam m/z 326.0 $\rightarrow$ 291.1, ESI+, cone voltage 46 V, collision energy 26 eV; diazepam m/z 285.1 $\rightarrow$ 154.1, ESI+, cone voltage 37 V, collision energy 26 eV; phenacetin m/z 180.1 $\rightarrow$ 110.1, ESI+, cone voltage 37 V, collision energy 19 eV; bufuralol m/z 262.2 $\rightarrow$ 188.2, ESI+, cone voltage 19 V, collision energy 19 eV. The dwell time for each transition was 80 ms.

Determination of intrinsic clearances

The concentration of each cocktail compound in the hepatocyte incubation was fit to a first-order elimination equation:

$$C = C_0 \cdot e^{-k \cdot \Delta t},$$

where C is the measured concentration at any time, C_0 is the concentration at zero-time and k is the elimination constant. Intrinsic clearance was calculated as follows:

$$CL_{\text{int}} = k \cdot V,$$

where V is the volume of the hepatocyte suspension.

All calculations were performed by employing an Excel-based standardized protocol.

Statistical analysis

Data are means $\pm$ standard deviations of the number of experiments indicated. Analysis of statistical significance, where appropriate, was performed using the Student's t -test.

Results

Incubation of cryopreserved human hepatocytes with the cocktail

The highest CL_{int} was observed with 7-hydroxycoumarin ($25 \pm 5.3 \mu\text{l min}^{-1}/10^6$ cells) and the lowest CL_{int} was seen with diazepam ($1.7 \pm 1.4 \mu\text{l min}^{-1}/10^6$ cells) when cryopreserved human hepatocytes were incubated with the cocktail. The order of the human CL_{int} values for the compounds in the cocktail was diazepam < bufuralol $\leq$ phenacetin $\leq$ diclofenac $\leq$ midazolam < 7-hydroxycoumarin (figure 1A). When the cocktail contained 7-ethoxycoumarin, the CL_{int} for phenacetin that was about half of the CL_{int} observed when 7-hydroxycoumarin was used in the cocktail (table 2). It was demonstrated that phenacetin was the only cocktail compound affected by inter-substrate interactions (data not shown).

Incubation of fresh rat hepatocytes with the cocktail

Of the six compounds in the cocktail tested with fresh rat hepatocytes, bufuralol demonstrated the highest CL_{int} ($70.6 \pm 10.8 \mu\text{l min}^{-1}/10^6$ cells), and the lowest CL_{int} was observed with phenacetin ($14.3 \pm 5.5 \mu\text{l min}^{-1}/10^6$ cells). As can be seen in figure 1B, the order of the rat hepatocyte CL_{int} values for the cocktail compounds was phenacetin < diclofenac $\leq$ diazepam < 7-hydroxycoumarin < midazolam < bufuralol. Initially, the cocktail contained 7-ethoxycoumarin, and this gave a CL_{int} for phenacetin that was 3.3 times lower than with 7-hydroxycoumarin (table 2).

Incubation of cryopreserved dog hepatocytes with the cocktail

When cryopreserved dog hepatocytes were incubated with the cocktail, the highest CL_{int} was observed with 7-hydroxycoumarin ($24 \pm 6.4 \mu\text{l min}^{-1}/10^6$ cells) and the lowest with diclofenac ($1.8 \pm 0.9 \mu\text{l min}^{-1}/10^6$ cells). For dog hepatocytes, the order of the CL_{int} values for the compounds of the cocktail was diclofenac < midazolam < phenacetin $\leq$ bufuralol $\leq$ diazepam < 7-hydroxycoumarin (figure 1C). As for the rat and human hepatocyte incubations, the dog hepatocyte incubations were also initially performed with a cocktail containing 7-ethoxycoumarin instead of 7-hydroxycoumarin. The ethoxycoumarin appeared

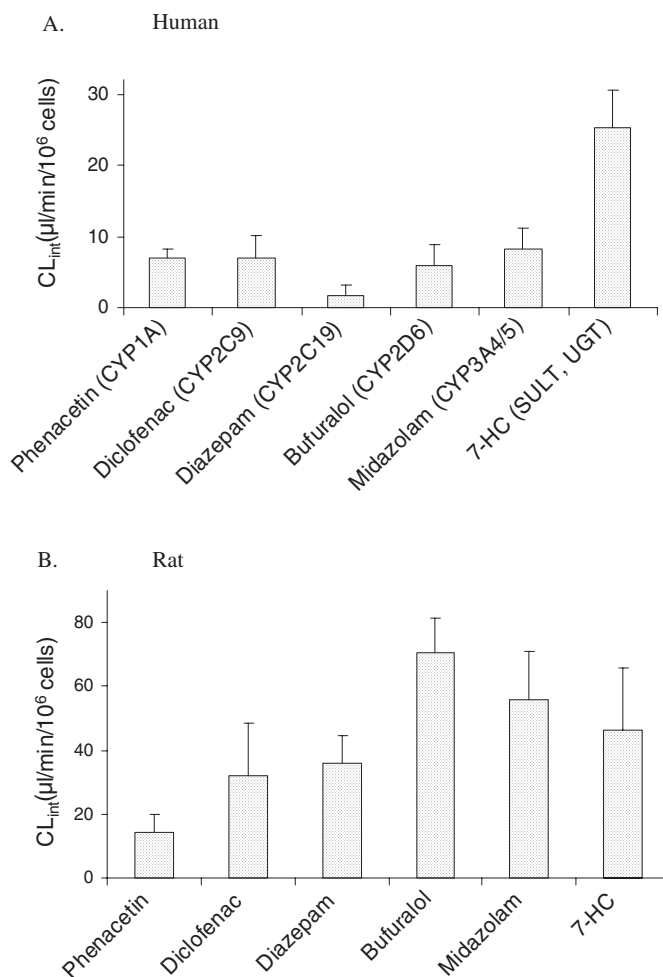


Figure 1. Intrinsic clearance data for the cocktail compounds in suspensions of human (A), rat (B), dog (C) and minipig (D) hepatocytes. The rat hepatocytes used were freshly prepared, whereas the human, dog and minipig hepatocytes were cryopreserved. Data are means $\pm$ standard deviations for 13 experiments with human hepatocytes (A), hepatocytes from 14 male rats (B), eight experiments with male dog hepatocytes (C) and four experiments with male minipig hepatocytes (D). The human enzymes that the probes assess are indicated in (A).

to have a slight but not significant inhibitory effect on the metabolism of phenacetin in the dog hepatocytes (table 2).

Incubation of cryopreserved minipig hepatocytes with the cocktail

In the case of minipig hepatocytes, bufuralol was the cocktail compound that was metabolized at the highest rate, and diclofenac was metabolized at the lowest rate (29 ± 2.9 and $2.7 \pm 1.5 \mu\text{l min}^{-1}/10^6$ cells, respectively). As can be seen in figure 1D, the order of hepatocyte minipig CL_{int} values for the cocktail compounds was diclofenac $\leq$ diazepam $<$ midazolam $<$ phenacetin $<$ 7-hydroxycoumarin $<$ bufuralol.

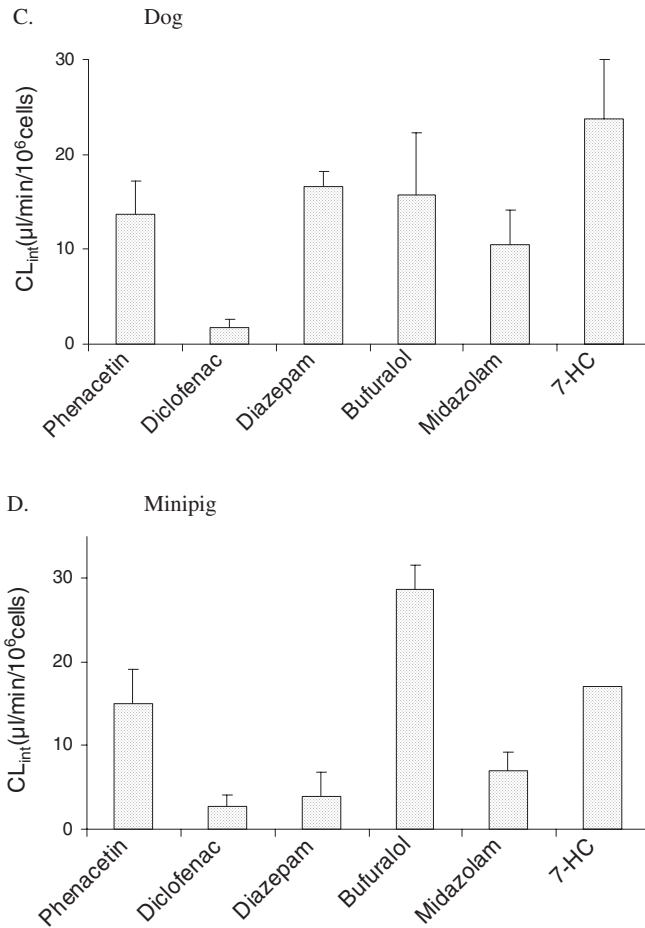


Figure 1. Continued.

Table 2. Effect of 7-ethoxycoumarin on the intrinsic clearance for phenacetin in incubations with fresh rat hepatocytes and cryopreserved human and dog hepatocytes.

Species	Intrinsic clearance rates (µl min ⁻¹ /10 ⁶ cells)	
	Phenacetin CL _{int} (number of experiments)	Phenacetin CL _{int} (number of experiments) + 7-ethoxycoumarin
Human	7.0 ± 1.3 (6)	3.4 ± 1.8 (6)**
Rat	14.3 ± 5.5 (7)	4.3 ± 2.1 (7)***
Dog	13.7 ± 3.5 (3)	8.4 ± 5.8 (5)

p* < 0.01, *p* < 0.001 compared with incubations without 7-ethoxycoumarin.

Incubation of cryopreserved human hepatocytes with the cocktail and ketoconazole

Human hepatocytes were incubated with the cocktail together with 1 µM ketoconazole. This resulted in a decrease in CL_{int} for midazolam to 7.5% of the control value (without the inhibitor), whereas the metabolism of the other cocktail compounds were virtually unaffected by this treatment (figure 2).

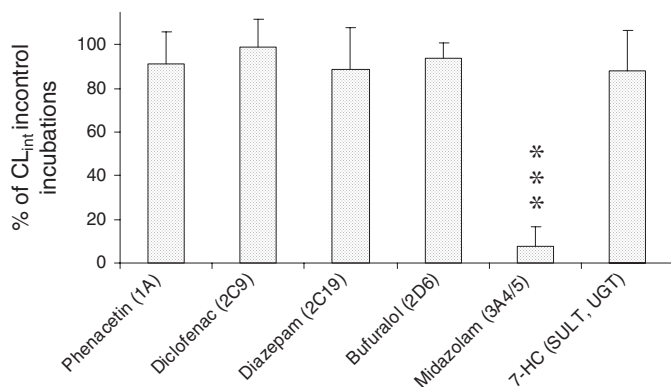


Figure 2. Effect of 1 μ M ketoconazole on the intrinsic clearance for the cocktail compounds in suspensions of cryopreserved human hepatocytes. Results are percentages of the intrinsic clearance in hepatocytes incubated without ketoconazole (control). Data are mean $\pm$ standard deviations of four experiments. Hepatocytes from one or two individuals were used in each experiment, giving a total number of five individuals. The enzymes assessed are indicated in parenthesis. *** $p < 0.001$ compared with the incubations without ketoconazole.

Discussion

The aim of this study was to develop a method for the simultaneous determination of several enzyme activities to be used as a quality control in hepatocyte experiments. This could be achieved by using a cocktail with specific probes for major human forms of cytochrome P450 and for UDP-glucuronosyltransferase and sulphotransferase. These specific probe substrates were phenacetin (for human CYP1A2), diclofenac (CYP2C9), diazepam (CYP2C19), bufuralol (CYP2D6), midazolam (CYP3A4/5) and 7-hydroxycoumarin (for glucuronidation and sulphation). An LC-MS/MS method for the analysis of all compounds in the same run was developed in an Open Access environment. Open Access is suitable for less experienced MS operators, since it allows them to login, submit and analyse sample sets at any time of the day without immediate attendance of an experienced analyst.

The CL_{int} values were calculated from the disappearance of the probe substrates from the incubations. Similar approaches have been applied earlier to both fresh and cryopreserved hepatocytes (Lave *et al.* 1996, Lau *et al.* 2002, Bachmann *et al.* 2003, Naritomi *et al.* 2003) as well as to liver slices and microsomes (Axelsson *et al.* 2003). For example, by measuring the disappearance of six xenobiotics from incubations with cryopreserved human hepatocytes, Bachmann *et al.* (2003) predicted hepatic clearances that were within twofold of *in vivo* clearances. Moreover, by applying a similar approach, Lau *et al.* (2002) could demonstrate a satisfactory correlation between *in vitro* CL_{int} in human cryopreserved hepatocytes and *in vivo* human clearance for 64 compounds.

The highest CL_{int} value estimated with human and dog hepatocytes, was observed for 7-hydroxycoumarin. For rat and minipig hepatocytes, the highest CL_{int} was observed for bufuralol. In incubations with dog and minipig hepatocytes, the lowest CL_{int} value was seen with diclofenac, whereas for human and rat hepatocytes the lowest value was observed with diazepam and phenacetin, respectively.

Completely different cocktail compound CL_{int} profiles were observed for the four species tested here (figure 1A–D). This clearly illustrates the difficulties with inter-species extrapolations of drug metabolism. The classification of cytochrome P450 enzymes into subfamilies is based on amino acid identity (Nelson *et al.* 1993). However, a high degree of sequence identity does not automatically mean similar catalytic activity (Guengerich 1997, Bogaards *et al.* 2000) and even a single amino acid substitution may result in a change in substrate specificity (Lindberg and Negishi 1989). Thus, it is conceivable that the same CYP probe substrate usually gives different CL_{int} values in different species. Detailed comparisons between the substrate specificities in different species would require more information about the specific reactions, including K_m and V_{max} determinations, preferably for different CYP enzymes. The objective of our present study was simply to ensure quality and reproducibility of experiments, and no further interpretations of the species differences found here have been made.

The use of multiple probe substrates to evaluate the metabolic capacity of drug-metabolizing enzymes requires that there are no inter-substrate interactions. By using low substrate concentrations, the risk of such interactions can be reduced. Initially, we used a cocktail with 7-ethoxycoumarin instead of 7-hydroxycoumarin. However, we found that the metabolism of phenacetin in human and rat hepatocytes was significantly inhibited in the presence of 7-ethoxycoumarin even at these low concentrations (table 2). This is most likely explained by the fact that the deethylation of 7-ethoxycoumarin can be catalysed by CYP1A2 (Yamazaki *et al.* 1996), which is the same CYP enzyme that catalyses the deethylation of phenacetin.

Although diazepam is commonly used as a specific probe substrate for human CYP2C19, it can also be metabolized by CYP3A4 (Andersson *et al.* 1994). It was, however, demonstrated that diazepam had no effect on the CL_{int} of the CYP3A4 probe substrate midazolam (data not shown) under the conditions used (with low substrate concentration). In addition, diazepam is easily analysed in the same assay as the other compounds of our cocktail.

Drug cocktails can be used for drug–drug interaction studies *in vivo* (Christensen *et al.* 2003, Blakey *et al.* 2004). Moreover, Dierks *et al.* (2001) have investigated the effect of specific CYP inhibitors on the metabolism of compounds in a cocktail in incubations with human hepatic microsomes. They concluded that their cocktail method could be a useful tool to determine CYP inhibition during drug development. The approach to employ our cocktail for drug–drug interaction studies with human hepatocytes was investigated here. In these experiments we could show that the metabolism of midazolam, which is metabolized by human CYP3A4/5, was significantly decreased by 1 μ M ketoconazole, whereas the metabolism of the other cocktail compounds were virtually unaffected by this specific CYP3A inhibitor under the conditions used.

In summary, our *in vitro* cocktail method saves time by the simultaneous determination of CL_{int} for six probe substrates of major human drug-metabolizing enzymes. We suggest that this cocktail can be used routinely to follow batch-to-batch variations in the metabolic capacity of hepatocyte preparations also in other species. In addition, the utilization of this cocktail could be of value for the assessment of CYP inhibition during drug development. Our cocktail is now included as a positive control to assess the quality of all our hepatocyte preparations.

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Metabolic properties of the acid and lactone forms of HMG-CoA reductase inhibitors

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1. To gain a better understanding of the metabolic properties between the open acid and lactone form of HMG-CoA reductase inhibitors (statins), the paper focused primarily on characterizing the metabolic properties of statins. We compared the metabolism of the acid and lactone forms of several statins, including atorvastatin, simvastatin, cerivastatin fluvastatin, pitavastatin and rosuvastatin with respect to metabolic clearance, CYP enzymes involved and drug–drug interactions.

2. A remarkable increase in metabolic clearance was noted for all lactones compared with all acids except for pitavastatin lactone. The metabolic clearances of the atorvastatin, simvastatin, cerivastatin, fluvastatin and rosuvastatin lactones were 73-, 70-, 30-, 7- and 64-fold higher, respectively, than those of the corresponding acids.

3. CYP2Cs were critically involved in the metabolism of cerivastatin, fluvastatin and pitavastatin acids. In contrast, CYP2Cs were not involved in the metabolism of the corresponding lactones and CYP3A4 was mainly involved. Moreover, a substantial difference in the metabolic inhibition of statins was found between acids and lactones.

4. Overall, the study demonstrates that CYP-mediated metabolism of lactones is also a common metabolic pathway for statins and that the CYP3A4-mediated metabolism of the lactone forms clearly will need to be taken into account in assessing mechanistic aspects of drug–drug interaction involving statins.

Introduction

Generally, elevated levels of low-density lipoprotein (LDL)-cholesterol are considered the most important risk factor for coronary artery disease (Brown *et al.* 1993). The most effective method of lowering LDL-cholesterol is the administration of HMG-CoA reductase inhibitors, namely statins (Havel and Rapaport 1995). Pitavastatin is a novel synthetic statin and has a persistent effect on serum lipids and its safety has been confirmed in clinical practice (Kajinami *et al.* 2000, 2003).

Marketed statins can be classified into the following two groups: those administered as a pro-drug, i.e. the lactone form including simvastatin and lovastatin, and those administered in the active form, i.e. the acid form such as atorvastatin, fluvastatin, pravastatin, rosuvastatin and pitavastatin. Pitavastatin is administered as the calcium salt of the active hydroxy acid. However, the areas under the concentration–time curves (AUCs) of pitavastatin lactone were similar in clinical studies (Kojima *et al.* 1999, Fujino *et al.* 1999a). Also, no marked

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difference was found in the AUC of atorvastatin and its lactone form in plasma following oral administration to humans (Kantola *et al.* 1998). Lactones have been reported to be a metabolite of the acid form statins (Dain *et al.* 1993, Boberg *et al.* 1998, Cooper *et al.* 2002). Recent studies have additionally demonstrated glucuronidation to be a novel metabolic route for statin clearance and glucuronidation occurs at the dihydroxy heptanoic or heptenoic acid side-chain, common to the active forms of all marketed statins (Prueksaritanont *et al.* 2002). UDP-glucuronosyl transferase (UGTs), such as UGT1A1 and UGT1A3, are critically involved in ester-type glucuronidation of these statins. Subsequently, the statin glucuronides are converted to the lactone form by an elimination reaction. We also clarified the mechanism for the lactonization of pitavastatin. In UGT studies, at least two UGT isoforms, UGT1A3 and UGT2B7, are principally responsible for the lactonization of pitavastatin (Fujino *et al.* 2003). On the other hand, lactonization increased the metabolic clearance of atorvastatin in human hepatic microsomes (Jacobsen *et al.* 2000). Also, the K_i values of lactones for CYP3A4-mediated mexazolam metabolism were much smaller than those of the acid forms (Ishigami *et al.* 2001). These results indicated that the metabolic properties of acids and lactones are substantially different.

The current study compared the metabolism of the acid and lactone forms of several statins, including atorvastatin, simvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin, with respect to metabolic clearance, the CYP enzymes involved and drug-drug interactions.

Materials and methods

Chemicals

[Fluorobenzene- U - ^{14}C] pitavastatin (specific radioactivity: 981 kBq mg $^{-1}$) was synthesized by Amersham Bioscience Co. (Little Chalfort, UK). ^{14}C -pitavastatin lactone was produced according to Yamada *et al.* (2003). [Ring- U - ^{14}C] tolbutamide (Amersham Bioscience Co., 2.26 GBq mmol $^{-1}$), and [2-benzoyl ring U - ^{14}C] paclitaxel (Sigma-Aldrich, 2.23 MBq mg $^{-1}$) were purchased commercially. The radiochemical purities of ^{14}C -labeled chemicals were more than 99%. Pitavastatin and pitavastatin lactone were synthesized by Nissan Chemical Industries (Chiba, Japan). The acids and lactones of atorvastatin, simvastatin, fluvastatin, cerivastatin and rosuvastatin were synthesized in our laboratories. The chemical purities of these statins were more than 98%. 4-Hydroxytolbutamide, 6- α -hydroxypaclitaxel and 3-hydroxypaclitaxel were purchased from Ultrafine Chemicals (Manchester, UK). All other chemicals were commercially available and of guaranteed purity.

Microsomes

Pooled and individual human hepatic microsomes were purchased from BD-GENTEST (Woburn, MA, USA). The recombinant microsomes derived from baculovirus expressing human CYPs (CYP1A2, CYP2C8, CYP2C9, CYP2D6 and CYP3A4) and control microsomes were also obtained from BD-GENTEST. The NADPH-regenerating system (β -NADP $^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were obtained commercially.

Incubation conditions

The incubation conditions were the same as in Fujino *et al.* (1999b, 2003). Briefly, the incubation mixture was prepared as follows: a reaction mixture containing 0.1–1 mg microsomal protein ml $^{-1}$ 1.3 mM β -NADP $^+$, 3.3 mM glucose 6-phosphate, 0.4 U ml $^{-1}$ glucose 6-phosphate dehydrogenase and 3.3 mM magnesium chloride in 100 mM sodium phosphate buffer (pH 7.4) (final incubation volume of 500 μ l) was incubated. After incubation at 37°C, the reaction was quenched by adding an equal volume of acetonitrile. The reaction-terminated samples were centrifuged at 6000 g and the supernatant was subsequently analysed for statin disappearance.

Metabolic clearance of statins in human hepatic microsomes

The acid and lactone forms, at a concentration of 2.5 μM , were incubated with human hepatic microsomes and the metabolic clearance (CL_{int}) determined. The residual acid and lactone remaining were measured 60 min after the addition of hepatic microsomes. In the determination of *in vitro* CL_{int} , the slope ($-k$) of the linear regression of the log concentration versus incubation time relationships was used in the calculation as follows:

$$\text{CL}_{\text{int}} = k \times (\text{ml incubation}) / (\text{mg microsomes}).$$

In the evaluation of the CYP-mediated pitavastatin lactone and rosuvastatin lactone clearances, the acid form, which was converted from the lactone non-enzymatically, was calculated as the amount of residual lactone.

Possible role of CYP enzymes in statin metabolism

Using five recombinant human CYP-expressing microsomes, the CYP enzymes participating in the metabolism of statins were investigated. The reaction mixtures were incubated for 20 min for all statins (2.5 μM). In addition, incubation with control microsomes (native insect cells) was performed in parallel. The incubation conditions were the same as for the human hepatic microsomes described above.

Correlation of statin metabolism with CYP activities

Using ten individual human hepatic microsomes, the correlation between pitavastatin lactone, rosuvastatin lactone or fluvastatin metabolism and CYP marker activity was studied. Assays described in the data sheet from BD-GENTEST for CYP2C9 (diclofenac 4'-hydroxylase), CYP2D6 (Bufuralol 1'-hydroxylase) and CYP3A4 (testosterone 6 β -hydroxylase) were used.

Effect of statins on the metabolism of several CYP markers.

The inhibitory effect of statins on CYP-mediated metabolism was examined using ^{14}C -tolbutamide and ^{14}C -paclitaxel in the concentration range of 15–200 μM and 2.5–40 μM , respectively. In the inhibition study, statins were co-incubated with ^{14}C -tolbutamide or ^{14}C -paclitaxel and the production of CYP-mediated metabolite, such as CYP2C9-mediated 4-hydroxytolbutamide, CYP2C8-mediated 6- α -hydroxypaclitaxel and CYP3A4-mediated 3-hydroxy-paclitaxel were evaluated, respectively. The apparent inhibition constants (K_i) in each experiment were estimated by non-linear regression. The incubation conditions of these CYP model substrates were the same as in Fujino *et al.* (1999b).

Analytical methods

Quantification of cerivastatin, simvastatin, fluvastatin, atorvastatin and rosuvastatin was performed according to previous reports using LC/MS/MS (Prueksaritanont *et al.* 1997, Jemal *et al.* 1999a,b, Hull *et al.* 2002). All statins were detected in the positive-ion mode. The precursor/product ion transitions monitored were m/z 437.3/303.0, 419.3/285.0 (for simvastatin acid and simvastatin), 460.8/356.5, 442.2/354.0 (for cerivastatin and cerivastatin lactone), 411.0/265.3, 393.5/289.5 (for fluvastatin and fluvastatin lactone), 559.0/440.6, 541.2/448.2 (for atorvastatin and atorvastatin lactone) and 482.0/258.0, 464.56/269.5 (for rosuvastatin and rosuvastatin lactone), respectively.

The measurement of pitavastatin was undertaken according to a previously described HPLC method (Kimata *et al.* 1998). The measurement of 4-hydroxytolbutamide was performed by a TLC method (Ludwig *et al.* 1998). In the case of paclitaxel, the simultaneous determination of 6- α -hydroxypaclitaxel and 3-hydroxypaclitaxel were undertaken according to a TLC method (Fujino *et al.* 2001). The amount of radioactive unchanged drug and its metabolites were determined using a BAS-2500 system (Fuji Photo Film Co., Tokyo, Japan).

Results*Metabolic properties of statins in human hepatic microsomes*

Figure 1 shows the metabolic clearance of several statins in human hepatic microsomes. The acid form of pitavastatin and rosuvastatin show little metabolism compared with other statins. On the other hand, extensive metabolism of the lactone form of statins was noted in human hepatic micorosmes compared with the acid forms, except for pitavastatin. The metabolic clearances of the acid form and lactone form of atorvastatin were 26 and 1892 $\mu\text{l min}^{-1} \text{mg}^{-1}$ protein, respectively (table 1). Also, the metabolic clearance of simvastatin was 28 $\mu\text{l min}^{-1} \text{mg}^{-1}$

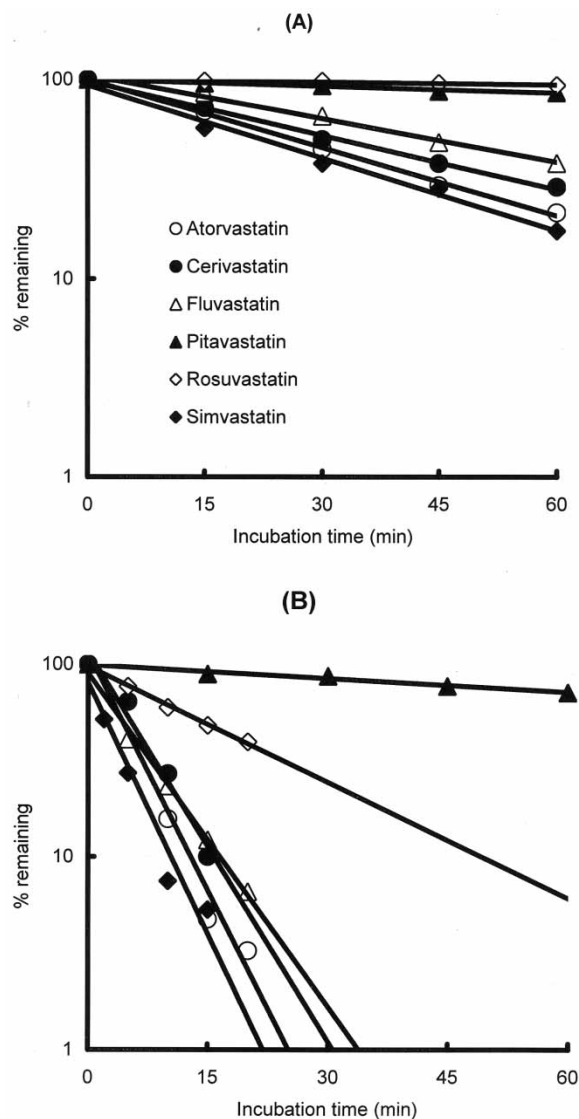


Figure 1. Metabolic stability of statins in human hepatic microsomes: (A) acid form and (B) lactone form (substrate concentration: $2.5 \mu\text{M}$, microsomal protein concentration for acid forms: $1 \text{ mg protein ml}^{-1}$, pitavastatin lactone: 1 mg protein/ml , rosuvastatin lactone: $0.5 \text{ mg protein ml}^{-1}$, fluvastatin lactone and cerivastatin lactone: $0.25 \text{ mg protein ml}^{-1}$, atorvastatin lactone and simvastatin: $0.1 \text{ mg protein ml}^{-1}$). Data are derived from duplicate determinations.

protein in the acid form and $1959 \mu\text{l min}^{-1} \text{mg}^{-1}$ protein in the lactone form. Rosuvastatin lactone also showed an extensive metabolism in human hepatic microsomes ($71 \mu\text{l min}^{-1} \text{mg}^{-1}$ protein) in contrast to the acid form. Taken collectively, the metabolic clearance of the lactones was up to 64-fold higher than that of the acids. In the case of cerivastatin and fluvastatin, the metabolic clearance of the statins was 30- and 7-fold higher than that of the acids, respectively. However, both pitavastatin lactone and acid showed little metabolism in human hepatic microsomes, with the metabolic clearances of $3 \mu\text{l min}^{-1} \text{mg}^{-1}$

Table 1. Metabolic properties of acid and lactone forms of statins.

Statin	Metabolic clearance* (CL_{int}) ($\mu\text{l min}^{-1} \text{mg}^{-1}$ protein)		CL_{int} ratio (lactone/acid)	CYP enzymes	
	Acid form	Lactone form		Acid form	Lactone form
Atorvastatin	26	1892	73	CYP3A4	CYP3A4
Simvastatin	28	1959	70	CYP3A4	CYP3A4
Cerivastatin	21	622	30	CYP2C8	CYP3A4
				CYP3A4	
Fluvastatin	33	226	7	CYP2C9	CYP3A4
				CYP3A4**	
Rosuvastatin	1.1	70.7	64	–	CYP3A4
					CYP2C9**
					CYP2D6**
Pitavastatin	2.5	5.4	2	CYP2C9	CYP3A4
				CYP2C8	CYP2D6**

* Substrate concentration: 2.5 μM ; ** minor involvement.

protein and $5 \mu\text{l min}^{-1} \text{mg}^{-1}$ protein for the acid and lactone, respectively. In addition, the appearance of the acid form, which was converted from the lactone non-enzymatically, was 30% for pitavastatin lactone and rosuvastatin lactone in human hepatic microsomes. However, the appearance of acids was negligible in other lactones examined (data not shown).

Identification of CYP enzymes responsible for statin metabolism

The CYP enzymes were identified by several human CYP expressing microsomes (figure 2). CYP3A4 plays a major role in the metabolism of atorvastatin and simvastatin acids. Also, no marked difference was shown in the involvement of CYP enzymes on the metabolism of lactones. Cerivastatin was metabolized by two CYPs, CYP2C8 and CYP3A4, respectively. On the other hand, cerivastatin lactone was metabolized by CYP3A4, and CYP2C8 was not involved. In the case of fluvastatin, CYP2C9 and CYP3A4 were involved the metabolism of the acid form. In contrast, fluvastatin lactone was metabolized by CYP3A4 and no involvement of CYP2C9 was apparent. For pitavastatin, CYP2Cs such as CYP2C9 and CYP2C8 were involved in the metabolism of the acid form. On the other hand, CYP2D6 and CYP3A4 were principally responsible for the metabolism of pitavastatin lactone and CYP2Cs showed no involvement. In the case of rosuvastatin, no involvement of CYP enzymes was apparent. In contrast, several CYP enzymes, including CYP2C9, CYP2D6 and CYP3A4 were involved in the metabolism of rosuvastatin lactone.

To gain a better understanding of the contribution of these CYPs to the metabolism of pitavastatin lactone, fluvastatin and rosuvastatin lactone, we investigated correlation studies using ten individual human hepatic microsomes. CYP3A4-mediated testosterone 6β -hydroxylase activities showed good correlation with pitavastatin lactone ($r^2=0.911$) and rosuvastatin lactone ($r^2=0.869$). These results indicated that CYP3A4 plays a major role in the metabolism of these lactones. Also, CYP2C9-mediated diclofenac metabolism showed a good correlation with fluvastatin metabolism ($r^2=0.845$). In contrast, a lower correlation was observed between other CYP-mediated metabolism and statin metabolism (data not shown).

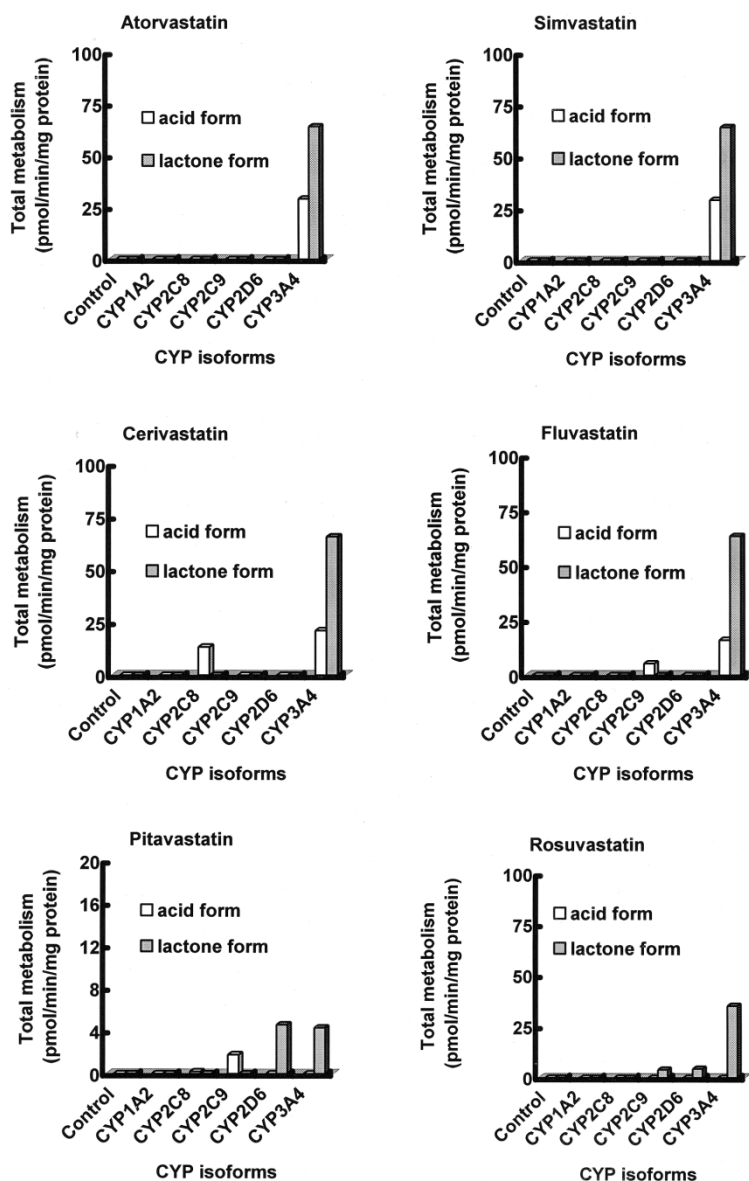


Figure 2. Identification of CYP enzymes metabolizing statins (Substrate concentration: 2.5 μ M, incubation time: 20 min) Data are derived from duplicate determinations.

Inhibitory effect of statins on CYP-mediated metabolism

Figure 3 shows the inhibitory effect of acids and lactones of rosuvastatin on CYP-mediated metabolism. In the presence of the acid form of rosuvastatin, parallel lines without a focal point were obtained for CYP-mediated metabolism. In contrast, in the presence of the lactone form, focal points were obtained in all experiments, demonstrating that there was inhibition of CYP2C9-, CYP2C8- and CYP3A4-mediated metabolism by rosuvastatin lactone (figure 3). The apparent K_i values for rosuvastatin lactone were 14.8, 9.8 and 5.8 μ M for CYP2C9, CYP2C8 and CYP3A4, respectively.

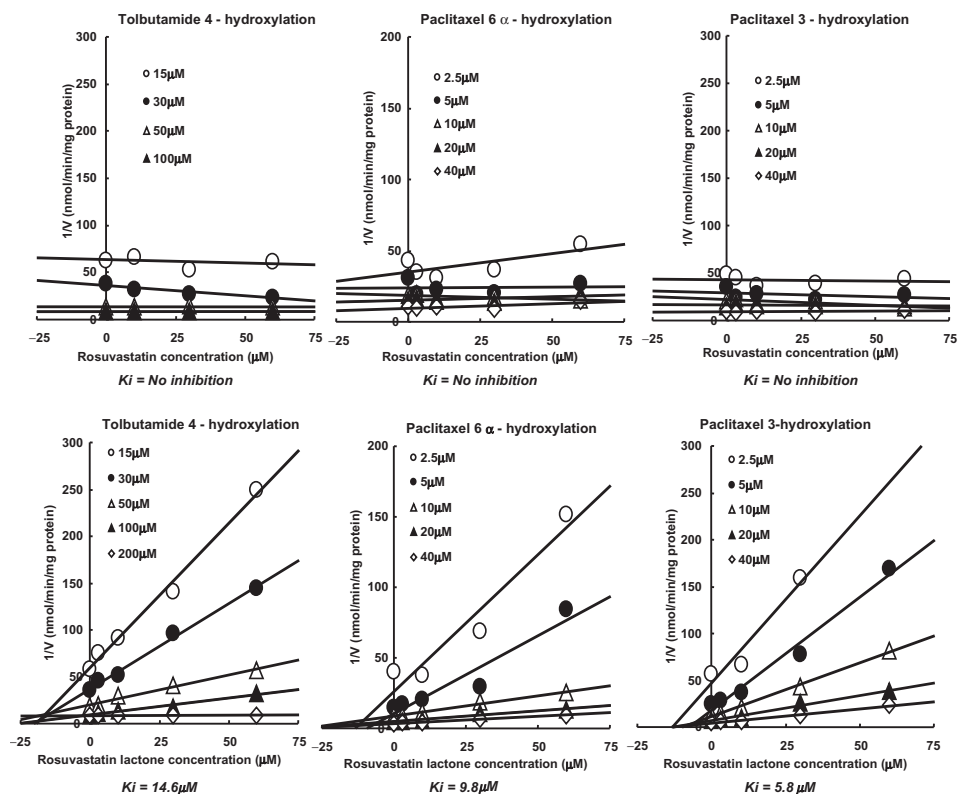


Figure 3. Inhibition of tolbutamide 4-hydroxylation, paclitaxel 6- α -hydroxylation and paclitaxel 3-hydroxylation by rosuvastatin in human hepatic microsomes. Data are derived from duplicate determinations.

The acid form of fluvastatin showed competitive inhibition for CYP2C9-mediated tolbutamide 4-hydroxylation and its apparent K_i value was 0.5 μM (table 2). In contrast, fluvastatin lactone showed no inhibition of tolbutamide metabolism. In addition, in the presence of either acid or lactone form of pitavastatin, an inhibitory effect was not observed on CYP2C9-mediated metabolism. Moreover, cerivastatin acid inhibited the 6- α -hydroxylation of paclitaxel and its apparent K_i value was 34.4 μM . In contrast, cerivastatin lactone did not inhibit the CYP2C8-mediated metabolism of paclitaxel but inhibited the CYP3A4-mediated 3-hydroxylation of paclitaxel ($K_i = 16.8 \mu\text{M}$).

Discussion

As reported, several CYPs, such as CYP3A4 and CYP2Cs, are involved in the metabolism of the acid form of statins (Christians *et al.* 1998, Fischer *et al.* 1999, Fujino *et al.* 1999a, b, Mück 2000). In the current study, we compare the metabolic properties of acid and lactone statins. First, a substantial CYP3A4-mediated metabolic clearance was found for all statins examined except for pitavastatin. The metabolic clearances (CL_{int}) of the lactones of atorvastatin, simvastatin, rosuvastatin, cerivastatin and fluvastatin were 73-, 70-, 64-, 30- and 7-fold higher, respectively, than those of the corresponding acids. Second, the switching of

Table 2. Metabolic inhibition of CYP-mediated metabolism by statins.

Metabolic pathway	CYP	Statins	Form	K_i (μ M)
Tolbutamide 4-hydroxylation	2C9	pitavastatin	acid	N.I.
			lactone	N.I.
		fluvastatin	acid	0.5
			lactone	N.I.
		rosuvastatin	acid	N.I.
Paclitaxel 6 α -hydroxylation	2C8	cerivastatin	lactone	14.6
			acid	34.4
		rosuvastatin	lactone	N.I.
			acid	N.I.
		rosuvastatin	lactone	9.8
Paclitaxel 3-hydroxylation	3A4	cerivastatin	acid	N.I.
			lactone	16.8
		rosuvastatin	acid	N.I.
			lactone	5.8

N.I.; no inhibition.

CYPs between acids and lactones was noted for cerivastatin, fluvastatin and pitavastatin, respectively. CYP2Cs were not involved, but CYP3A4 was involved, in the metabolism of these lactones. Interestingly, CYP3A4 was principally responsible for the metabolism of all lactones examined. Taking the above into consideration, we hypothesize that CYP3A4 inhibitors would block CYP3A4-mediated lactone metabolism, leading to an increase in the plasma concentration. In fact, grapefruit juice increases the plasma concentration of statins; however, the increased ratio of lactone AUCs is much higher than that of acid form of simvastatin and atorvastatin (Lilja *et al.* 1998, 1999). Third, substantial differences in the metabolic inhibition of statins were shown between acids and lactones, except for pitavastatin. These results showed a good agreement with the switching of CYP enzymes between acid and lactones.

Generally, the hydrophobic substrate access channels of CYPs are positioned near the membrane surface and hydrophobic compounds dissolved in the membrane can directly enter the access channels from the membrane (Peterson *et al.* 1995). It seems that the more lipophilic lactone is a better CYP substrate than the acid form. Indeed, Greenspan *et al.* (1988) reported that the lactone form of lovastatin is subjected to metabolism rather than its open acid form, which in agreement with our findings. Taking these into consideration, our findings may be explained by the fact that lipophilic lactones have a high affinity for CYP3A4. In addition, there are numerous reports in the literature documenting the drug-drug interactions of statins (Christians *et al.* 1998, Paoletti *et al.* 2002, Williams and Feely 2002, Kajinami *et al.* 2003); however, almost all of these reports described an increase or decrease in the acid statins. Our findings indicate that future studies assessing the metabolism and drug-drug interactions of statins should include the lactone form.

The present results suggest that the metabolism of statins is complex, involving acid/lactone interconversion by various pathways, as proposed in figure 4. The acid forms of statins are converted to the corresponding lactones by a glucuronide intermediate (Prueksaritanont *et al.* 2002, Fujino *et al.* 2003). We did not measure the metabolites of statins, however, Jacobson *et al.* (2000) reported that the lactone form of atorvastatin did not result in the generation of

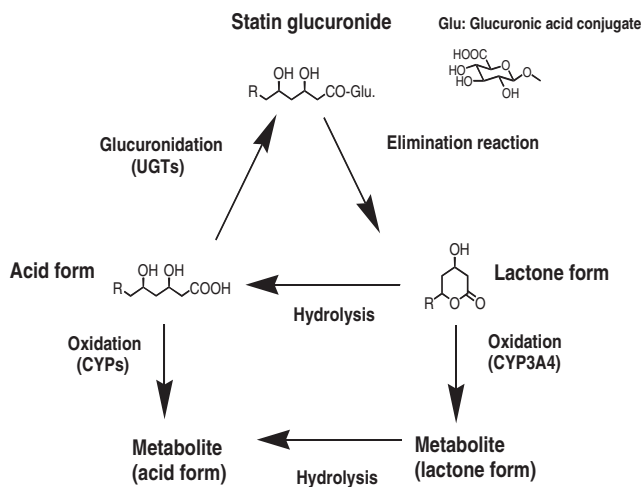


Figure 4. Proposed metabolic pathways of statins.

different metabolite patterns in human hepatic microsomes. We believe that the other statin lactones did not generate different metabolites. In human hepatic microsomes, a portion of pitavastatin lactone was metabolized by CYP3A4; however, it was mainly hydrolysed to the open acid form. In contrast, as the other lactones are metabolized by CYP3A4 with an extensive metabolic clearance, it can be expected that the metabolism of lactones is the relevant pathway for the elimination of statins. Indeed, the oxidative metabolites of atorvastatin lactone and cerivastatin lactone have been identified in animals and humans (Black *et al.* 1999). Also, the oxidative metabolites of atorvastatin were detected as both lactone and acid forms in human plasma with the concentrations of the lactone metabolites exceeding those of the corresponding acid metabolites (Kantorla *et al.* 1998). These reports suggested that after metabolism by CYP3A4, some of the resulting lactone metabolites are converted to the corresponding acid forms. In addition, metabolic profiling studies indicated that rosuvastatin undergoes relatively little metabolism by hepatic CYPs with no significant involvement of CYP3A4 (Nezaka *et al.* 2002). On the other hand, N-desmethyl rosuvastatin and rosuvastatin lactone were detected as major metabolites in human plasma (Cooper *et al.* 2002). Taking these reports and our findings into consideration, we hypothesize that most of the metabolites of statins present in human plasma are generated by interconversion of lactone metabolites, which are formed by CYP3A4.

In conclusion, the present study demonstrates that in addition to the well-known CYP- or UGT-mediated metabolism, CYP-mediated metabolism of lactones is also a common metabolic pathway for statins. Also, the metabolic properties of lactones will clearly need to be taken into account in assessing mechanistic aspects of drug-drug interaction involving statins.

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Role of human cytochrome P450 3A4 in the metabolism of DA-8159, a new erectogenic[#]

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1. The purpose of this paper was to characterize cytochrome P450 (CYP) enzymes involved in *N*-dealkylation of a new oral erectogenic, DA-8159 to DA-8164, a major circulating active metabolite, in human liver microsomes and to investigate the inhibitory potential of DA-8159 on CYP enzymes.

2. CYP3A4 was identified as the major enzyme responsible for DA-8159 *N*-dealkylation to DA-8164 based on correlation analysis and specific CYP inhibitor and antibody-mediated inhibition study in human liver microsomes, and DA-8159 metabolism in cDNA expressed CYP enzymes. There is the possibility of drug-drug interactions when prescribing DA-8159 concomitantly with known inhibitors or inducers of CYP3A4.

3. DA-8159 was found to be only a very weak inhibitor of eight major CYPs (1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4), the largest inhibition occurring against CYP2D6 (IC₅₀ 67.7 µM) in human liver microsomes. Drug-drug interactions would not be predicted on the basis of DA-8159 inhibiting the metabolism of coadministered drugs.

Introduction

DA-8159, 5-[2-propoxy-5-(1-methyl-2-pyrrollyldinylethylamidosulfonyl)-phenyl]-1-methyl-3-propyl-1,6-dihydro-7H-pyrazolo(4,3-d)pyrimidin-7-one, is a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5) (Oh *et al.* 2000). The data obtained from a phase I clinical trial demonstrated that DA-8159 is safe and well tolerated after a single oral dose in healthy subjects (Asian and Caucasian males for up to 300 mg or 400 mg without severe adverse events) (Amakye *et al.* 2004). DA-8159 is now being evaluated in phase III clinical trials as an oral therapy for erectile dysfunction.

Based on *in vitro* metabolism of DA-8159 in rat liver microsomes and rat isolated perfused liver, DA-8159 is biotransformed to three metabolites: hydroxy-DA-8159, *N*-demethyl-DA-8159 and DA-8164 (figure 1) (Choi *et al.* 2002). Following oral doses of DA-8159 to humans, plasma concentrations of DA-8164 are approximately 50% of those seen for DA-8159 (Kim *et al.* 2003, Amakye *et al.* 2004). DA-8164 has a half lower *in vitro* potency for the inhibition of PDE5 compared to that of DA-8159 and has been also identified as a major metabolite

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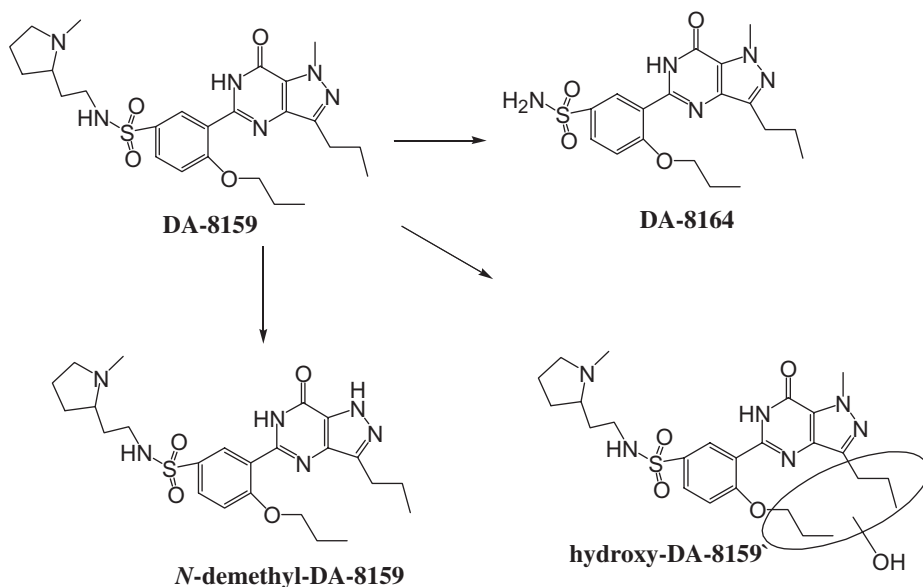


Figure 1. Metabolic pathways of DA-8159 in rat and human liver microsomes.

in the incubations with human liver microsomes. The mean apparent elimination half-life of DA-8159 and an active metabolite DA-8164 were similar (12 h) (Amakye *et al.* 2004).

The oxidative metabolism of a large number of therapeutic agents is mediated by CYP. The information on specific CYP enzyme(s) involved in the biotransformation of a drug can be of considerable clinical impact in regard to potential drug interactions and inter-individual variation in drug metabolism. Consequently, the evaluation of human CYP enzyme(s) responsible for the metabolism of a given drug can be useful in understanding the disposition and the *in vivo* behaviour of the drug and may have clinical implications.

The present study describes the *in vitro* investigation into the metabolism of DA-8159 to identify the human CYP enzyme(s) responsible for the biotransformation of DA-8159 to the active metabolite DA-8164 and to investigate the inhibitory effects of DA-8159 and DA-8164 on major human CYP enzymes in human liver microsomes.

Materials and methods

Materials

DA-8159 and DA-8164 [5-(2-propoxy-5-aminosulfonylphenyl)-1-methyl-3-propyl-1, 6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one] were synthesized by Dong-A Pharmaceutical Co. (Yongin, Korea) with >99.8% purity. NADP⁺, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, α -naphthoflavone, quercetin, tranlycypromine, chlorzoxazone, sulfaphenazole, diethyldithiocarbamate, ketoconazole, and quinidine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, acetonitrile and methylene chloride (spectroscopy grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available.

Human liver microsomes (coded H003, H023, H042, H043, H056, H066, H070, H089, H093, H112, HK23, HK34 and a pooled microsome H161) and microsomes derived from baculovirus infected insect cells, transfected with human P450 cDNA over-expressing CYP-1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, were purchased from the Gentest (Woburn, MA, USA). Human selective monoclonal antibodies for the immunoinhibition of human CYP, *i.e.*, anti-CYP1A2, anti-CYP2D6, anti-CYP2B6, anti-CYP2E1 and anti-CYP3A4, and polyclonal antisera of CYP2C (anti-2C) were obtained from Gentest.

Assay for DA-8159 N-dealkylation

The metabolism of DA-8159 to DA-8164 by human liver microsomes or cDNA-expressed CYP enzymes was determined according to the following method. Each incubation contained 50 mM potassium phosphate buffer (pH 7.4), human liver microsomes (0.2 mg protein ml⁻¹) or one of eight cDNA-expressed CYP enzymes (5 pmol), 3 mM magnesium chloride, NADPH generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate and 0.8 U ml⁻¹ glucose-6-phosphate dehydrogenase) and DA-8159 (5 μ M, added in acetonitrile, final solvent concentration not exceeding 0.5%, v/v) in a final volume of 200 μ l. The incubation mixture was incubated for 10 min at 37 °C, and the reaction was terminated by the addition of 800 μ l of dichloromethane followed by internal standard (sildenafil 500 ng ml⁻¹, 10 μ l). Following mixing with a vortex mixer and centrifugation, an aliquot of the organic layer (700 μ l) was transferred and evaporated under a nitrogen stream. The residue was dissolved in 40 μ l of 30% acetonitrile and an aliquot (10 μ l) was analyzed by LC/MS/MS.

The concentrations of DA-8164 in the microsomal incubates were analysed by a slight modification of LC/MS/MS method developed from our laboratories (Kim *et al.* 2003). The separation was performed on a Luna-phenylhexyl column (3 μ m, 2.0 mm i.d. $\times$ 100 mm, Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile and 5 mM ammonium formate (55:45, v/v) at a flow rate of 0.2 ml min⁻¹. The column and autosampler tray temperature were 30 °C and 4 °C, respectively. The eluent was introduced directly into a tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, Manchester, UK) through the positive ionization electrospray interface. The ion source and desolvation temperature were held at 120 °C and 350 °C, respectively. The optimum cone voltage for ionization of DA-8164 and sildenafil (internal standard) were 40 V. The molecular ions of DA-8164 and sildenafil were fragmented at collision energy of 30 and 13 eV, respectively, using argon as collision gas. Detection of the ions was performed by monitoring the transitions of m/z 406 to m/z 299 for DA-8164 and m/z 475 to m/z 100 for sildenafil. Peak areas for all components were automatically integrated using MassLynx 3.5 software (Micromass UK Ltd). The amount of DA-8164 formed in the incubations was determined by interpolation from standard curves constructed in microsomes with 0.5 to 400 pmol DA-8164. The rate of DA-8164 formation was expressed as pmol DA-8164 formed min⁻¹ mg⁻¹ microsomal protein.

DA-8159 kinetics in human liver microsomes

The kinetic study for the formation of DA-8164 was conducted over a DA-8159 range of 1–200 μ M in three human liver microsomes (H003, H042 and H112) and cDNA-expressed 3A4 enzyme. The rates of DA-8164 formation were determined in duplicate to obtain values for K_m and V_{max} .

Correlation with probe substrate activities

A bank of 12 human liver microsomes was used to assess DA-8159 metabolism. A correlation was performed between the rate of DA-8164 formation at 5 μ M DA-8159 and each CYP activity across these liver microsomes reported by Gentest using the Pearson Product Moment Correlation.

Inhibition experiments

Immunoinhibition studies were performed by incubating human liver microsomes with various amounts of human selective antibodies, anti-CYP1A2, anti-CYP2C, anti-CYP2D6, anti-CYP2E1 or anti-CYP3A4 for 15 min on ice before the addition of buffer, DA-8159 (5 μ M) and NADPH. As a control, comparable incubations were done with microsomes and 25 mM Tris buffer. The influence of CYP antibodies on the rate of DA-8164 formation was investigated.

The effects of selective chemical inhibitors for individual CYP enzymes on DA-8159 metabolism were investigated in pooled human liver microsomes (H161). Well characterized inhibitors of specific CYP enzymes, that is, α -naphthoflavone (CYP1A2), quercetin (CYP2C8), sulfaphenazole (CYP2C9), tranlylcypromine (CYP2C19), quinidine (CYP2D6), diethyldithiocarbamate (CYP2E1) and ketoconazole (CYP3A4) (Bourrie *et al.* 1996, Eagling *et al.* 1998, Dierks *et al.* 2001) were incubated for 10 min at 37 °C with 5 μ M DA-8159, pooled human liver microsome (H161) and the NADPH-generating system. Diethyldithiocarbamate was preincubated for 10 min with microsomes and the NADPH-generating system before addition of DA-8159 to initiate the reaction. For all of the inhibitors, a comparison was made to the activity of control incubations that did not contain the inhibitor.

Inhibitory potential of DA-8159 on CYP Activities in human liver microsomes

The inhibitory potential of DA-8159 was investigated by performing assays for specific cytochrome P450 activities in the absence and presence of DA-8159 (final concentrations of 1-400 μ M with acetonitrile concentration less than 0.5% v/v) using pooled human liver microsomes (H161). All incubations were performed in duplicate. Assays for specific cytochrome P450 activities were performed, as described in earlier report as follows. Phenacetin *O*-deethylase, coumarin 7-hydroxylase, paclitaxel 6 α -hydroxylase, diclofenac 4-hydroxylase, (*S*)-mephénytoin 4-hydroxylase, bufuralol 1'-hydroxylase, chlorzoxazone hydroxylase and testosterone 6 β -hydroxylase were determined as probe activities for CYP1A2 (Tassaneeyakul *et al.* 1993), CYP2A6 (Yamano *et al.* 1990), CYP2C8 (Crespi *et al.* 1998), CYP2C9 (Leemann *et al.* 1993), CYP2C19 (Wrighton *et al.* 1993), CYP2D6 (Kronbach *et al.* 1991), CYP2E1 (Guengerich *et al.* 1991) and CYP3A4 (Arlotto *et al.* 1991), respectively. The effect of DA-8159 on midazolam 1'-hydroxylase (von Moltke *et al.* 1996, Perloff *et al.* 2000) also was studied to further investigate the potential for interaction with CYP3A4.

Data analysis

All results are presented as mean $\pm$ SD. Several enzyme kinetic equations were fitted to the untransformed data using Enzyme kinetics software (version 1.1, SPSS Science Inc., Richmond, CA, USA). The equation, $V = V_{\max} \bullet S / (K_m + S)$ best described the kinetics of DA-8164 formation in human liver microsomes and cDNA-expressed CYP3A4 enzyme. V is the velocity of the reaction

at substrate concentration $[S]$; $V_{\max}$ the maximum velocity; K_m , the substrate concentration at which the reaction velocity is 50% of $V_{\max}$. The intrinsic clearance (Cl_{int}) of *in vitro* incubation was calculated as $V_{\max}/K_m$. Correlation analysis was carried out in SigmaStat software (version 2.0, SPSS Science Inc.).

Results

The rate of formation of DA-8164 in human liver microsomes was linear with respect to both time over 30 min and liver microsomal protein concentration (0.1–1.0 mg ml⁻¹, data not shown). The *N*-dealkylation of DA-8159 to DA-8164 in three human liver microsomes followed single-enzyme Michaelis-Menten kinetics (figure 2a). The apparent kinetic parameters for the formation of DA-8164 in three human liver microsomes were K_m values of 91.4 (± 19.8) μM and $V_{\max}$ values of 749 (± 533) pmol mg⁻¹ min⁻¹, respectively. Cl_{int} for *N*-dealkylation of DA-8159 to DA-8164 was 9.3 (± 7.8) $\mu\text{l mg}^{-1} \text{min}^{-1}$ (table 1).

The *N*-dealkylation of DA-8159 (5 μM) to DA-8164 was investigated in microsomes derived from baculovirus infected insect cells transfected with human P450 cDNA over-expressing CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 or 3A4. At 5 μM DA-8159, only CYP3A4 formed DA-8164 (0.50 pmol min⁻¹ pmol⁻¹ CYP) and none of the other CYP isoforms catalyzed *N*-dealkylation (< 0.01 pmol min⁻¹ pmol⁻¹ CYP).

The formation of DA-8164 in CYP3A4 microsomes was consistent with single-enzyme Michaelis-Menten kinetics with a K_m of 58.9 μM and a $V_{\max}$ of 959 pmol mg⁻¹ min⁻¹ (figure 2b).

The rates of DA-8164 formation from DA-8159 in 12 human liver microsomes ranged from 5.80 to 63.8 pmol/min/mg protein. As shown in table 2, a significant correlation was observed between rates of testosterone 6 β -hydroxylation, a marker of CYP3A4 and the formation of DA-8164 ($\gamma = 0.933$, $p < 0.001$). Poor correlations were observed with phenacetin *O*-deethylase (CYP1A2), paclitaxel 6 α -hydroxylase (CYP2C8), diclofenac 4'-hydroxylase (CYP2C9), *S*-mephenytoin 4'-hydroxylase

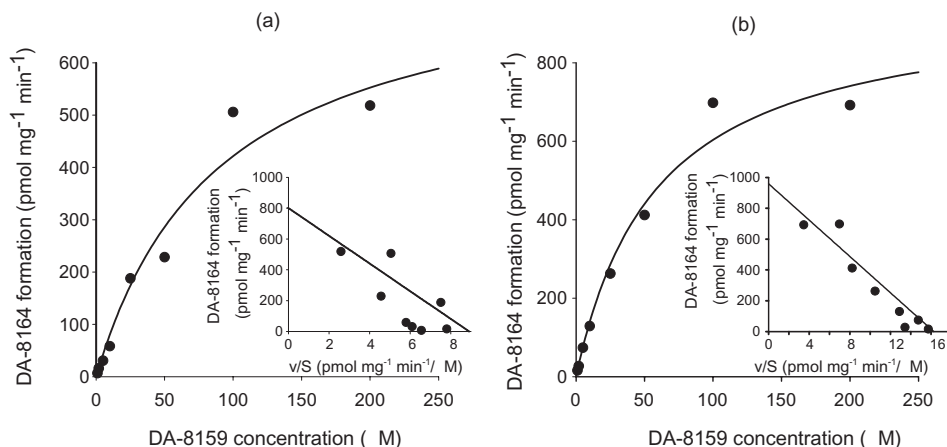


Figure 2. Michaelis-Menten plots for the biotransformation of DA-8159 to DA-8164 in (a) human liver microsomes H042 and (b) microsome expressing CYP3A4 with inset Eadie-Hoestel plots demonstrating single enzyme kinetics. Each data point represents the average of two determinations.

Table 1. Kinetic parameters of DA-8159 *N*-dealkylation to DA-8164 in human liver microsomes.

Human liver microsomes	K_m (μM)	$V_{\max}$ ($\text{pmol mg}^{-1} \text{min}^{-1}$)	Cl_{int} ($\mu\text{l mg}^{-1} \text{min}^{-1}$)
H003	111.9	192	1.71
H042	89.9	801	8.91
H112	72.4	1255	17.3
Mean $\pm$ S.D.	91.4 ± 19.8	749.2 ± 533	9.32 ± 7.822

Table 2. Correlation of CYP activities and the rates of formation of DA-8164 from DA-8159 in 12 different human liver microsomes.

Enzymatic activity	CYP isozymes	Correlation coefficient (<i>p</i> value)
Phenacetin <i>O</i> -deethylase	1A2	0.0707 (0.827)
Coumarin 7-hydroxylase	2A6	0.252 (0.429)
Paclitaxel 6 α -hydroxylase	2C8	0.231 (0.470)
Diclofenac 4'-hydroxylase	2C9	0.518 (0.0844)
<i>S</i> -mephenytoin 4'-hydroxylase	2C19	0.425 (0.169)
Bufuralol 1'-hydroxylase	2D6	0.495 (0.102)
Chlorzoxazone 6-hydroxylase	2E1	0.0119 (0.971)
Testosterone 6 β -hydroxylase	3A4	0.933 (0.0000961)

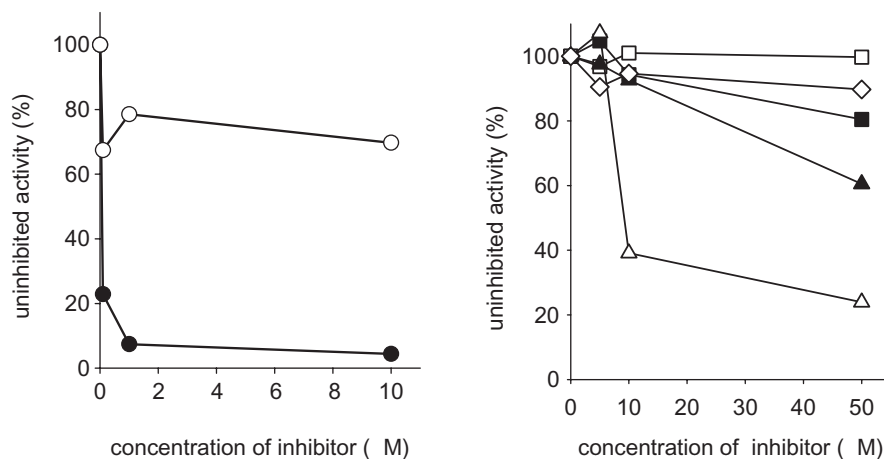


Figure 3. Inhibition of DA-8159 *N*-dealkylation to DA-8164 in human liver microsomes by several chemicals. Human liver microsome H161 was used, with the preincubation in the presence of NADPH-generating system and the indicated concentration of chemicals. ●: ketoconazole; ○: quinidine; ▲: sulfaphenazole; △: α -naphthoflavone; ■: tranlylcypramine; □: diethyldithiocarbamate; ◇: quercetin. Data are derived from two determinations. Rate of control reaction is 48.5 pmol/mg protein/min.

(CYP2C19), bufuralol 1'-hydroxylase (CYP2D6) and chlorzoxazone 6-hydroxylase (CYP2E1) activities.

The effects of selective CYP450 inhibitors on DA-8159 metabolism were investigated in pooled human liver microsomes (figure 3). Ketoconazole, a selective CYP3A4 inhibitor (Baldwin *et al.* 1995) and α -naphthoflavone, a CYP1A2 and CYP3A inhibitor (Boeke-Dohalska *et al.* 2001) effectively inhibited *N*-dealkylation of DA-8159 to DA-8164. There was no inhibition in the presence of quercetin (CYP2C8), sulfaphenazole (CYP2C9), tranlylcypramine (CYP2C19),

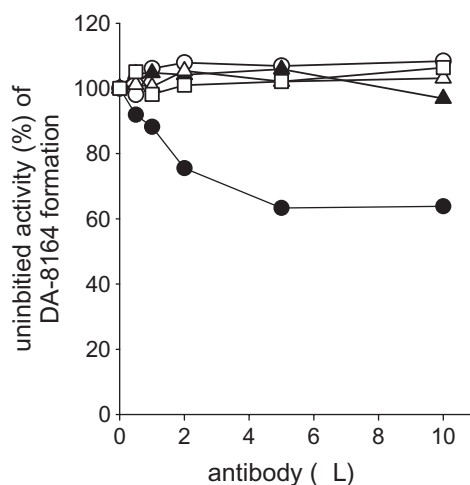


Figure 4. Effect of anti-CYP antibodies on the DA-8159 *N*-dealkylation to DA-8164. Pooled human liver microsomes H161 were pre-incubated with anti-CYP3A4 (●), anti-CYP1A2 (△), anti-CYP2C (○), anti-CYP2D6 (□) or anti-CYP2E1 (▲). Data are derived from two determinations. Rate of control reaction is 48.5 pmol/mg protein/min.

Table 3. Inhibitory potential of DA-8159 on specific CYP activities in human liver microsomes.

Enzyme activity	CYP	IC ₅₀ (μM)
Phenacetin <i>O</i> -deethylase	1A2	> 400
Coumarin 7-hydroxylase	2A6	> 400
Paclitaxel 6α-hydroxylase	2C8	> 400
Diclofenac 4-hydroxylase	2C9	> 400
[S]-mephenytoin 4'-hydroxylase	2C19	> 400
Bufuralol 1'-hydroxylase	2D6	67.7
Chlorzoxazone 6-hydroxylase	2E1	> 400
Testosterone 6β-hydroxylase	3A4	> 400
Midazolam 1'-hydroxylase	3A4	210.5

quinidine (CYP2D6) and diethyldithiocarbamate (CYP2E1). Ketoconazole inhibited DA-8159 *N*-dealkylation with an approximate IC₅₀ of 0.088 μM and a *K*_i value of 0.028 μM (data not shown).

To further confirm which CYP is primarily responsible for the formation of DA-8164 from DA-8159, immuno-inhibition studies with anti-CYP1A2, anti-CYP2D6, anti-CYP2E1, anti-CYP3A4 or anti-CYP2C were conducted. Only anti-CYP3A4 significantly inhibited DA-8164 formation in pooled human liver microsome H161 (figure 4), whereas other antibodies did not inhibit DA-8159 *N*-dealkylation activity. These results strongly suggest that CYP3A4 is responsible for almost all of DA-8159 *N*-dealkylation in human liver.

The inhibitory potential of DA-8159 on specific cytochrome P450 activities was also evaluated in human liver microsomes to investigate the effect of DA-8159 on CYP-mediated drug interactions. DA-8159 was a weak inhibitor of bufuralol 1'-hydroxylase (CYP2D6) and midazolam 1'-hydroxylase (additional CYP3A4 marker), respectively, with IC₅₀ values of 68 μM and 210 μM (table 3). DA-8159 at a concentration of 400 μM had no inhibitory effects on phenacetin *O*-deethylase (CYP1A2), paclitaxel 6α-hydroxylase (CYP2C8), diclofenac

4-hydroxylase (CYP2C9), (S)-mephenytoin 4-hydroxylase (CYP2C19), coumarin 7-hydroxylase (CYP2A6), chlorzoxazone 6-hydroxylase (CYP2E1) and testosterone 6 β -hydroxylase (CYP3A4).

Discussion

DA-8159 is metabolized to DA-8164, which is the major circulating active metabolite of DA-8159 in humans (Kim *et al.* 2003, Amakye *et al.* 2004). In order to identify the specific enzymes responsible for *N*-dealkylation of DA-8159 to DA-8164, a combination of chemical inhibition, immunoinhibition, correlation analysis in human liver microsomes and metabolism by recombinant cDNA-expressed CYP enzymes was employed. Correlation analysis between known CYP activities and the rate of DA-8164 formation showed significant correlation with testosterone 6 β -hydroxylase activity (a marker of CYP3A4) in the 12 human liver microsomes investigated. Ketoconazole, a selective CYP3A4 inhibitor and CYP3A4 monoclonal antibodies potently inhibited the DA-8159 oxidation to DA-8164. In addition, CYP3A4 microsomes mediated the formation of DA-8164 from DA-8159 with a K_m of 58.9 μ M and a V_{max} of 959 pmol mg⁻¹ min⁻¹. These results collectively suggest that DA-8159 is metabolized to an active metabolite DA-8164 predominantly by the CYP3A4 hepatic microsomal enzyme.

CYP3A4 is the most abundant CYP expressed in human liver and small intestine and contributes to the metabolism of approximately half the drugs in use today (Guengerich 1999). Expression of CYP3A4 has been highly variable among human liver samples (Watkins *et al.* 1985, Shimada *et al.* 1994, Gibson *et al.* 2002). CYP3A4 has been shown to be inducible by barbiturates, rifampin and dexamethasone or to be inhibited by ketoconazole, itraconazole and protease inhibitors such as ritonavir and indinavir (Baldwin *et al.* 1995; Lillibridge *et al.* 1998; von Moltke *et al.* 1996, 1998; Wang *et al.* 1999, Wrighton and Thummel, 2000, Plant and Gibson 2003). Sildenafil, the first oral therapeutic for erectile dysfunction, is *N*-demethylated to an active metabolite UK-103,320 by CYP3A4, a major route, and by CYP2C9, a minor route (Warrington *et al.* 2000, Hyland *et al.* 2001). Potent CYP3A4 inhibitors such as erythromycin and indinavir, ritonavir and saquinavir have been shown to produce significant increases in peak plasma concentrations and the AUC of sildenafil (Merry *et al.* 1999, Muirhead *et al.* 2000, 2002) in close agreement with *in vitro* findings (Warrington *et al.* 2000, Hyland *et al.* 2001). Therefore, coadministration of known inhibitors or inducers of CYP3A4 with DA-8159 can alter the metabolism of DA-8159 and may lead to significant changes in clinical pharmacokinetics of DA-8159.

Furthermore, the effects of DA-8159 on the activities of clinically significant human CYPs (1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) were also investigated in human liver microsomes. DA-8159 was found to be a weak inhibitor of all these enzymes; the most potent inhibition of DA-8159 was against CYP2D6 activity with an IC₅₀ values of 68 μ M. Since the maximum plasma concentrations of clinical doses (100 mg) of DA-8159 in volunteers are 250 ng ml⁻¹ ($\sim$ 0.5 μ M), it is very unlikely that DA-8159 will significantly alter the clearance of other compounds metabolized by these eight CYP enzymes.

This paper provides the evidences for CYP3A4-mediated metabolism of DA-8159. There is the possibility that the changes in plasma concentrations of DA-8159 could occur with co-administration of known CYP3A4 inducers

or inhibitors. Marked inhibition of the metabolism of the substrates for CYPs 3A4, 2D6, 1A2, 2A6, 2C8, 2C9, 2C19 and 2E1 would not be predicted, as the oral administration (100 mg) of DA-8159 to volunteers produces peak plasma concentrations of approximately 0.5 μ M.

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***In vitro* metabolism of gefitinib in human liver microsomes**

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1. The *in vitro* metabolism of gefitinib was investigated by incubating [^{14}C]-gefitinib, as well as M537194, M387783 and M523595 (the main metabolites of gefitinib observed in man), at a concentration of 100 μM with human liver microsomes (4 mg ml $^{-1}$) for 120 min. These relatively high substrate and microsomal protein concentrations were used in an effort to generate sufficient quantities of metabolites for identification.

2. HPLC with ultraviolet light, radiochemical and mass spectral analysis, together with the availability of authentic standards, enabled quantification and structural identification of a large number of metabolites. Although 16 metabolites were identified, metabolism was restricted to three regions of the molecule.

3. The major pathway involved morpholine ring-opening and step-wise removal of the morpholine ring and propoxy side chain. O-demethylation of the quinazoline methoxy group was a quantitatively less important pathway, in contrast to the clinical situation, where O-desmethyl gefitinib (M523595) is the predominant plasma metabolite. The third metabolic route, oxidative defluorination, was only a minor route of metabolism. Some metabolites were formed by a combination of these processes, but no metabolism was observed in other parts of the molecule.

4. Incubation of gefitinib produced ten identified metabolites, but the use of the three main *in vivo* metabolites as additional substrates enabled a more comprehensive metabolic pathway to be constructed and this has been valuable in supporting the more limited data available from the human *in vivo* study.

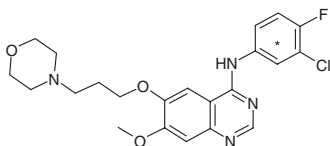
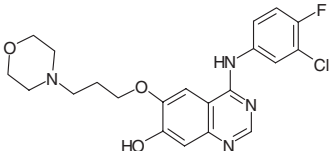
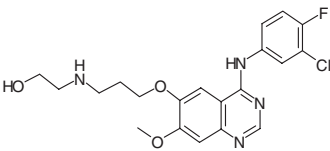
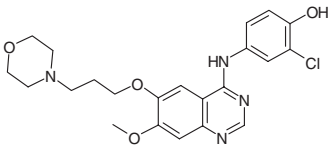
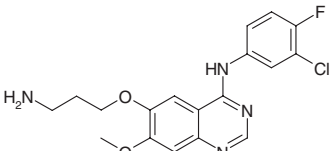
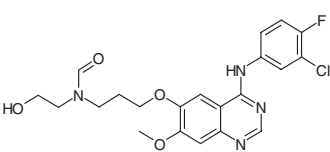
Introduction

Gefitinib (IressaTM) is an orally active inhibitor of the epidermal growth factor receptor (EGFR; erbB1) tyrosine kinase (TK), involved in signal transduction processes and implicated in the proliferation and maintenance of cancer cells (Woodburn 1999, Ciardiello and Tortora 2002). Since mutation or over-expression of EGFR, as well as erbB2, can lead to cellular transformation and has been linked to poor prognosis in cancer (Sainsbury *et al.* 1985, Di Fiore *et al.* 1987, Slamon *et al.* 1987), these erb receptors have been viewed as an attractive target for novel antitumour therapies and there are now a number of agents in development which target EGFR (Ennis *et al.* 1991, Wakeling *et al.* 1997, Woodburn 1999).

A novel low molecular weight anilino-quinazoline compound, gefitinib (table 1) is a potent inhibitor of EGFR-TK activity (IC $_{50}$, 0.033 μM) in isolated enzyme preparations and inhibits EGF-stimulated tumour cell growth with an IC $_{50}$ of 0.054 μM (Wakeling *et al.* 2002). Gefitinib blocks autophosphorylation of EGFR in a concentration-dependent manner in a number of cell lines and inhibits tumour

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Table 1. Chromatographic and structural characteristics of reference materials.

Reference compound	Structure	Retention time (min)	[M+H] ⁺	Fragment ions
[¹⁴ C]-gefitinib	 (* shows position of the radiolabel)	42.5	447	128
M523595		35.1	433	128
M537194		31.2	421	320, 102
M387783		27.8	445	128
M605212		31.5	377	320
M608236		36.2	449	320, 130

[M+H]⁺ ions were generated using a cone voltage of 25 V, while fragment ions were generated primarily using a cone voltage of 50 V.

growth when administered orally to mice bearing a range of human tumour xenografts (Sirotnak *et al.* 2000, Wakeling *et al.* 2002). Anti-tumour activity has been observed in a range of Phase I and II clinical studies (Herbst *et al.* 2002, Ranson *et al.* 2002, Fukuoka *et al.* 2003, Kris *et al.* 2003) and gefitinib (Iressa) is now approved in a number of countries for the treatment of non-small-cell lung cancer.

Preclinical studies, conducted during development, showed that gefitinib is cleared primarily by metabolism in rat and dog (McKillop *et al.* 2004a). Morpholine ring oxidation was the predominant route in the rat and this pathway, together with O-demethylation of the quinazoline methoxy group, were the main

routes of metabolism in the dog. A metabolism study in healthy human volunteers generated urinary and faecal metabolism profiles similar to those in dog, with the major metabolites being formed by O-demethylation and morpholine ring oxidation (McKillop *et al.* 2004a). However, the extent of the information generated from this human study was limited by the relatively low oral dose, prolonged elimination phase and by the relatively low extraction of drug-related material from faeces, the major route of excretion. Although a number of metabolites were also observed in human plasma, O-desmethyl gefitinib (M523595) was the predominant plasma metabolite and was present at concentrations similar to gefitinib (McKillop *et al.* 2004b).

This *in vivo* information was preceded by the findings of an earlier study in human liver microsomes, in which two metabolites were shown to be produced by CYP3A4-dependent metabolism as a result of morpholine ring oxidation (M537194) and oxidative defluorination (M387783) (Swaisland *et al.* 2004). The present study was initiated in an effort to further delineate the metabolism of gefitinib by examining metabolism in human liver microsomes, as a cleaner sample matrix, using relatively high substrate (100 μM) and microsomal protein (4 mg ml⁻¹) concentrations to facilitate metabolite identification, and by using the main *in vivo* metabolites (M537194, M523595 and M387783) as additional substrates.

Materials and methods

Chemicals

[¹⁴C]-gefitinib, N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholino-propoxy)quinazolin-4-amine, was synthesized with the [¹⁴C]-label in the chlorofluoroaniline ring (see table 1 for gefitinib structure) by the Isotope Chemistry Unit, AstraZeneca, Alderley Park. Initial radiochemical purity was more than 99% and specific activity was 115 $\mu\text{Ci mg}^{-1}$. A range of metabolites (M387783, M523595, M537194, M594557, M605207, M605212 and M608236; structures are shown in table 1) were also synthesized within AstraZeneca, with their structure and purity being assessed by high performance liquid chromatography with UV detection (HPLC-UV), mass spectrometry and nuclear magnetic resonance spectroscopy. Details of synthetic routes to a range of these aniline-quinazoline compounds have been reported (Gibson *et al.* 1997, Barker *et al.* 2001).

Study design

Microsomal incubation. Frozen human liver samples from four male and four female organ donors were obtained from the International Institute for the Advancement of Medicine (IIAM), Exton, PA, USA; no phenotypic or genotypic information on the cytochrome P450 status of these samples was available. These samples were pooled and used to prepare hepatic microsomes as described previously (McKillop *et al.* 1998). [¹⁴C]-gefitinib, M387783, M523595 and M537194, dissolved in 20 mM HCl, were added to human hepatic microsomes (4 mg protein ml⁻¹ of 0.1 M phosphate buffer at pH 7.4) to give final substrate concentrations of 100 μM . Flasks were pre-warmed for approximately 5 min at 37°C in a shaking water bath and NADPH (final concentration 5 mM) was added; the total volume of each incubation was 30 ml. Aliquots were collected into an equal volume of ethanol at 0, 30, 60 and 120 min and samples were stored at -20°C until analysed. As controls, [¹⁴C]-gefitinib, M387783, M537194 and M523595 were also incubated in the absence of microsomes and in the absence of NADPH.

Sample analysis

Analysis of reference compounds. All substrates and reference compounds were dissolved in acidified water at appropriate concentrations before analysis by HPLC with radiochemical, UV or HPLC-MS detection. The radiochemical purity of a 10 $\mu\text{g ml}^{-1}$ [¹⁴C]-gefitinib solution was assessed by HPLC with radiochemical detection using a Jones Hypersil ODS BDS 5 μm (25 cm $\times$ 4.6 mm) column and eluted with 0.05 M ammonium acetate in water (A) and acetonitrile (B). A linear gradient was used, increasing from 10% B to 45% B over 45 min, then increasing to 100% B over an additional 0.5 min and held there for a further 9.5 min, at a flow rate of 1 ml min⁻¹. Radiochemical peaks were measured using a Packard

500TR radiochemical monitor. Levels of radioactivity in formulation and incubation samples were determined by liquid scintillation counting. The purities of M387783, M537194 and M523595 ($10\text{ }\mu\text{g ml}^{-1}$ solutions) were assessed by the same method but using UV absorbance to monitor the compound peaks.

An alternative HPLC system was also developed to enhance the separation of some metabolites. This method used a Hichrom HIRPB-150A ($15\text{ cm} \times 4.6\text{ mm}$) column, eluted at 0.75 ml min^{-1} with water (A) and acetonitrile (B), with both containing 0.1% (v/v) trifluoroacetic acid. Following injection of each sample, a linear gradient was run increasing from 10% B to 25% B over 45 min, then increasing to 40% B over a further 15 min and, finally, increasing to 100% B over an additional 15 min. Compound-related peaks were detected as previously indicated.

Metabolite profiling. After addition of ethanol to stop the incubation reaction, the microsomal samples were centrifuged at $2000g$ for 10 min to precipitate microsomal debris and aliquots of the supernatant were reduced to dryness under nitrogen at 35°C . Residues were reconstituted in initial HPLC mobile phase and then analysed using the above HPLC method.

Metabolite identification. Aliquots of the larger samples, collected into ethanol after 120 min incubation, were centrifuged and the supernatants dried and redissolved as before. Samples were analysed by HPLC-MS, using a similar HPLC system but linked to a Micromass single quadrupole ZMD mass spectrometer, operating in electrospray/positive ion mode. A cone voltage of 25 V was used to minimize fragmentation and 50 V to induce fragmentation, whilst monitoring spectra over a mass range of 20–700 Daltons.

Data analysis. Quantitative assessment of [^{14}C]-gefitinib, M387783, M537194 and M523595 metabolite profiles was made by analysis of the UV chromatograms. The retention time of each component was defined and the proportion of the total integrated UV chromatogram represented by each component was assessed. UV absorption proved to be more sensitive than radiochemical detection and was also used for quantitation of [^{14}C]-gefitinib metabolite profiles. Standard solutions ($10\text{ }\mu\text{g ml}^{-1}$ in methanol) of gefitinib, M387783, M523595 and M537194 had mean UV absorbances (at 250 nm) of 0.690, 0.750, 0.628 and 0.703, respectively. The extinction coefficient of subsequent metabolites was assumed to be similar to that of each substrate. Assessment of the metabolite profiles generated by HPLC-MS was achieved by measuring the ion current at masses between 20 and 700 Daltons, as well as by monitoring for selected ions corresponding to putative metabolites. As ionization was by means of positive ion electrospray, molecular ions were protonated, hence $(\text{M}+\text{H})^+$, and recorded at 1 Dalton above the non-ionized state.

Results

Supporting data

Characterization of reference compounds. [^{14}C]-gefitinib, M387783, M537194 and M523595 were examined by HPLC with radiochemical or UV detection and by HPLC-MS. Retention times (from HPLC-UV), $(\text{M}+\text{H})^+$ and ion fragments for each compound are given in table 1. Similar information is also presented for M605212 and M608236, other potential metabolites available as reference compounds.

Formulated [^{14}C]-gefitinib was approximately 97% pure, as determined by HPLC with radiochemical detection, while the purities of M387783, M537194 and M523595 were 93, 87 and 96% respectively by HPLC with UV detection. Although some minor impurities were observed by HPLC-UV, there was no evidence from proton NMR analysis to suggest that these were compound-related contaminants. No decomposition of any of the substrates was observed during 120 min incubation in human microsomal samples in the absence of NADPH or in phosphate buffer incubated in the absence of microsomal protein (data not shown).

Table 2. HPLC metabolite profile generated following incubation of [^{14}C]-gefitinib (100 μM) with human hepatic microsomes.

Retention time (RT)	Metabolite	Incubation time (min)				[M+H] $^{+}$	Fragment ions
		0	30	60	120		
26.8–26.9	E		1.3	1.5	1.9	447	318,130
30.6	H (M387783)		3.5	2.8	2.7	445	318,128
31.2–31.3	F		1.2	2.1	2.8	363	
34.7–34.8	I (M537194)		5.8	5.6	9.2	421	320,102
	J (M605212)					377	320
40.0–40.1	N (M608236)		2.4	3.2	3.3	449	320,130
41.7–41.8	P (M295820)		4.8	5.0	6.7	320	
45.1–45.2	Gefitinib	100	77.4	76.8	68.5	447	128

HPLC retention times and quantitative analysis are from HPLC with ultraviolet light detection.

Metabolite identification was by HPLC-MS. (M+H) $^{+}$ ions were generated using a cone voltage of 25 V, while fragment ions were generated primarily using a cone voltage of 50 V.

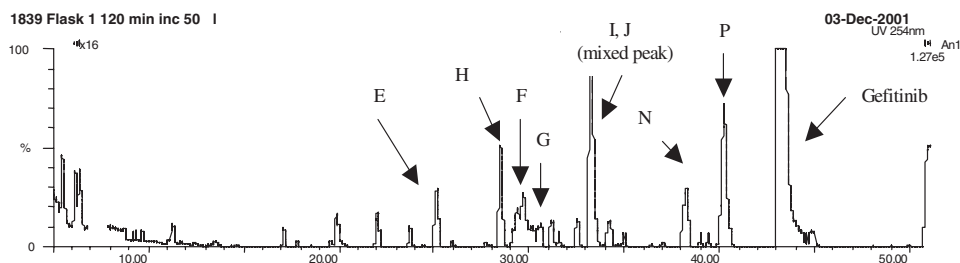


Figure 1. HPLC metabolite profile generated following incubation (120 min) of [^{14}C]-gefitinib (100 μM) with human liver microsomes. x-axis, retention time (min); y-axis, intensity (arbitrary units).

Metabolism of [^{14}C]-gefitinib, M387783, M523595 and M537194

Quantitative and qualitative analysis of the metabolite profiles of [^{14}C]-gefitinib, M387783, M523595 and M537194 was made by HPLC with UV detection. Since a large number of metabolites were observed in the HPLC profiles, those identified were assigned a code letter for ease of reporting. Identification was generally based on a comparison of HPLC retention time and mass spectral characteristics with authentic reference material.

Metabolism of [^{14}C]-gefitinib

Following incubation of [^{14}C]-gefitinib (100 μM), the proportion of parent compound decreased from 100% at the start of the incubation to 69% after 120 min (table 2). A number of components were observed in the sample chromatogram and these all eluted before gefitinib (figure 1). The most abundant of these accounted for approximately 9% of the integrated chromatogram at the end of the incubation period and six components were identified by HPLC-MS, as well as another not resolved by this HPLC system. The structures of these (metabolites E, F, H, I, J, N and P) are shown in the proposed metabolic pathway for gefitinib (figure 2) and the mass spectral data for each component are summarized in table 2.

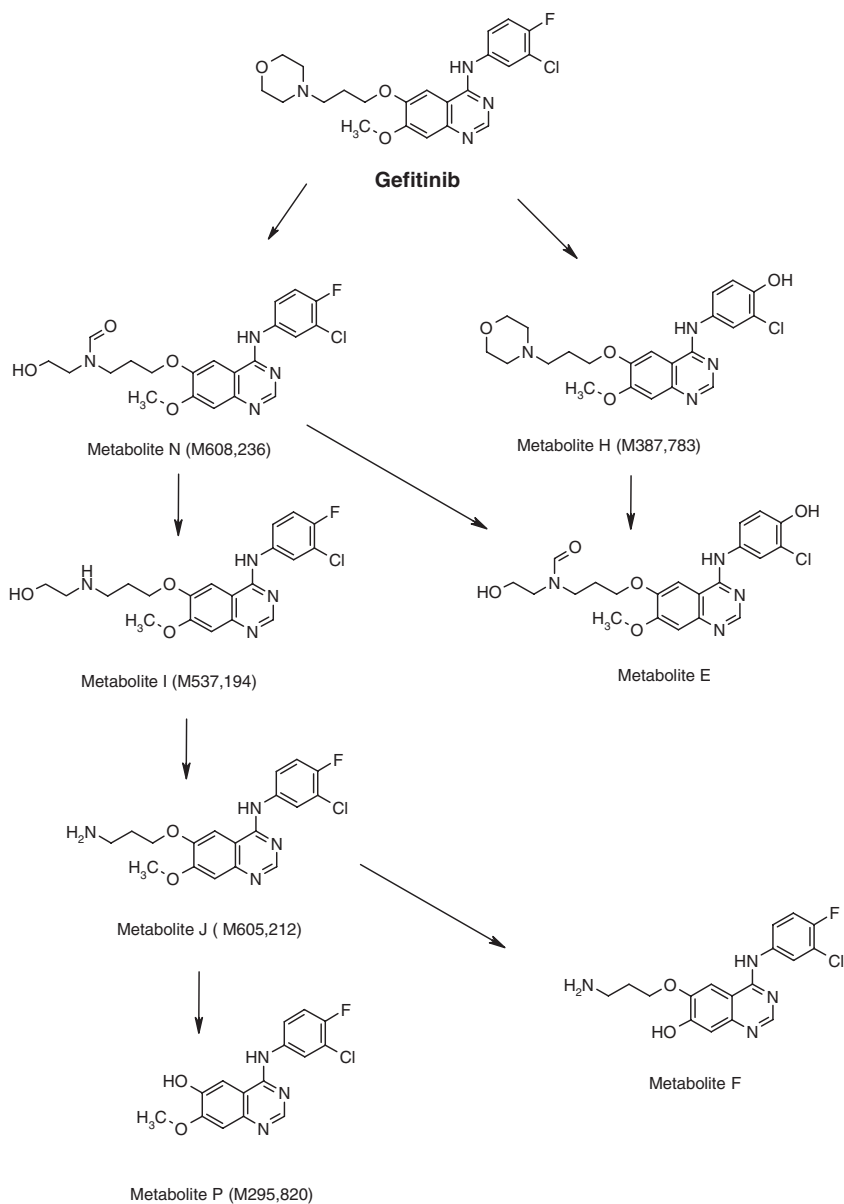


Figure 2. Gefitinib metabolic pathways in human liver microsomes.

The component with retention time (RT) of approximately 40 min (metabolite N) had similar mass spectral properties (m/z 449, 320 and 130) and co-chromatographed with authentic M608236, indicating metabolism of gefitinib by O-dealkylation at the morpholine group. The major peak at RT 34.7 min consisted of two components, metabolite I and metabolite J, and these had similar chromatographic and mass spectral properties to authentic standards of M537194 (m/z 421, 320 and 102) and M605212 (m/z 377 and 320) respectively. Metabolite I (M537194) was probably formed by N-dealkylation of metabolite N at the morpholine nitrogen and metabolite J by further N-dealkylation of metabolite I

at the same site (figure 2). One further component (RT 42 min), metabolite P (m/z 320), was structurally identical to M295820, a minor *in vivo* metabolite also available as a reference material. This could have been formed from metabolite J by O-dealkylation of the propoxy side chain, although there is a possibility that metabolite P was produced directly from [^{14}C]-gefitinib by O-dealkylation of the intact morpholine ring propoxy side chain.

Metabolite J (M605212) also appears to have been further metabolized to metabolite F (RT 31 min) by O-demethylation of the quinazoline methoxy group. Whereas the absence of fragment ions in the mass spectrum made definitive assignment of this structure difficult, the reduction in m/z of the $(\text{M}+\text{H})^+$ ion from 377 to 363 is characteristic of the conversion of methoxy to a hydroxyl group. Formation of this metabolite indicated that metabolism had occurred at two separate sites within the molecule. The component at RT 30.6 min (metabolite H) had similar chromatographic and mass spectral properties to M387783. The molecular ion for this component (m/z 445) was 2 Daltons less than that of [^{14}C]-gefitinib (m/z 447), which is indicative of replacement of a fluorine atom with a hydroxyl group, whilst comparison of fragment ions for metabolite H (m/z 318 and 128) and gefitinib (m/z 128) indicated that metabolism had not occurred on the propoxy morpholine side chain. These data support the view that metabolite H was formed by oxidative defluorination of gefitinib. One further component, metabolite E (RT 27 min), had fragment ions (m/z 318 and 130) analogous with those of metabolite N (m/z 130) and metabolite H (m/z 318). It is likely that metabolite E was formed either by O-dealkylation of metabolite H, or by oxidative defluorination of metabolite N, again demonstrating metabolism at two discrete sites within the molecule (figure 2). Only two components (RT 36.4 and 41 min) were unidentified and these accounted for less than 2% of the gefitinib metabolite profiles.

Quantitatively, the propoxy-morpholine ring was the most important region of gefitinib for metabolism, since the combined proportion of the chromatogram represented by metabolites E, F, N, I, J and P at the end of the incubation period (23.9%) was much greater than that represented by metabolites H and E (4.6%), which had arisen by oxidative defluorination, or metabolite F (2.8%), generated by O-demethylation of the quinazoline methoxy group. Although, at this stage, seven metabolites of gefitinib have been identified, there was no evidence of a primary metabolite in the morpholine ring oxidation pathway, nor was M523595, the predominant plasma metabolite, detected. Since previous microsomal work (Swaisland *et al.* 2004) using a different HPLC system had shown the presence of metabolites with a longer retention time than gefitinib, further analysis in this system was conducted as detailed below.

Analysis in an alternative HPLC system. Using a trifluoroacetic acid/acetonitrile mobile phase, a number of components now had longer HPLC retention times than [^{14}C]-gefitinib (RT 38.7 min) and HPLC-MS of the terminal incubation (scan range 250–600 Da) indicated that four (metabolites N, P, Q and R) were derived from [^{14}C]-gefitinib. The proportion of the UV chromatogram represented by these metabolites, together with their $(\text{M}+\text{H})^+$ and fragment ions, is presented in table 3. Two of these components (RT 44.4 and 48.2 min) were identified as metabolite P (M295820) and metabolite N (M608236), observed and characterized using the previous HPLC system. The other two components

Table 3. Late-running metabolites separated by the second HPLC system following 120-min incubation of [^{14}C]-gefitinib (100 μM) with human hepatic microsomes.

Retention time (min)	Metabolite	Structure	Proportion of ultraviolet light chromatogram (%)	[M+H] $^{+}$	Fragment ions
39.5	Gefitinib				
43.0	Q (M594557)		1.6	463	360, 320
44.4	* P (M295820)		3.7	320	ND
48.2	* N (M608236)		3.3	449	320, 130
53.3	R (M605211)		0.4	461	320, 142

* Metabolites N and P were identified and characterized in the previous HPLC system (table 2). ND, scanned at cone voltages of 25 and 50 V, but no fragmentation observed at 50 V.

were only minor metabolites and together accounted for approximately 2% of the UV chromatogram. The component at RT 43.0 min (metabolite Q) had similar chromatographic and mass spectral properties (m/z 463, 360 and 320) to M594557 (gefitinib N-oxide), formed by N-oxidation of the morpholine ring. The component at RT 53.3 min (metabolite R) accounted for only 0.4% of the UV chromatogram and had similar chromatographic and mass spectral properties to M605211 (m/z 461, 320 and 142). It seems likely that this minor metabolite, formed by oxidation of the morpholine ring, would be a precursor for subsequent morpholine ring-opening.

Metabolism of M537194. Following incubation of M537194 with human hepatic microsomes, parent compound decreased from 100% to 74% after 120 min incubation. Four components other than M537194 exceeded 1% of the chromatogram, with the most abundant (metabolite G, RT, 28.7 min) accounting for 13.9% after 120 min incubation (figure 3 and table 4). Using HPLC-MS analysis, structures were derived for these components (metabolites C, F, G and P), two of which (metabolites F and P) had been produced during gefitinib metabolism. A further component, metabolite J (also formed from gefitinib), was also observed on HPLC-MS analysis, but co-eluted with M537194 and could not be quantified separately. The structures of these components and a proposed metabolic pathway are presented in figure 4, while the ions detected by mass spectrometry are shown in table 4.

Metabolite G was formed by O-demethylation of the quinazoline methoxy group of M537194, since the fragment ions (m/z 306 and 102) indicated both the loss of the methoxy group from M537194 and the presence of the intact propoxy side chain. One further metabolite of M537194 formed in this incubation was metabolite C (RT 19 min), formed by oxidative defluorination in a manner similar to that observed with gefitinib. The (M+H) $^{+}$ ion (m/z 419) was only two Daltons

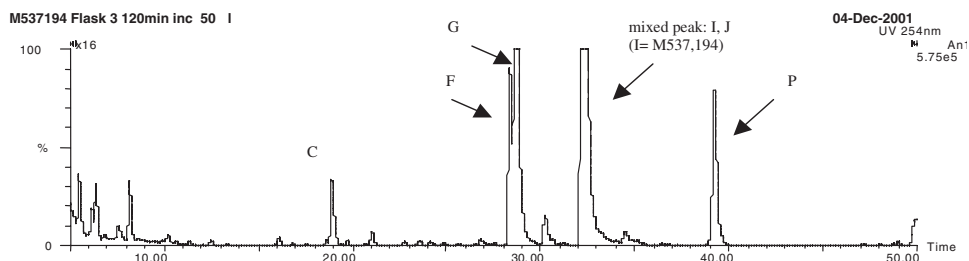


Figure 3. HPLC metabolite profile generated following incubation (120 min) of M537194 (100 μ M) with human liver microsomes. *x*-axis, retention time (min); *y*-axis, intensity (arbitrary units).

Table 4. HPLC metabolite profile generated following incubation of M537194 (100 μ M) with human hepatic microsomes.

Retention time (min)	Metabolite	Incubation time (min)				$[M+H]^+$	Fragment ions
		0	30	60	120		
18.8	C		1.3	1.5	1.7	419	318, 102
28.3	F		0.9	2.4	4.1	363	
28.7	G		7.1	10.7	13.9	407	306,102
32.3	I (M537194)	100	85.1	79.9	74.0	421	320, 102
	J (M605212)					377	320
39.8	P (M295280)		3.4	4.2	4.8		

HPLC retention times and quantitative analysis are from HPLC with ultraviolet light detection. Metabolite identification was by HPLC-MS. $(M+H)^+$ ions generated using a cone voltage of 25 V,

less than that of M537194 while the presence of fragment ions (m/z 318 and 102) indicated that the propoxy side chain was intact and that the loss of 2 Daltons had come from the remaining part of the molecule.

Metabolism of M387783. Following incubation of M387783 with human hepatic microsomes, its proportion decreased from 100 to 64.9% after 120 min. A number of components other than M387783 were observed (figure 5), with the most abundant accounting for 11.2% of the chromatogram; four of these (metabolites B, C, D and E) were identified by HPLC-MS (table 5 and figure 6). Metabolite E (RT 27 min) had also been observed with gefitinib metabolism and metabolite C had been formed from M537194. It appears likely that metabolite E was further metabolized in this incubation of M387783 to the secondary amine, metabolite C, and then to the primary amine, metabolite B (RT 21.1 min) by N-dealkylation. The structure of metabolite B was assigned from the $(M+H)^+$ ion (m/z 375).

The mass spectral data for metabolite D (RT 25.3 min) indicated loss of the propoxy side chain and, while this metabolite was probably formed by O-dealkylation of metabolite B, it could also have been produced by direct O-dealkylation of M387783 (figure 6). Four components quantified in the M387783 profiles were not identified, although the most abundant of these accounted for no more than 2.5% of the integrated chromatogram.

Metabolism of M523595. Incubation of M523595 with human hepatic microsomes resulted in the formation of several metabolites (figure 7). M523595 decreased from 100% to 75.6% over the course of the 120 min incubation, with the

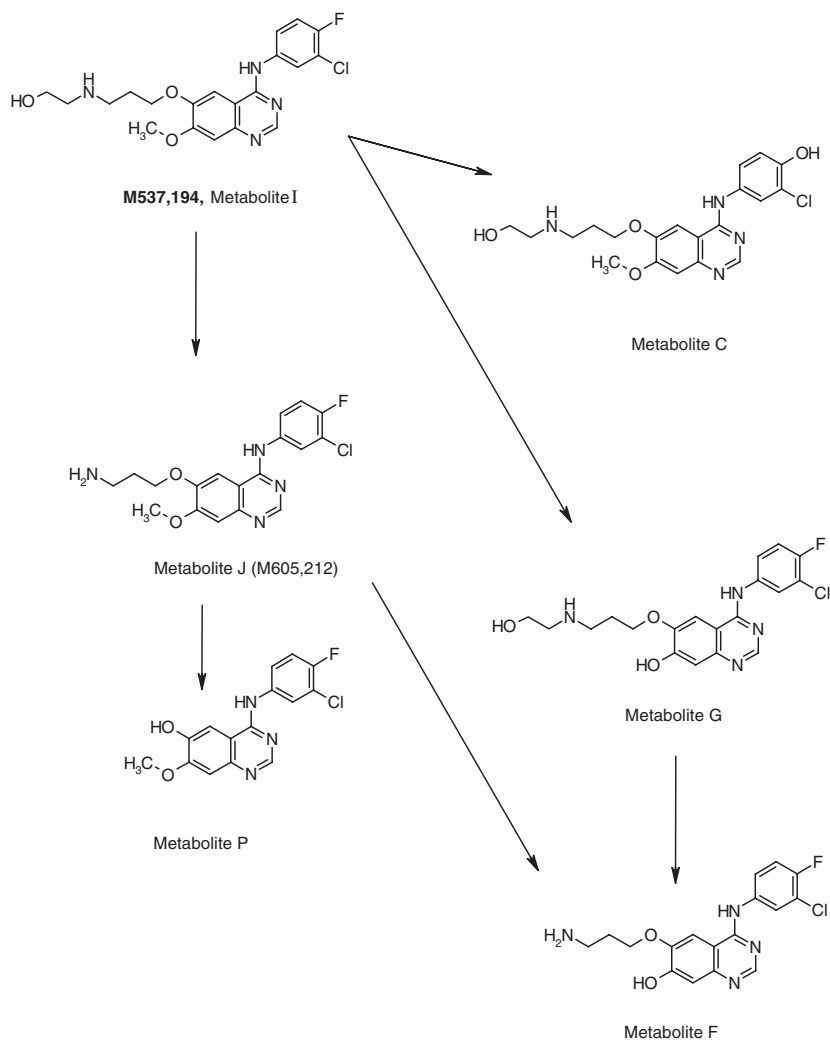


Figure 4. M537194 metabolic pathways in human liver microsomes.

most abundant metabolite (metabolite G) accounting for 9.2% of the chromatogram (table 6). Three other components (metabolites A, F and K) were also identified and their structures are shown in the proposed metabolic pathway (figure 8), with the related mass spectral data presented in table 6. Metabolism of M523595 occurred by similar routes to those seen with gefitinib, M537194 and M387783.

The structural assignment of metabolite K (RT 33 min), formed by O-dealkylation of the morpholine ring, was based on the $(M+H)^+$ ion (m/z 435), together with the characteristic fragment ion (m/z 130), also observed with metabolite N, formed in an analogous manner from gefitinib. Metabolite G (RT 29 min) and metabolite F (RT 28 min) were probably formed by sequential N-dealkylation of metabolite K, although it is also possible that these components were formed by alternative pathways. M523595 also underwent oxidative defluorination to form metabolite A (RT 21 min), in a manner analogous to gefitinib and

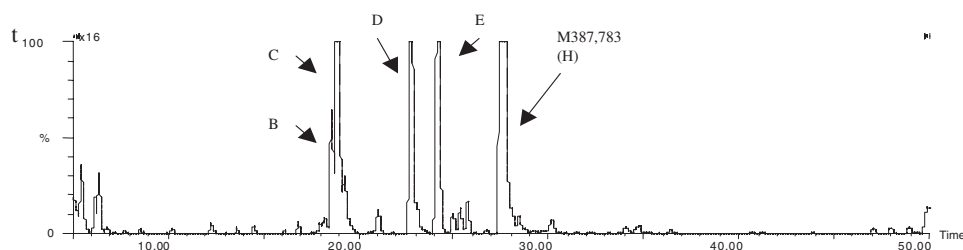


Figure 5. HPLC metabolite profile generated following incubation (120 min) of M387783 (100 μ M) with human liver microsomes. *x*-axis, retention time (min); *y*-axis, intensity (arbitrary units).

Table 5. HPLC metabolite profile generated following incubation of M387783 (100 μ M) with human hepatic microsomes.

Retention time (min)	Metabolite	Incubation time (min)				$[M+H]^+$	Fragment ions
		0	30	60	120		
21.1	B		1.7	2.4	3.8	375	
21.4	C		7.9	9.7	11.2	419	318, 102
25.3	D		4.5	6.7	7.5	318	303
26.9	E		5.3	6.2	7.2	447	318, 130
30.0	H (M387783)	100	78.9	70.0	64.9		

HPLC retention times and quantitative analysis are from HPLC with ultraviolet light detection.

Metabolite identification was by HPLC-MS. $(M+H)^+$ ions generated using a cone voltage of 25 V, while fragment ions were generated primarily using a cone voltage of 50 V.

M537194. Five quantifiable components detected in the M523595 chromatograms were not identified and the most abundant of these accounted for no more than 2.4% of the integrated chromatogram.

Discussion

The study investigated the metabolism of gefitinib in human liver microsomes by incorporating the use of M387783, M537194 and M523595 (the main metabolites of gefitinib found in man), as well as $[^{14}\text{C}]$ -gefitinib, as starting substrates in an effort to enhance the more limited metabolism data available from a clinical study (McKillop *et al.* 2004a). All four compounds showed fairly extensive metabolism, particularly considering the relatively high initial substrate concentration (100 μ M). The most important pathway of metabolism, quantitatively, involved oxidative degradation of the propoxy-morpholine side chain (as demonstrated when $[^{14}\text{C}]$ -gefitinib, M387783 and M523595 were incubated) and this resulted in ring-opening, with partial and complete removal of the morpholine ring and propoxy side chain. The extensive range of metabolites formed was similar to that found during metabolism of other morpholine ring containing compounds, including timolol (Tocco *et al.* 1980), moclobemide (Mayersohn and Guentert 1995) and the renin inhibitor, A-74273 (Denissen *et al.* 1994).

While the initial analysis indicated extensive metabolism to a number of ring-opened metabolites, no primary oxidation products were observed, which was also the case with A-74273 (Denissen *et al.* 1994), where the role of primary oxidation

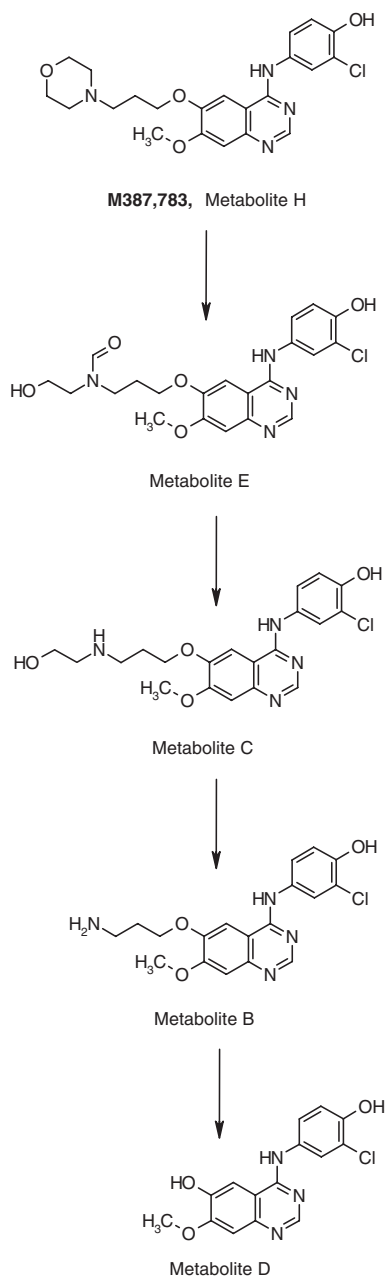


Figure 6. M387783 metabolic pathways in human liver microsomes.

metabolites was hypothesized. However, subsequent HPLC-MS examination of the gefitinib samples, together with the availability of further standards, provided evidence for the formation of two relatively minor products demonstrating direct oxygenation of the morpholine ring. These metabolites, M605211 and M594557, were produced by keto-substitution and N-oxidation of the morpholine ring

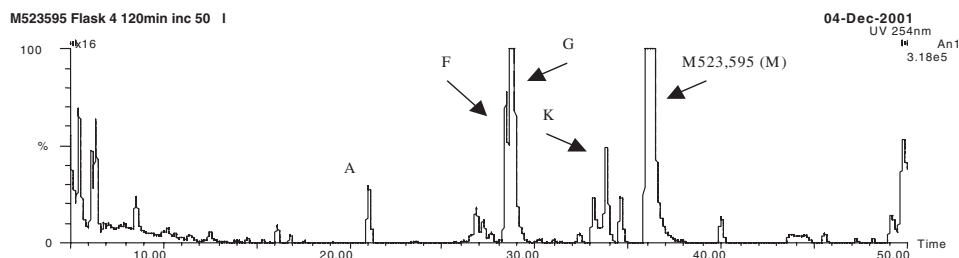


Figure 7. HPLC metabolite profile generated following incubation (120 min) of M523595 (100 μ M) with human liver microsomes. *x*-axis, retention time (min); *y*-axis, intensity (arbitrary units).

Table 6. HPLC metabolite profile generated following incubation of M523595 (100 μ M) with human hepatic microsomes.

Retention time (min)	Metabolite	Incubation time (min)				$[M+H]^+$	Fragment ions
		0	30	60	120		
20.8	A		0.3	0.3	0.6	431	128
28.1–28.6	F		1.5	2.3	6.8	363	
28.5–28.8	G		6.9	9.9	9.2	407	306, 102
33.2–33.4	K		1.1	1.3	1.6	435	130
36.4–36.6	M (523595)	100	85.4	79.6	75.6	433	128

HPLC retention times and quantitative analysis are from HPLC with ultraviolet light detection.

Metabolite identification was by HPLC-MS. $(M+H)^+$ ions generated using a cone voltage of 25 V, while fragment ions were generated primarily using a cone voltage of 50 V.

respectively (table 3). Interestingly, a similar keto-substitution intermediate has been found during morpholine ring oxidation with timolol (Tocco *et al.* 1980), moclobemide (Mayersohn and Guentert 1995), linezolid (Slatter *et al.* 2001) and aprepitant (Huskey *et al.* 2004), although N-oxidation of the morpholine ring was only reported with moclobemide (Mayersohn and Guentert 1995).

O-demethylation of the quinazoline methoxy group represented the second most important metabolic change, although, while this pathway was prominent with M537194 as substrate, it did not occur when the morpholine ring was intact with [^{14} C]-gefitinib and M387783 as substrates. However, once oxidation of the morpholine ring had occurred, O-demethylation of the methoxy group became a more important route of metabolism and resulted in the formation of metabolites F and G during incubation of [^{14} C]-gefitinib and M537194. This *in vitro* profile, however, contrasts markedly with the *in vivo* situation in man, where M523595 (O-desmethyl gefitinib) was the predominant metabolite observed in plasma following administration of Iressa to cancer patients (McKillop *et al.* 2004b). This difference may be due to lack of M523595 formation under the applied incubation conditions (100 μ M substrate and 4 mg ml $^{-1}$ microsomal protein), although the observation of metabolite F indicates that the liver microsomes used were capable of O-demethylation of the quinazoline methoxy group. Further work (data not shown), using gefitinib substrate concentrations (1–3 μ M) more closely related to therapeutic plasma concentrations (about 0.5 μ M), produced a very similar overall metabolic profile, where M523595, although formed to a slightly greater extent, was still a relatively minor product.

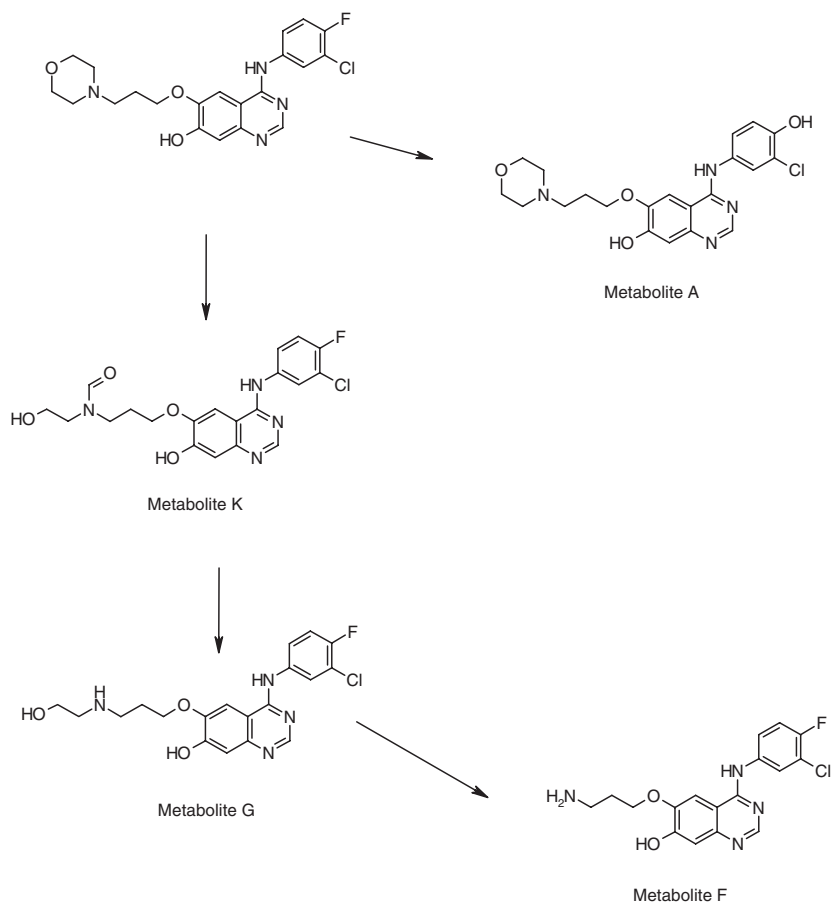


Figure 8. M523595 metabolic pathways in human liver microsomes.

The third metabolic process observed, oxidative defluorination, was only a minor route of metabolism, demonstrated by formation of low amounts of metabolites A, C, E and H. In contrast to the other two pathways, oxidative defluorination is a relatively unusual route of metabolism, but was also observed to a minor extent *in vivo* following administration of gefitinib to rat, dog and man (McKillop *et al.* 2004a). However, oxidative defluorination of aromatic ring structures has been reported for a number of compounds in the literature (Park *et al.* 1991, Scarfe *et al.* 1999, Chen *et al.* 2003). Recent studies have shown that this pathway is catalyzed by cytochrome P450 in man for a quinazoline antiviral compound (GW420867X) (Mutch *et al.* 2001) and oxidative defluorination of the reverse transcriptase inhibitor, DPC963, is CYP3A4-dependent (Chen *et al.* 2003). As with the intact morpholine group, metabolic replacement of the fluorine atom of gefitinib with a hydroxyl group also appeared to confer additional metabolic stability on the quinazoline methoxy group, which was not metabolized following defluorination. This regional stability was illustrated by the incubation with M387783 where, despite extensive metabolism and removal of the morpholine containing side chain, the methoxy group remained intact. This

contrasts with the situation with [^{14}C]-gefitinib and M537194 where, in the presence of the fluorine atom, limited metabolism of the morpholine ring enabled O-demethylation of the methoxy group. Apart from the three regions of the molecule discussed, no other area of the compound appeared to be involved in metabolism, although some minor chromatographic components were unidentified.

The large number of gefitinib metabolites observed in this study is in marked contrast to the findings of an earlier *in vitro* study, which showed that gefitinib was rapidly and extensively metabolized to three distinct metabolites following incubation with human hepatic microsomes (Swaisland *et al.* 2004). Two of the metabolites were identified as M537194 and M387783, while the third, apparently less polar, metabolite was not identified. This apparent inconsistency is probably related to the higher substrate concentration (100 μM), the longer incubation period (120 min), the availability of many more reference standards and the more sensitive analytical technique (HPLC-UV-MS) of the current study compared with the previous study design, where 10 μM gefitinib, 30 min incubation and HPLC-radiochemical detection were used (Swaisland *et al.* 2004).

Following incubation of [^{14}C]-gefitinib, ten metabolites were identified and a fairly comprehensive pathway of metabolism was delineated. However, the data from the other three substrates clearly complemented the original metabolic mechanism and enabled a further six structures to be used to enhance the overall metabolic pathway (figure 9). M537194 underwent metabolism in the same three regions of the molecule as gefitinib and, at the end of the incubation, metabolites that had lost the methoxy group (metabolite G and F) accounted for approximately 18% of the chromatogram area, at least 9% was represented by components that had undergone further N- and O-dealkylation of the propoxy side chain (metabolites F, J and P) and oxidative defluorination was still a minor route of metabolism (metabolite C represented less than 2% of the chromatogram). Interestingly, metabolites C and G were produced from M537194 but not from [^{14}C]-gefitinib. This was probably due to the limited sensitivity of the analytical technique although it is possible that additional metabolic pathways were involved at the higher concentration of M537194 present in this incubation. Oxidation of the propoxy-morpholine side chain is also the major site of M523595 metabolism (metabolites K, G and F represented approximately 19% of the chromatogram) and oxidative defluorination is again a minor route (metabolite A represented <1% of the chromatogram). With M387783 as substrate, oxidation of the propoxy-morpholine side chain was the only route of metabolism observed.

In summary, metabolism of gefitinib was extensive, fairly rapid and complex. Metabolism was restricted to three regions of the molecule and involved the formation of a range of metabolites. Whereas metabolism was frequently observed in two different regions of the molecules examined, there was no evidence that all three pathways of metabolism were used in the generation of any of the metabolites observed. The ability to use known metabolites of gefitinib as additional *in vitro* substrates substantially enhanced the delineation of the overall metabolic pathway of gefitinib, an extensively metabolized compound. This information will facilitate a greater understanding of the more limited *in vivo* metabolism data available from human studies.

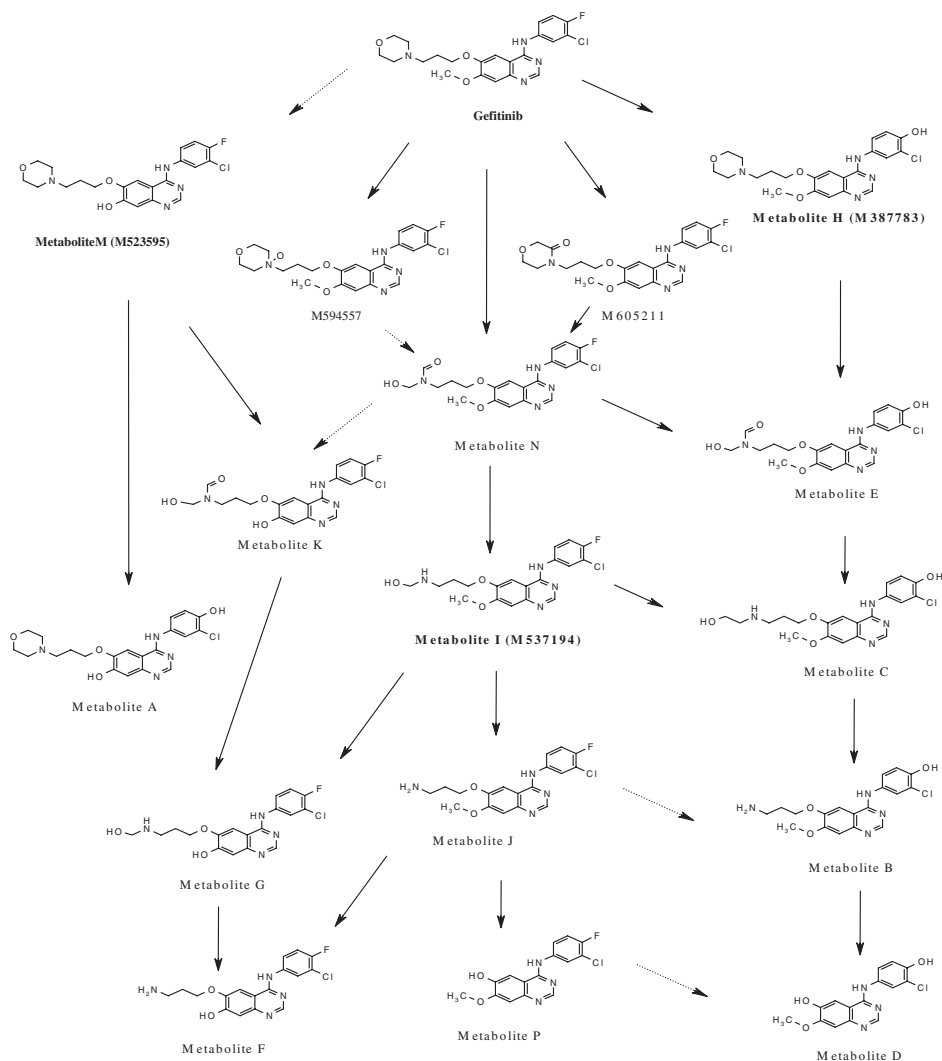


Figure 9. Proposed metabolic pathways of gefitinib in human liver microsomes.

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***In vitro* metabolism of quazepam in human liver and intestine and assessment of drug interactions**

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1. The study was carried out to identify and characterize kinetically the cytochrome P450 (CYP) enzymes responsible for the major metabolite formation of quazepam.

2. In *in vitro* studies using human liver and intestinal microsomes and cDNA-expressed human CYP and FMO isoenzymes, quazepam was rapidly metabolized mainly by CYP3A4 and to a minor extent by CYP2C9, CYP2C19 and FMO1 to 2-oxoquazepam (OQ), which was then further biotransformed to *N*-desalkyl-2-oxoquazepam (DOQ) and to 3-hydroxy-2-oxoquazepam (HOQ) mainly by CYP3A4 and CYP2C9. CYP3A4 is the enzyme predominantly responsible for all the metabolic pathways of quazepam.

3. Itraconazole inhibited the formation of OQ from quazepam, HOQ from OQ and DOQ from OQ in human liver microsomes with K_i values of 8.40, 0.08 and 0.39 μ M, respectively. However, the K_i for OQ formation was greater than the peak plasma itraconazole concentration following a clinically relevant 200-mg oral dose to healthy volunteers. In addition, CYP2C9 and CYP2C19 inhibitors failed to inhibit OQ formation from quazepam.

4. In conclusion, clinically relevant drug interaction with CYP inhibitors seem unlikely for the major metabolic pathway of quazepam to OQ.

Introduction

Quazepam [7-chloro-5-(2-fluorophenyl)-1,3-dihydro-1-(2,2,2-trifluoroethyl)-2H-1,4-benzodiazepine-2-thione] (figure 1) is a trifluoroethyl benzodiazepine hypnotic and widely used in the treatment of insomnia (Ankier and Goa 1988). It is rapidly and extensively metabolized in humans. Substitution of the sulfur group of quazepam with oxygen yields 2-oxoquazepam (OQ), which is pharmacologically active. OQ is then biotransformed by *N*-dealkylation to *N*-desalkyl-2-oxoquazepam (DOQ) and by hydroxylation to 3-hydroxy-2-oxoquazepam (HOQ) (figure 1) (Zampaglione *et al.* 1985). One simple *in vitro* experiment with cDNA-expressed human cytochrome P450 (CYP)s suggested that the formation of OQ from quazepam is mediated mainly by CYP3A4 and CYP2C9, and that the OQ formed is further metabolized to DOQ by CYP3A4 and HOQ by CYP2C9 and CYP3A4 (Fujisaki *et al.* 2001). These data would predict that a drug interaction would occur when quazepam and itraconazole, a potent inhibitor of CYP3A4, are co-administered, resulting in an increased in quazepam plasma concentration. However, Kato *et al.* (2003) reported that the biotransformation to OQ after a single oral dose of quazepam was not affected by itraconazole. Until now, although the identity of the species of CYP enzymes responsible for

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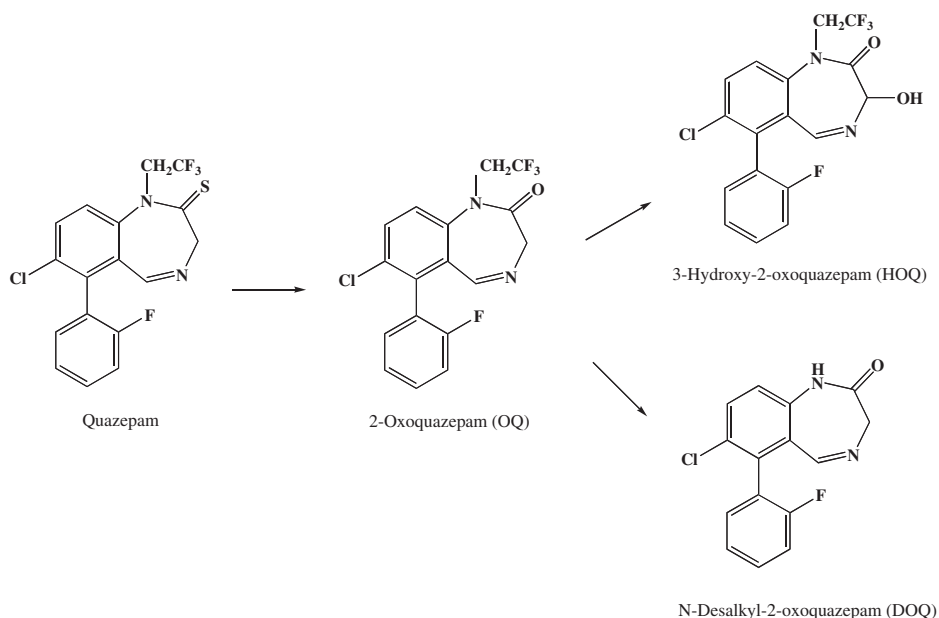


Figure 1. Metabolic pathways for quazepam.

the major metabolic pathways of quazepam has been clarified, information on kinetics such as the affinities and intrinsic clearance in human liver microsomes for the replacement of the sulfur group of quazepam by oxygen have not been performed. The aim of this study was to characterize kinetically the CYP enzymes responsible for the biotransformation of quazepam.

Materials and methods

Reagents and chemicals

Quazepam and its metabolites, 2-oxoquazepam (OQ), 3-hydroxy-2-oxoquazepam (HOQ) and N-desalkyl-2-oxoquazepam (DOQ), were donated by Mitsubishi Welpharma Co. Ltd (Osaka, Japan). The chemical purities of quazepam, OQ, HOQ and DOQ were > 98% (data was provided by Mitsubishi Welpharma). Midazolam was kindly donated by Yamanouti Pharmaceutical (Tokyo, Japan). Sep-Pak CN cartridges were purchased from Waters (Bedford, MA, USA). All solvents used were of HPLC grade (Wako Pure Chemical Industries, Osaka, Japan). Omeprazole, sulphaphenazole, ticlopidine, methimazole and erythromycin were purchased from Sigma (St Louis, MO, USA). Itraconazole was donated from Jansen Pharmaceutical Co. (Beerse, Belgium). The cofactors (nicotinamide adenine dinucleotide phosphate [NADP⁺], glucose 6-phosphate dehydrogenase, and glucose 6-phosphate) were purchased from Sigma. Human liver microsomes, human intestinal microsomes, microsomes prepared from human B lymphoblastoid cells expressing human CYPs 1A2, 2A6, 2C9, 2C19, 2D6, 2E1 or 3A4 and microsomes prepared from baculovirus-infected insect cells expressing human flavin-containing monooxygenase 1 (FMO1) or FMO3 were purchased from GENTEST Corporation (Woburn, MA, USA).

Analytical procedures

The concentrations of quazepam and its metabolites in microsomal sample were measured by a reverse-phase HPLC method. The apparatus used for HPLC was a Model PU-980 chromatography pump (JASCO, Tokyo, Japan) equipped with a JASCO UV-970 ultraviolet detector. The wavelength was set at 254 nm. Test samples were introduced using a JASCO AS-1555-10 auto sampler with an effective volume of 50 µl. The HPLC column used a Grand 120 C2-5 CN stationary phase (150 mm × 4.6 mm i.d., Owani, Japan). The mobile phase consisted of 0.5% KH₂PO₄ (pH 6.5)-acetonitrile (75:25, v/v), which was degassed in an ultrasonic bath before use. A flow-rate

of 1.0 ml min^{-1} was used at ambient temperature. The detection limits of quazepam, OQ, HOQ and DOQ were 2.6, 2.7, 2.7 and 3.6 nM , respectively.

Incubation conditions

Incubations were carried out in duplicate with the reconstituted human liver microsomes in 5-ml test tubes, on three separate occasions ($n = 3$), using a shaking water bath at 37°C . A typical incubation mixture consisted of a cofactor solution ($100 \mu\text{l}$), microsomal preparation ($50 \mu\text{l}$, 0.5 mg microsomal protein) and substrate ($5 \mu\text{l}$, $0\text{--}64.6 \mu\text{M}$ for quazepam and $0\text{--}67.4 \mu\text{M}$ for OQ) in a total volume of 0.2 ml . The cofactor solution consisted of NADP^+ (1.3 mM), glucose 6-phosphate (3.3 mM), glucose 6-phosphate dehydrogenase (0.4 units) and magnesium chloride (3.3 mM) in sodium phosphate buffer (0.1 M , $\text{pH } 7.4$). The metabolic reaction was initiated by the addition of the cofactor solution and terminated by immersing in an ice bath and the addition of 5.0 ml of 1.0 M sodium chloride. Before extraction, midazolam (50 ng) in methanol ($10 \mu\text{l}$) was added as an internal standard to the incubation mixture. The reactions with pooled human liver microsomes were incubated for 5 min for quazepam and 60 min for OQ. OQ formation from quazepam by human liver microsomes was linear for up to 5 min at a substrate concentration range of $12.9 \text{ nM}\text{--}64.6 \mu\text{M}$ quazepam; therefore, on the basis of this data, subsequent kinetic studies were carried out at 5 min . On the other hand, the formation of HOQ and DOQ from OQ by human liver microsomes appears to be linear over 60 min at substrate concentrations of $13.5 \text{ nM}\text{--}67.4 \mu\text{M}$; therefore, subsequent kinetic studies were carried out at 60 min . Microsomal enzymes were inactivated by heat treatment in control incubations. The reactions with CYP enzymes expressed in a cell line were also carried out for 5 min for quazepam and 60 min for OQ under the same condition described above. Controls for human CYP enzymes were expressed in a cell line incubated with control microsomes transfected with a control vector. For examination of FMO1 and FMO3 involvement in quazepam and OQ metabolism, microsomal incubations were carried out for 5 min for quazepam and 60 min for OQ under experimental condition similar to those described above.

Inhibition studies

Five different selective inhibitors were used in order to define the role of individual CYP enzymes in the metabolism of quazepam and OQ. The CYP inhibitors used were sulfaphenazole (1 , 10 , 50 and $100 \mu\text{M}$) for CYP2C9, omeprazole ($50 \mu\text{M}$) and ticlopidine ($100 \mu\text{M}$) for CYP2C19, and itraconazole (0.1 , 1 , 5 and $10 \mu\text{M}$) and erythromycin (1 , 10 , 50 and $100 \mu\text{M}$) for CYP3A4 (Bourrie *et al.* 1996, Collier *et al.* 1999, Echizen *et al.* 1993, Giancarlo *et al.* 2001, Halpert *et al.* 1994, Mancy *et al.* 1996, Newton *et al.* 1995, Venkatakrishnan *et al.* 2001). On the other hand, methimazole ($100 \mu\text{M}$) was used for inhibition of FMOs present in liver microsomes (Chung and Cha 1997, Rodriguez and Acosta 1997). The inhibitors were pre-incubated with human liver microsomes and the NADPH-generating system for 5 min at 37°C under the same conditions described above. The reaction was started by addition of the substrate quazepam or OQ (final concentration: $64.6 \text{ nM}\text{--}64.6 \mu\text{M}$ for quazepam and $67.4 \text{ nM}\text{--}67.4 \mu\text{M}$ for OQ). After incubation, reactions were terminated as described above.

Extraction method

The mixture was applied to a Sep-Pak CN cartridge that had previously been activated with 5.0 ml acetonitrile followed by 5.0 ml water. The cartridge was then washed with 5.0 ml water, and then eluted with 5.0 ml 40% acetonitrile in water. The eluate was evaporated to dryness in vacuum at 50°C by a rotary evaporator (Iwaki, Tokyo, Japan). The residue was dissolved in $50 \mu\text{l}$ acetonitrile and $50 \mu\text{l}$ HPLC mobile phase and injected into the HPLC apparatus.

Data analysis

The calibration graphs were constructed from the peak-height ratio of each metabolite to midazolam as an internal standard of the HPLC chromatograms, and plotted against the nominal concentration of each corresponding metabolite.

The kinetics of the biotransformations of quazepam and OQ by human liver microsomes and cDNA expressed CYP enzymes were described by the Michaelis-Menten equation for a one-enzyme system using a non-linear least-squares regression program, MULTI (Yamaoka *et al.* 1981), and apparent K_m , $V_{\max}$ and enzyme inhibition constant (K_i) values were estimated. A Student's t -test was used for statistical calculation of metabolite formation from quazepam or OQ by reaction with CYP enzymes expressed in a cell line. $p < 0.05$ was considered statistically significant.

Results

Biotransformation of quazepam to OQ

Quazepam was rapidly metabolized to OQ. The kinetics for OQ formation from quazepam by human liver and intestinal microsomes was consistent with

the Michaelis–Menten model (figure 2A). Eadie–Hofstee plots for OQ formation showed a biphasic profile in human liver and a monophasic profile in human intestinal due to undetectable OQ formation at lower substrate concentrations (figure 2B). In human liver microsomes, this indicates that several enzymes, high- and low-affinity enzymes, could be involved in OQ formation: namely, high

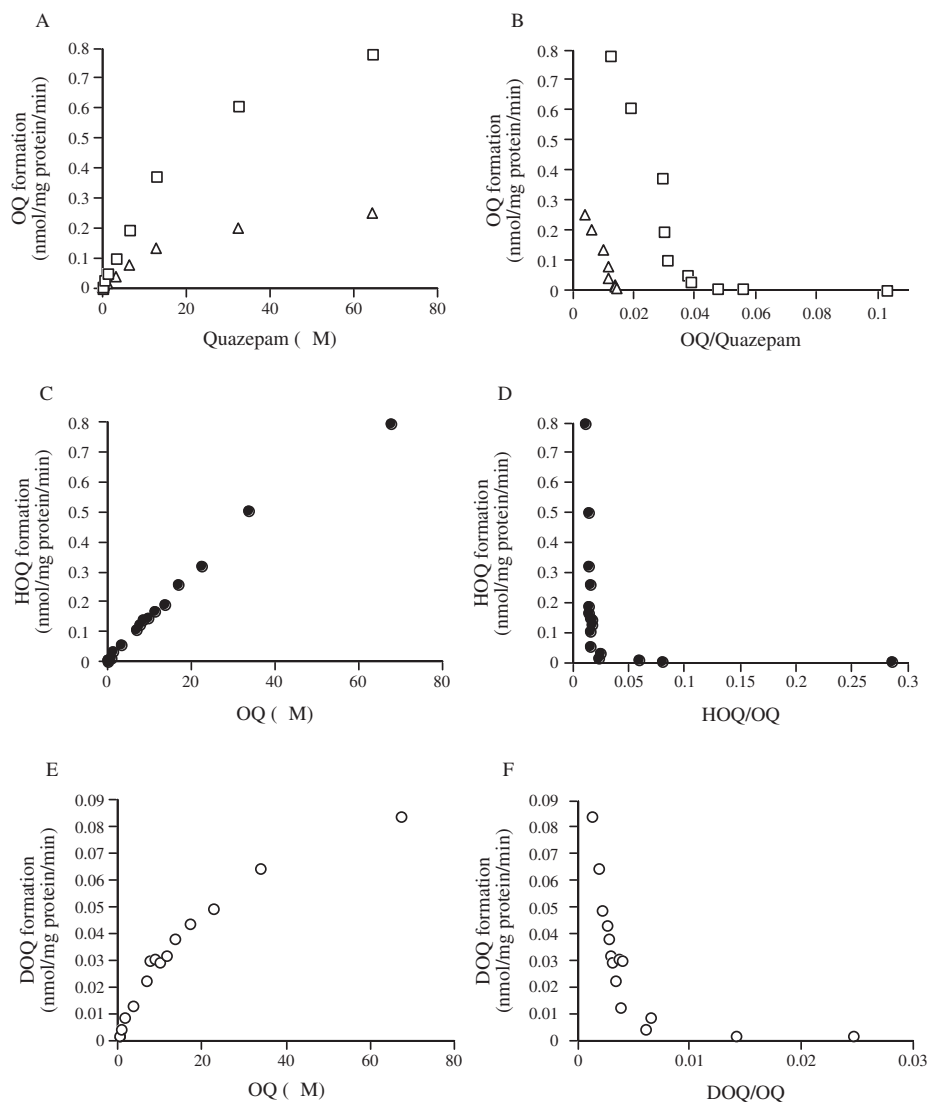


Figure 2. Kinetics of quazepam metabolism. (A) Velocity versus substrate concentration plots for OQ formation from quazepam in human liver (open squares) and intestinal (open triangles) microsomes. (B) Eadie–Hofstee plots for velocity of OQ formation versus the velocity/concentration of quazepam in same liver sample. (C) Velocity versus substrate concentration plots for HOQ formation from OQ in human liver microsomes. (D) Eadie–Hofstee plots for velocity of HOQ formation versus the velocity/concentration of OQ in same liver sample. (E) Velocity versus substrate concentration plots for DOQ formation from OQ in human liver microsomes. (F) Eadie–Hofstee plots for velocity of DOQ formation versus the velocity/concentration of OQ in same liver sample. Symbols are experimental data points representing an average of three incubations.

Table 1. Kinetic parameters for the metabolite formation of quazepam in human liver microsomes.

Metabolites	Samples	K_{m1}	V_{max1}	V_{max}/K_m	K_{m2}	V_{max2}	V_{max}/K_m
OQ from quazepam	HLM	0.11	12	114	22	860	40
	HIM	—	—	—	24	343	15
HOQ from OQ	HLM	0.21	9.5	45	19	467	24
DOQ from OQ	HLM	0.11	4.0	38	27	117	4.3

HLM; Human liver microsomes, HIM; Human intestinal microsomes

K_m and V_{max} are expressed as μM and $\text{pmol}/\text{min}/\text{mg}$ protein, respectively. V_{max}/K_m is expressed as $\mu\text{l}/\text{min}/\text{mg}$ protein.

All data are means from three experiments.

affinity with an apparent K_{m1} of $0.11\mu\text{M}$ and lower affinity with K_{m2} of $22\mu\text{M}$ (table 1). On the other hand, the estimated apparent K_m and V_{max} values for OQ formation in human intestinal microsomes were $24\mu\text{M}$ and $343\text{pmol mg}^{-1}\text{protein min}^{-1}$, respectively (table 1).

The effects of selective CYPs 2C9, 2C19 and 3A4 inhibitors and an FMO inhibitor on OQ formation from quazepam in human liver microsomes are shown in table 2. As shown in table 2, no appreciable change was observed in OQ formation at a concentration of $12.9\mu\text{M}$ of quazepam in human liver microsomes when the CYP2C9 and CYP2C19 inhibitors, sulfaphenazole ($50\mu\text{M}$, CYP2C9 inhibitor), omeprazole ($50\mu\text{M}$, CYP2C19 inhibitor), ticlopidine ($100\mu\text{M}$, CYP2C19 inhibitor), and the FMO inhibitor; methimazole ($100\mu\text{M}$), were added. However, itraconazole ($10\mu\text{M}$, CYP3A4 inhibitor) and erythromycin ($100\mu\text{M}$, CYP3A4 inhibitor) inhibited OQ formation from quazepam to 49 and 43% of control activity, respectively. The apparent K_i values for inhibition by itraconazole and erythromycin of OQ formation in human liver microsomes were 8.4 and $26\mu\text{M}$, respectively.

Furthermore, the abilities of cDNA-expressed P450 and FMO enzymes to form OQ following incubations with $12.9\mu\text{M}$ of quazepam were determined (figure 3 and table 3). As shown in figure 3, CYPs 3A4, 2C9 and 2C19 catalysed OQ formation, but not CYPs 1A2, 2A6, 2D6 and 2E1 (detection limit for OQ was 2.7nM). CYP3A4 displayed the highest activity for the formation of OQ. The apparent mean K_m values obtained were $23\mu\text{M}$ for CYP2C9, $8.9\mu\text{M}$ for CYP2C19 and $16\mu\text{M}$ for CYP3A4, and the mean V_{max} values were 6.4 , 0.39 and $12\text{pmol min}^{-1}\text{pmol}^{-1}$ of P450 for CYPs 2C9, 2C19 and 3A4, respectively (table 3). On the other hand, FMO1 exhibited activity toward the OQ formation, but not FMO3. Mean K_m and V_{max} values for FMO1 were $26\mu\text{M}$ and $1.9\text{pmol min}^{-1}\text{pmol}^{-1}$ of FAD, respectively. The activity towards OQ formation by CYP3A4 was significantly greater than that by FMO1 (figure 3).

Biotransformation of OQ to HOQ and DOQ

The apparent kinetic parameters for the enzymes responsible for HOQ and DOQ were examined using human liver microsomes. The kinetics for the formation of HOQ and DOQ from OQ by human liver microsomes was also consistent with the Michaelis–Menten model (figure 2C, E). Using Eadie–Hofstee representation, it appeared that the P450 enzymes involved in the formation of HOQ and DOQ displayed two K_m values (figure 2D, F and table 1).

As shown in table 2, selective CYP2C19 inhibitors did not have effect on OQ formation at a concentration of $13.5\mu\text{M}$ of OQ; however, sulfaphenazole ($50\mu\text{M}$),

Table 2. Effects of various CYP and FMO inhibitors on the metabolism of quazepam and OQ in human liver microsomes.

Incubation time			OQ formation from quazepam		HOQ formation from OQ		DOQ formation from OQ	
			5 min		10 min		10 min	
Inhibitors	μM	CYP isozyme targeted	% of control ¹	Ki (μM) ²	% of control ¹	Ki (μM) ²	% of control ¹	Ki (μM) ²
None	–	–	100		100		100	
Sulphaphenazole	50	CYP2C9	91	–	82	6.4 ± 0.3	74	7.0 ± 0.6
Omeprazole	50	CYP2C19	93	–	97	–	101	–
Ticlopidine	100	CYP2C19	110	–	94	–	98	–
Itraconazole	10	CYP3A4	49	8.4 ± 0.3	12	0.08 ± 0.06	30	0.39 ± 0.17
Erythromycin	50	CYP3A4	43	26 ± 6	23	1.1 ± 0.6	43	1.7 ± 0.3
Methimazole	100	FMO	105	–	–	–	–	–

1. Data are expressed as mean values obtained from three incubations at a concentration of 12.9 μM for quazepam or 13.5 μM for OQ.
2. Data are expressed as mean (±SD) obtained from three incubations.

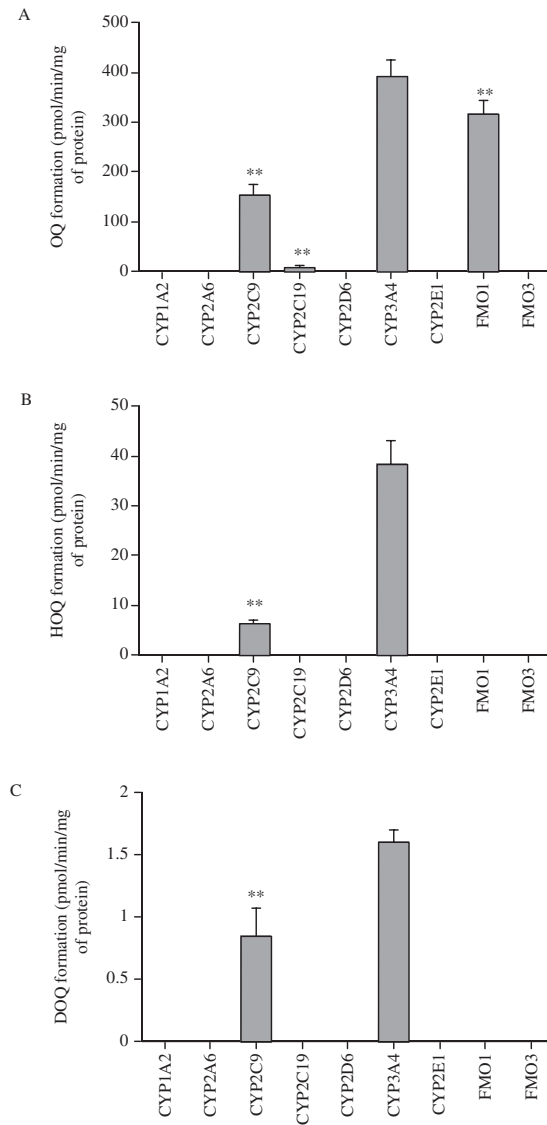


Figure 3. Enzymology of quazepam metabolism. (A) OQ formation from quazepam. (B) HOQ formation from OQ. (C) DOQ formation from OQ in cDNA-expressed human CYP and FMO enzymes at a substrate concentration of 12.9 μ M for quazepam or 13.5 μ M for OQ. Data are means $\pm$ SD obtained from three incubations. ** $p < 0.01$ compared with the metabolite formation activity by CYP3A4.

itraconazole (10 μ M) and erythromycin (50 μ M) inhibited HOQ formation to 82, 23 and 12% of control activity, respectively, and DOQ formation to 74, 30 and 43%, respectively. The apparent mean K_i values for inhibition by sulfaphenazole, itraconazole and erythromycin of HOQ formation in human liver microsomes were 6.4, 0.08 and 1.1 μ M, respectively, and those for DOQ formation were 7.0, 0.39 and 1.7 μ M, respectively.

HOQ and DOQ formation from OQ were also studied using the human cDNA-expressed P450 enzymes (figure 3 and table 3). As shown in figure 3,

Table 3. Kinetic parameters for the metabolite formation from quazepam and OQ in human cDNA-expressed P450s.

Metabolites	Expressed enzymes	K_m	V_{max}	V_{max}/K_m
OQ from quazepam	CYP2C9	23	6.4	277
	CYP2C19	8.9	0.39	44
	CYP3A4	16	12	722
	FMO1	26	1.9	75
HOQ from OQ	CYP2C9	20	0.18	9.0
	CYP3A4	15	0.67	44
DOQ from OQ	CYP2C9	31	0.03	1.1
	CYP3A4	25	0.07	2.8

K_m and V_{max} are expressed as μM and $\text{pmol/min/pmol P450}$ or FAD isoform , respectively.
 V_{max}/K_m is expressed as $\text{nl/min/pmol P450 isoform for CYP}$ and $\text{nl/min/pmol FAD for FMO1}$
 All data are means from three experiments.

CYPs 3A4 and 2C9 exhibited activity toward both HOQ and DOQ formation, but not CYPs 1A2, 2A6, 2C19, 2D6 and 2E1 (detection limit for HOQ and DOQ were 2.7 and 3.6 nM, respectively). The apparent mean K_m values for the formation of HOQ were 20 and 15 μM for CYP2C9 and CYP3A4, respectively, and the mean V_{max} values were 0.18 and 0.67 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 for CYP2C9 and CYP3A4, respectively. K_m values for the formation of DOQ were 31 and 25 μM for CYP2C9 and CYP3A4, respectively, and the V_{max} values were 0.03 and 0.07 $\text{pmol min}^{-1} \text{pmol}^{-1}$ P450 for CYP2C9 and CYP3A4, respectively.

Discussion

The metabolism of quazepam was examined using human liver microsomes as well as microsomes expressing individual human CYP enzymes in order to characterize kinetically the CYP enzymes in the metabolic pathways of quazepam and OQ, a pharmacologically active metabolite. Following quazepam administration, OQ appears in plasma rapidly with a plasma concentration–time pattern closely resembling that of unchanged drug (Zampaglione *et al.* 1985). Itraconazole did not change the pharmacokinetics such as peak plasma concentration (C_{max}), the area under plasma concentration–time curve (AUC) and elimination half-life of quazepam, although it significantly decreased the C_{max} and AUC for DOQ from OQ (Kato *et al.* 2003). Plasma levels of itraconazole during usual clinical use generally exceed 0.05–0.3 μM (Hardin *et al.* 1988, Heykants *et al.* 1989, Negroni and Arechavala 1993). Since the calculated K_i values (8.4 μM) for inhibition of OQ formation by itraconazole are considerably higher than typical clinical plasma concentrations, the inhibition of OQ biotransformation by itraconazole is likely to be of little clinical importance. On the other hand, even at the highest concentration examined for the specific CYP2C9 inhibitor, it failed to inhibit OQ formation from quazepam. This result indicates that the formation of OQ was almost completely dependent on CYP3A4. Thus, our data infer that a drug interaction with CYP3A4 and CYP2C9 inhibitors is not important in the major metabolic pathway to OQ from quazepam.

On the other hand, quazepam is a selective substrate of FMO1, which is present in human small intestine, but not detectable in human liver (Yeung *et al.* 2000). Therefore, we could not confirm inhibition of OQ formation by methimazole, an FMO inhibitor, in human liver microsomes. It is reported that FMO1

content in human small intestine is 2.9 (range 1.1–6.4) pmol mg⁻¹ protein (Paine *et al.* 1997), and CYP3A content in human duodenum is 30.6 (range 3.0–90.8) pmol mg⁻¹ protein (Yeung *et al.* 2000). Although the contribution of FMO1 for quazepam metabolism has very little clinical significance, this result is very interesting. CYPs 3A4, 2C9 and 2C19 exist in the small intestine in addition to FMO1 (Kolars *et al.* 1994, Zhang *et al.* 1999, Lapple *et al.* 2003). In the present study, the intrinsic clearance for OQ formation in human intestinal microsomes was 2.7-fold lower compared with that in liver microsomes (15 and 40 µl min⁻¹ mg⁻¹ protein, respectively). However, this OQ formation activity by intestinal microsomes predicts a contribution of the intestine at the first-pass metabolism of quazepam. We therefore conclude that quazepam is efficiently metabolized to OQ by CYPs 3A4, 2C9, 2C19 and FMO1 in the small intestine and CYPs 3A4, 2C9 and 2C19 in the liver. Although the expression and activity of CYP2C9 and CYP2C19 is lower in the intestine than in the liver, the surface area of the proximal small intestine is large and CYP2C9 and CYP2C19 may well contribute to the first-pass metabolism of quazepam (Obach *et al.* 2001, Lapple *et al.* 2003).

In the present data, Eadie–Hofstee plots for the formation of OQ, HOQ and DOQ in human liver microsomes showed a biphasic kinetic profile. We could not clearly differentiate whether these profiles indicate an enzyme with a low- and high-affinity binding component or indicating a dual-enzyme, low- and high-affinity enzymes, because the formation of OQ, HOQ and DOQ did not exhibit biphasic Eadie–Hofstee plots with cDNA-expressed P450 enzymes and the affinities of quazepam and OQ for cDNA-expressed P450 were relatively low. However, similar to our present results, Fujisaki *et al.* (2001) has also reported that only CYP3A4 and CYP2C9 contribute to the metabolism of quazepam in the therapeutic plasma concentration range of quazepam (15–150 nM).

In our present data, OQ is metabolized mainly by CYP3A4 and to a minor extent by CYP2C9 to both HOQ and DOQ with the major metabolite being HOQ. HOQ is excreted as a glucuronide in humans, and HOQ glucuronide becomes the major urinary metabolite of quazepam (Zampaglione *et al.* 1985). The formation of HOQ and DOQ from OQ were inhibited by itraconazole with K_i values of 0.08 and 0.39 µM, respectively. Our *in vitro* data suggest that itraconazole inhibits the metabolic pathway to DOQ from OQ at therapeutic levels, resulting in a decreased plasma concentration of DOQ and an increased plasma concentration of OQ. However, Kato *et al.* (2003) reported that the $C_{\max}$ and the AUC of DOQ were significantly decreased by itraconazole treatment and those for OQ were also decreased. This previously reported interpretation of *in vivo* data is not consistent with the current *in vitro* data. Furthermore, our inhibitory study showed that although OQ formation from quazepam was inhibited by itraconazole for up to 10 min after commencement of incubation ($K_i = 8.4$ µM), it increased conversely after 20 min (data not shown). This finding is also the result of the following metabolic pathways to HOQ and DOQ from OQ being substantially inhibited by itraconazole. Both OQ and DOQ are also pharmacologically active. Although the concentration of DOQ in human brain was reported to be much higher than those of quazepam and OQ (Hilbert *et al.* 1986, Anker and Goa 1988), the extent of contribution of DOQ to the hypnotic activity of quazepam is not established and the clinical importance of DOQ is also unknown. Moreover, the degree of the pharmacological activity of OQ is also not unknown. Kato *et al.* (2003)

reported that itraconazole treatment did not affect psychomotor function parameter of quazepam, though the plasma concentrations of OQ and DOQ significantly decreases by itraconazole. Possibly only the plasma concentration of unchanged drug itself may relate to the pharmacological activity of quazepam. Then our *in vitro* data suggest clearly that clinically significant drug interaction between quazepam and other CYP substrates will not occur. However, further studies on the pharmacological properties of OQ and DOQ are necessary.

In conclusion, quazepam is rapidly metabolized to OQ mainly by CYP3A4 and to a minor extent by CYPs 2C9, 2C19 and FMO1, and OQ is further biotransformed to HOQ and DOQ mainly by CYP3A4 and CYP2C9. There is almost no possibility that a drug interaction will occur in the major metabolic pathway of quazepam to OQ at therapeutic doses.

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Identification of the cytochrome P450 enzymes involved in the metabolism of domperidone

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1. The objective was to identify the major cytochrome P450 enzyme(s) involved in the metabolism of domperidone.
2. Experiments were performed using human liver microsomes (HLMs), recombinant human cytochrome P450 enzymes, cytochrome P450 chemical inhibitors and monoclonal antibodies directed against cytochrome P450 enzymes.
3. Four metabolites were identified from incubations performed with HLMs and excellent correlations were observed between the formation of domperidone hydroxylated metabolites (M1, M3 and M4), N-desalkylated domperidone metabolite (M2) and enzymatic markers of CYP3A4/5 ($r^2 = 0.9427, 0.951, 0.9497$ and 0.8304 , respectively).
4. Ketoconazole (1 μM) decreased the formation rate of M1, M2, M3 and M4 by 83, 78, 75 and 88%, respectively, whereas the effect of other inhibitors (quinidine, furafylline and sulfaphenazole) was minimal. Important decreases in the formation rate of M1 (68%), M2 (64%) and M3 (54%) were observed with anti-CYP3A4 antibodies.
5. Formation of M1, M2 and M3 in HLMs exhibited Michaelis–Menten kinetics (K_m : 166, 248 and 36 μM , respectively). Similar K_m values were observed for M1, M2 and M3 when incubations were performed with recombinant human CYP3A4 (K_m : 107, 273 and 34 μM , respectively).
6. The data suggest that CYP3As are the major enzymes involved in the metabolism of domperidone.

Introduction

Domperidone is a selective antagonist of dopamine D2 receptors located outside of the blood–brain barrier (Meuldermans *et al.* 1981, Prakash and Wagstaff 1998, Barone 1999). The drug is structurally related to butyrophenones and has gastrokinetic and anti-emetic properties similar to those of metoclopramide, cisapride and certain neuroleptic drugs (Brogden *et al.* 1982, Prakash and Wagstaff 1998, Barone 1999). It regulates smooth muscle functions in the stomach, the small intestine and the oesophagus (Barone 1999). It increases the frequency and duration of antral and duodenal contractions, thus decreasing transit time of food through the gastrointestinal tract (Prakash and Wagstaff 1998). Since domperidone does not readily cross the blood–brain barrier, it is not likely to cause extrapyramidal effects (Brogden *et al.* 1982, Prakash and Wagstaff 1998, Barone 1999).

Domperidone is rapidly and almost completely absorbed following oral, intramuscular and rectal administration (Barone 1999). In comparison with

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and furafylline and ketoconazole were purchased from Gentest Corporation (Woburn, MA, USA). Domperidone (> 99% purity) was purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). Other chemicals were obtained from commercial sources and were of analytical grade.

Biological materials

Liver microsomes from 15 human liver samples were obtained from Human Biologics International (Scottsdale, AZ, USA). Microsomes from lymphoblasts expressing CYP3A4 protein were purchased from Gentest. This latter protein was derived from a human AHH-1-TK^{+/−} cell line transfected with cDNA encoding human CYP3A4. Monoclonal antibodies (MAB) directed against CYP3A4 and CYP2B6 were also purchased from Gentest. All preparations were stored at -80°C .

HPLC assay

The determination of domperidone and its metabolites in the incubation mixture was performed by HPLC with combined fluorescence and ultraviolet detection methods. The HPLC system consisted of a SpectraSystem P4000 pump, a SpectraSystem AS3000 autosampler, a model FL3000 fluorescence detector, a model UV3000 ultraviolet detector, a model PC1000 System Software, all from Thermo Separation Product (Fremont, CA, USA). Liquid chromatography was carried out using a Beckman Ultrasphere ODS column (5 μm , 25 cm $\times$ 4.6 mm: Fullerton, CA, USA). Separation of the metabolites was achieved using a mixture of methanol, acetonitrile and 0.01 M disodium citrate pH 3.4 (35:10:55 v/v) and containing 1.5 ml l^{−1} triethylamine. The mobile phase was delivered at a flow rate of 1 ml min^{−1}. The excitation and emission wavelengths of the fluorescence detector were set at 282 and 328 nm, respectively, and the absorption wavelength of the ultraviolet light detector was set at 254 nm. Intra- and interassay coefficients of variation for precision and accuracy were < 8%.

Microsomal incubations

Correlation analyses with HLMs. Standard incubations (final volume of 500 μl) consisted of 5 μl of the substrate solution (10 mM solution in DMSO; final concentration of domperidone in incubation mixture was 100 μM), 20 μl of microsomes from human livers (400 μg proteins), 375 μl potassium phosphate buffer 50 mM (pH 7.4), 100 μl of an NADPH-generating system solution (14.93 mg NADP⁺, 15.05 mg glucose 6-phosphate and 6 units glucose 6-phosphate dehydrogenase in 3.0 ml MgCl₂ 25 mM). Under aerobic conditions, tubes containing buffer, NADPH-generating system solution and microsomes were pre-incubated for 8 min at 37°C; then substrate was added. Incubations were performed in duplicate for 45 min at 37°C in a shaking water bath. The enzymatic process was stopped by adding 500 μl ice-cold acetonitrile and 60 μl water solution of the internal standard (encainide at a concentration of 100 μg ml^{−1}). Then the mixture was centrifuged at 12 000 rpm (10 000 g) for 5 min and the supernatant (10 μl) was injected onto the HPLC apparatus described above.

Determination of K_m and V_{max}

Kinetic parameters for the formation of domperidone metabolites were obtained by incubating a range of domperidone concentrations (5–500 μM) with human liver microsomes (400 μg proteins) or human microsomes expressing high levels of CYP3A4 protein (100 μg protein).

Identification of domperidone metabolites

Supernatant from incubations performed with HLMs was evaporated to dryness and reconstituted in acetonitrile. An aliquot (1–5 μl) was injected onto a capillary 1100 HPLC system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA). The LC system was coupled to a QSTAR — pulsar time-of-flight (TOF) mass spectrometer, model QSTAR — Pulsar (MDs Sciex, Concord, Ontario) equipped with an electrospray ionization (ESI) source. The mass spectrometer was calibrated using a two-point calibration mode, with the octapeptide 'ALILTLVS' of exact mass 829.5398 amu, and caesium iodide of exact mass 132.9054 amu. The instrument was considered ready for sample injection when the mass drift from the theoretical readings was < 2 ppm. A Zorbax C-18 capillary column 300 SB, 3–5 μm , 150 $\times$ 0.3 mm i.d. (Agilent Technologies) was used to chromatograph the compounds of interest. The mobile phase was a mixture of A; double-distilled and de-ionized water containing 0.1% TFA and B; acetonitrile with 0.1% TFA. A gradient of 20-min 90% to 70% A was employed at a flow rate of 4 μl min^{−1} followed by an isocratic flow of 70% A and 30% B maintained for 30 min at the same flow rate.

Inhibition studies

Ketoconazole (1 μM), quinidine (3 μM), sulfaphenazole (10 μM) or furafylline (20 μM) were pre-incubated 8 min at 37°C with 10 μl (200 μg proteins) of pooled liver microsomal preparations (prepared from equal volumes of microsomes obtained from the 15 human liver samples), buffer (380 μl) and NADPH-generating system (100 μl) before the addition of domperidone (5 μl)

at a final concentration of 100 μ M. Final volume was 500 μ l and incubation procedure was as described above. Incubations were performed in duplicate for each inhibitor.

Immunoinhibition studies

Mouse monoclonal antibodies raised against purified human CYP3A4 and CYP2B6 were used for immuno-inhibition studies. Pooled human liver microsomes (10 μ l; 200 μ g proteins) were pre-incubated with Tris buffer (25 μ M) containing 0, 5, 10, 20, 50 and 100 μ g anti-CYP3A4 or anti-CYP2B6 on ice for 15 min. Subsequently, the NADPH-generating system plus the potassium phosphate buffer were added to the incubation mixture. An 8-min pre-incubation was performed at 37°C before adding the substrate solution (domperidone 100 μ M). Other procedures were as previously described.

Statistical analysis

Data are expressed as the mean $\pm$ SD. Formation rates in the presence of each chemical inhibitor were compared using one-way ANOVA separately. The normality assumption was verified with the Shapiro-Wilk's test and the Brown and Forsythe's variation of Levene's test statistic was used to verify the homogeneity of variances. Relationships between metabolite formation rates and specific activities in human liver microsomal preparations were expressed using the Pearson correlation coefficient. The results were considered significant with $p \leq 0.05$. The data were analysed using the statistical package program SAS v8.2 (SAS Institute, Inc., Cary, NC, USA).

Results

Identification of domperidone metabolites

Four metabolites were detected in analysis performed by HPLC with fluorescence detection. However, a fifth (M5) could be detected when analyses were performed using the HPLC system equipped with a diode array detector and coupled to a time-of-flight mass spectrometer (table 1). Four hydroxylated domperidone metabolites M1, M3, M4 and M5 with molecular weights of 442 amu were identified. M1 and M3 are the major hydroxylated metabolites detected with a relative retention time of 0.735 and 0.923, respectively. M4 and M5 are minor metabolites with a relative retention time of 0.999 and 1.141, respectively. M4 co-eluted with domperidone and M5 eluted slightly more slowly than domperidone. Their exact position of hydroxylation remains however to be established. M2 was identified as 5-chloro-4-pipridazole-1,3-dihydro-benzimidazol-2-one (*N*-dealkylated metabolite) with a molecular weight of 252 amu and a relative retention time of 0.420. The molecular weight of the metabolites was determined with mass accuracies varying from 1 to 10 ppm for both the ^{35}Cl and ^{37}Cl isotopic molecular ions of all metabolites except M2, which corresponding molecular isotopic ions were measured with mass accuracies of about 15 and 27 ppm, respectively. Given the high mass accuracy of these experimental measurements, it can be asserted that M1–M5 were actually generated from hydroxylation and *N*-dealkylation reactions and not by other metabolic processes.

Correlation analyses using HLMs

Table 2 shows the correlation measured between the formation rates of domperidone metabolites M1–4 and the respective cytochrome P450 enzyme activities measured from each of these human microsomal samples. M1–4 were detected in all human liver microsomal preparations studied. Highest correlations were observed between the formation of each domperidone metabolites and testosterone 6 β -hydroxylase activity (a substrate marker of CYP3A4/5 activity). Strong correlations were also observed with dextromethorphan *N*-demethylase (CYP3A4) and good correlations were observed with *S*-mephenytoin *N*-demethylase (CYP2B6) activities.

Table 1. Identification of the major metabolites of domperidone.

Compound	MH ⁺	Relative retention time	Theoretical MH ⁺ (Cl-35) (amu)	Measured MH ⁺ (amu)	Theoretical and observed (amu)	Theoretical and observed (ppm)
Domperidone	C ₂₂ H ₂₅ N ₅ O ₂ Cl	1.0000	426.1699	426.1690	0.0009	2.11
M1	C ₂₂ H ₂₅ N ₅ O ₃ Cl	0.735	442.1648	442.1648	0.0009	2.04
M2	C ₁₂ H ₁₅ N ₃ OCl	0.420	252.0905	252.0868	0.0037	14.68
M3	C ₂₂ H ₂₅ N ₅ O ₃ Cl	0.923	442.1648	442.1603	0.0045	10.18
M4	C ₂₂ H ₂₅ N ₅ O ₃ Cl	0.999	442.1648	442.1604	0.0044	9.95
M5	C ₂₂ H ₂₅ N ₅ O ₃ Cl	1.141	442.1648	442.1612	0.0036	8.14
Theoretical MH ⁺ (Cl-37) (amu)						
Domperidone	C ₂₂ H ₂₅ N ₅ O ₂ Cl	1.0000	428.1669	428.1675	0.0008	1.87
M1	C ₂₂ H ₂₅ N ₅ O ₃ Cl	0.735	444.1617	444.1582	0.0035	7.88
M2	C ₁₂ H ₁₅ N ₃ OCl	0.420	254.0875	254.0836	0.0033	27.16
M3	C ₂₂ H ₂₅ N ₅ O ₃ Cl	0.923	444.1617	444.1584	0.0005	7.43
M4	C ₂₂ H ₂₅ N ₅ O ₃ Cl	0.999	444.1617	444.1622	0.0007	1.13
M5	C ₂₂ H ₂₅ N ₅ O ₃ Cl	1.141	444.1617	444.1624	0.0069	1.58

M1–5, metabolites 1–5.

Table 2. Correlation coefficients between the formation rate of M1, M2, M3 and M4 and cytochrome P450-specific activities in liver microsomes of 15 human samples.

CYP isoform	Enzyme activity	Correlation (r^2) with the formation rates of domperidone metabolites			
		M1	M2	M3	M4
CYP1A2	Caffeine N3-demethylation	0.2112	0.1656	0.1744	0.2424
CYP2A6	Coumarin 7-hydroxylation	0.0038	0.0262	0.0105	0.0067
CYP2B6	<i>S</i> -mephenytoin N-demethylaiton	0.583*	0.5344*	0.5721*	0.5903*
CYP2C9	Tolbutamide methyl-hydroxylation	0.0876	0.0866	0.0798	0.1237
CYP2C19	<i>S</i> -mephytoin 4-hydroxylation	0.2386	0.3318	0.2501	0.2218
CYP2D6	Dextrometorphan <i>O</i> -demethylation	0.2989	0.4097	0.269	0.2454
CYP2E1	Chlorzoxazone 6-hydroxylation	0.3518	0.2248	0.3396	0.3552
CYP3A4	Dextrometorphan N-demethylation	0.9244*	0.8122*	0.8981*	0.9221*
CYP3A4/5	Testosterone 6 β -hydroxylation	0.9427*	0.8304*	0.951*	0.9497*

* $p \leq 0.05$.

Determination of K_m and V_{max}

Experiments were performed to characterize kinetic parameters (K_m and V_{max}) for the formation of domperidone metabolites using human liver microsomes and microsomes from human lymphoblasts expressing high levels of CYP3A4 protein (table 3). Kinetic parameters could not be estimated for M4 since detection was below limit of quantification at low substrate concentration. K_m and V_{max} values measured for the formation of M1–3 were similar in both enzymatic preparations used suggesting that CYP3A4 is a major enzyme involved in the formation of these metabolites. Finally, K_m values for the formation of M3 was much lower than those determined for the formation of M1 and M2, indicating the preferential formation of this metabolite by CYP3A4.

Inhibition studies

To characterize further the involvement of various cytochrome P450 enzymes in the formation of M1–4, studies were performed with well-known semi-selective inhibitors of these enzymes. Figure 2 reports effects of furafylline, sulfaphenazole, quinidine and ketoconazole on the amount of M1–4 formed by human liver microsomes. The presence of ketoconazole in the incubation mixture caused a significant decrease in the formation of M1–4: 83, 78, 75 and 88%, respectively. In contrast, formation of M1–4 was not significantly reduced by sulphafenazole, quinidine and furafylline.

Under the chromatographic conditions used, no chromatographic peaks that might have interfered with the determination of domperidone, its metabolites or the internal standard (i.e. encainide) were observed in the presence or absence of the four inhibitors.

Figure 3 reports on the effects of various amounts of antibodies directed against CYP3A4 and CYP2B6 on the formation of M1–3. M4 could not be quantified in these experiments because of small interfering peaks. Decreases (14, 18, 15%) in the formation of M1–3 were observed even with the lowest amount of anti-CYP3A4 added to the incubation mixture (5 µg). Formation of all metabolites was decreased to a greater extent, but not completely (M1, 68%; M2, 64%; M3, 54%), with 100 µg anti-CYP3A4. On the other hand, the formation rate of metabolites M1–3 was not decreased by the addition of anti-CYP2B6 (0–100 µg) to the incubation mixture.

Table 3. K_m and V_{max} values determined during *in vitro* metabolism of domperidone to M1, M2 and M3 using human liver microsomes and lymphoblastoid cells expressing high level of CYP3A4.

	Metabolite 1		Metabolite 2		Metabolite 3	
	$K_m^\dagger$	$V_{max}^\ddagger$	K_m	V_{max}	K_m	V_{max}
HLMs (pool)	166	2.4	248	1.42	36	5.6
CYP3A4	107	3.1	273	1.3	34	8.1

HLMs, human liver microsomes.

† µM; ‡ nmol nmol⁻¹ P450 min⁻¹.

Kinetic parameters for M4 are not given since the amount of metabolite formed could not be estimated at low substrate concentrations.

Data were derived from incubations performed in duplicate at 10 concentrations of domperidone (5–500 µM).

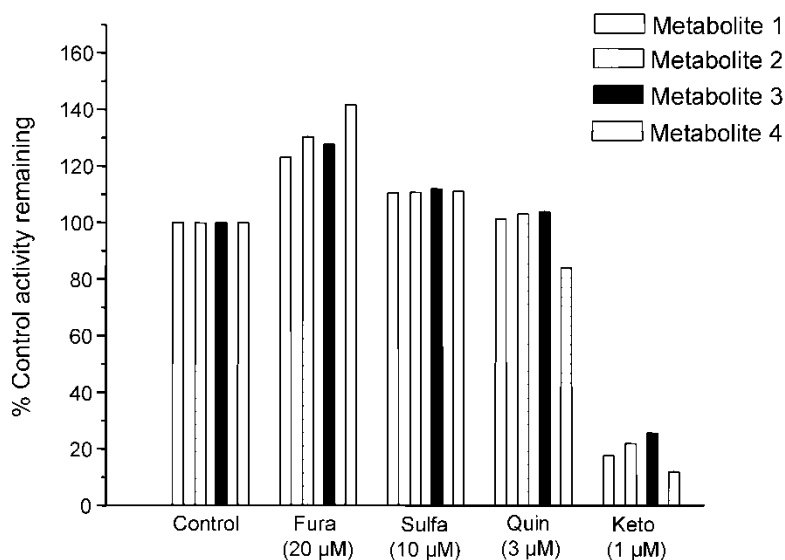


Figure 2. Relative formation rate of domperidone metabolites in the presence of chemical inhibitors (mean $\pm$ SD). Incubations were performed in duplicate with pooled microsomes prepared from 15 human liver samples. Control activities were 3.23 ± 0.08 , 3.13 ± 0.11 , 9.15 ± 0.54 and 0.52 ± 0.06 nmol nmol⁻¹ P450 min⁻¹ for M1, M2, M3 and M4, respectively). Final inhibitor concentrations used are indicated in parentheses.

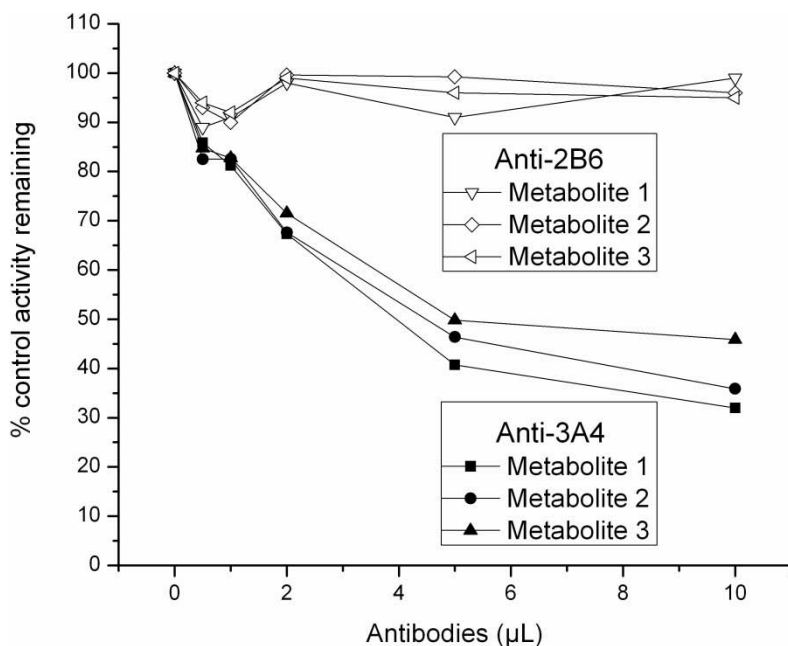


Figure 3. Inhibition of M1, M2 and M3 formation by antibodies directed against CYP3A4 and CYP2B6. Incubations were performed in duplicate with pooled microsomes prepared from 15 human liver samples. Control activities were 1.88 ± 0.08 , 1.91 ± 0.07 and 5.73 ± 0.17 nmol nmol⁻¹ P450 min⁻¹ for M1, M2 and M3, respectively, for CYP3A4 and 1.45 ± 0.05 , 1.58 ± 0.09 and 5.43 ± 0.2 nmol nmol⁻¹ P450 min⁻¹ for M1, M2 and M3, respectively, for CYP2B6.

Discussion

This study investigated the metabolism of domperidone by human liver microsomes and cDNA-expressed human hepatic cytochrome P450 enzymes. Results suggest that CYP3As are the major cytochrome P450 enzymes involved in the formation of M1–4 in man. This contention is based on the following observations: (1) experiments with human liver microsomes demonstrated a very good correlation between the formation rate of M1–4 and testosterone 6 β -hydroxylation activity (CYP3A4/5) or dextrometorphan N-demethylation (CYP3A4) activity; (2) ketoconazole, a known selective inhibitor of CYP3A4, extensively inhibited the formation of M1–4 in human liver microsomes, and (3) the formation of M1–3 was substantially decreased by the addition of CYP3A4 antibodies.

Results obtained from correlation analysis are supported by results of the present chemical and antibody inhibition studies. Previous studies have demonstrated that furafylline, sulfaphenazole, quinidine and ketoconazole are relatively selective inhibitors of CYP1A2, CYP2C9, CYP2D6 and CYP3A4, respectively (Baldwin *et al.* 1995, Newton *et al.* 1995, Clarke 1998, Lessard *et al.* 1999). Inhibition of domperidone metabolism to M1–4 was 83, 78, 75 and 88%, respectively, by the CYP3A4 inhibitor ketoconazole. In contrast, inhibitors of CYP1A2, CYP2C9 and CYP2D6 studied had no marked effect on domperidone metabolism to either M1, M2, M3 or M4.

It was also observed that a significant correlation occurred between the formation rate of M1–4 (0.583, 0.5344, 0.5721 and 0.5903, respectively) and CYP2B6 activity determined in HLMS. To determine whether CYP2B6 has an important role in the metabolism of domperidone, experiments were performed with antibodies directed against CYP2B6. Formation rate of three metabolites (M1–3) was slightly decreased but remained > 90% of control. The significant correlation between the formation rate of M1–4 and *S*-mephenytoin N-demethylation, a selective marker of CYP2B6 activity, may be derived from the fact that we noticed a significant correlation between CYP2B6 and CYP3A4 and CYP3A4/5 activities in the panel of liver microsomes used ($r=0.87$, $p<0.05$ and $r=0.58$, $p<0.05$ respectively, data not shown). A similar correlation between CYP activities was not seen between any other enzymes. Thus, it is concluded that CYP2B6 has no significant impact on the metabolism of domperidone.

Data from the present experiments also suggest that CYP3A5 may be involved in the formation of these metabolites. First, correlation with domperidone metabolites formation was highest with testosterone 6 β -hydroxylase activity (CYP3A4/5) compared with dextrometorphan N-demethylase activity (CYP3A4). These coefficients were 0.9427, 0.8304, 0.951 and 0.9497 compared with 0.9244, 0.8122, 0.8981 and 0.9221 for M1–4, respectively. Inhibition studies with anti-CYP3A4 also suggest a probable role of another cytochrome P450 enzyme in the formation of M1–3. Indeed, inhibition of the formation of these metabolites was not complete in the presence of microsomes containing several CYP450 enzymes.

The characterization of CYP450 enzymes involved in the metabolism of domperidone may help predict potential interaction of domperidone with other drugs metabolized by CYP3A4/5. As demonstrated by Drolet *et al.* (2000), domperidone blocks one of the main potassium channel (HERG) responsible for repolarization of the human heart. An important block of this channel

could lead to an excessive prolongation of QT interval and eventually to torsades de pointes and death. Inhibitions of the metabolism of domperidone by drugs that are also metabolized by the same enzyme or by a potent inhibitor of CYP3A4/5 are therefore expected to cause important drug–drug interactions. Moreover, CYP3A4/5 polymorphisms may play a role in the onset of adverse effects (Chou *et al.* 2001, Kuehl *et al.* 2001).

Conclusion

The results indicate that domperidone is metabolized in human via N-dealkylation (M2) and aromatic hydroxylations (M1, M3 and M4), and that these metabolic steps are catalysed primarily by the enzymes from the CYP3A subfamily.

Acknowledgements

The study was supported by the Canadian Institute of Health Research and Heart and Stroke Foundation of Quebec. C. S. was the recipient of a studentship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) and MDS Pharma Services. V. M. was the recipient of a studentship from MDS Pharma Services and the Canadian Institute for Health Research. The authors also thank Michel Blouin and Nathalie Morin for technical assistance, and Serge Simard, MSc, for statistical analysis.

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Incorporation of xenobiotic carboxylic acids into lipids by cultured 3T3-L1 adipocytes

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1. The study was established to assess the potential for a variety of xenobiotic aromatic carboxylic acids to be incorporated into glycerolipids.

2. The ^{14}C -labelled xenobiotic acids were included in incubations of cultured 3T3-L1 adipocytes under defined conditions. Lipids were extracted and identified by TLC and radioscanning.

3. Ibuprofen, 4-(2,4-dichlorophenoxy)-butanoic acid (2,4-DB), 4-(2-methyl-4-chlorophenoxy)-butanoic acid (MCPB) and 2-(2-methyl-4-chlorophenoxy)-propanoic acid (MCPP) (all 0.5 mM) were incorporated into lipid extracts at rates of 220, 227, 199 and 21 pmol μg^{-1} phospholipid/h, respectively. 2,4-Dichlorophenoxyacetic acid (2,4-D), indomethacin, naproxen and fluroxypyr were incorporated at rates lower than MCPP or not at all. The incorporation of acids was first order to at least 1 mM acid (except MCPB: 300 μM). Triacylglycerol analogues were the major products with incorporation into diacylglycerol and phosphatidylcholine also observed. After digestion with pancreatic lipase, ibuprofen-containing triacylglycerol was unusual in that the main product was the monoacylglycerol analogue, suggesting that esterification had been at the *sn*-2 position.

4. Incubation with cultured 3T3-L1 adipocytes is a useful and easy method to assess whether xenobiotic compounds can be incorporated into glycerolipids; of eight acids assessed, four (of which three have not previously been reported) were shown to form xenobiotic triacylglycerols.

Introduction

It has been known for some time that some foreign compounds such as drugs, food additives or pesticides, which bear a carboxylic acid moiety, can form hydrophobic conjugates that resemble natural lipids in which the xenobiotic acid has replaced a natural fatty acid (Fears *et al.* 1978, Crayford and Hutson 1980). About 40 such acids have been identified of which about 30 can form analogues of triacylglycerol (TAG) (xenobiotic triacylglycerols) and 11 can form xenobiotic cholesteryl esters. A smaller number of reports of phospholipid and diacylglycerol analogues being formed have also appeared (Dodds 1991, 1995).

The formation of xenobiotic TAGs may be regarded as benign metabolism in the short term as the product is likely to be less toxic than the precursor. Over an extended period, however, a method of bio-accumulation is suggested. Xenobiotic phospholipids have the potential to disrupt membrane function. Xenobiotic diacylglycerols are intermediates in the synthesis and degradation of xenobiotic TAGs and some xenobiotic phospholipids. By analogy with natural diacylglycerols and with phorbol diesters, the xenobiotic diacylglycerols could

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have effects on the regulation of the cell cycle: at least one example, a *sn*-1,2 diacylglycerol in which the fatty acid at the *sn*-2 position was replaced with 3-phenoxybenzoic acid, has been shown to stimulate protein kinase C (Moorhouse *et al.* 1989) and preliminary results suggest malignant transformation of cultured fibroblasts can also be provoked (Parry *et al.* 1997).

Experiments to assess the magnitude of xenobiotic lipid formation from individual acids have been performed under a variety of conditions, ranging from whole animal experiments (Quistad *et al.* 1976, Crayford and Hutson 1980) to assays of individual enzymes (Imhof *et al.* 1985, Moorhouse *et al.* 1990), making comparisons between the acids difficult or impossible. Fears *et al.* compared a number of acids using a liver slice preparation (Fears *et al.* 1978) and isolated hepatocytes have also been used (Moorhouse *et al.* 1991). More recently, we reported the use of cultured 3T3-L1 adipocytes to study the lipid metabolism of the non-steroidal anti-inflammatory drug fenbufen (Dodds *et al.* 1995). Here, we adopt this method and use it to compare the formation of xenobiotic glycerolipids from eight different xenobiotic acids.

Materials and methods

Materials

Dulbecco's minimum essential medium (DMEM) (containing 22 mM glucose, pyridoxine HCl and NaHCO₃ but without L-glutamine), Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin/glutamine mixed solution, dexamethasone, isobutyl methyl xanthine, oleic acid, phosphatidylcholine, phosphatidylethanolamine, ammonium thiocyanate, iron(III) chloride, anhydrous sodium taurocholate, (2,4-dichlorophenoxy)acetic acid (2,4-D), 4-(2,4-dichlorophenoxy)butanoic acid (2,4-DB), (±)-ibuprofen, indomethacin and lipase (crude Type II, from porcine pancreas) were all obtained from Sigma Chemical Co. (Poole UK). (+)-Naproxen was from Aldrich (Gillingham, UK); (±)-2-(4-chloro-2-methylphenoxy)propanoic acid (mecoprop or MCPP) and fluroxypyr were from Riedel de Haën (Seelze, Germany). 4-(4-Chloro-2-methyl phenoxy)butanoic acid (MCPB) was recrystallized from stock obtained from May & Baker (Dagenham, UK). The structures of the xenobiotic acids are given in figure 1. Whatman LK6DF silica gel TLC plates, 20 × 20 cm, pre-ruled into 19 lanes and having a 3.5 cm pre-concentration layer, were obtained from Merck Ltd (Poole, UK). Porcine insulin was from Calbiochem (Nottingham, UK), foetal bovine serum from Biochrom KG (Berlin, Germany) and Ecolite scintillation fluid from ICN Biomedicals (Basingstoke, UK). Rosiglitazone was a generous gift from SmithKline Beecham (Welwyn Garden City, UK).

[Ring-¹⁴C]-MCPB, [ring-¹⁴C]-2,4-DB, [ring-¹⁴C]-MCPP, [3-¹⁴C-propanoate]-ibuprofen and [carboxy-¹⁴C]-2,4-D were obtained from Amersham Biosciences (Chalfont St Giles, UK). [Pyrolyring-¹⁴C]-indomethacin and [methoxy-¹⁴C]-naproxen were synthesized 'in house' by Shell Research Ltd from whom they were a gift; similarly, [2,6-¹⁴C]-fluroxypyr was a gift from Dow-Elanco (Wantage, UK). Radioactive acids were re-purified, if necessary, by TLC and adjusted to appropriate specific radioactivities using the unlabelled acids before use.

Xenobiotic TAGs, diacylglycerols and phosphatidylcholines, for use as TLC standards, were prepared from dioleoylglycerol or lyso-phosphatidylcholine and the respective xenobiotic acid using a carbonyl dimidazole method (Bergelson 1980, Dodds *et al.* 1995). The compounds were deemed to be pure as judged by consistent integrals after proton NMR and by the production of a single spot in the TLC systems used.

Cells

Pre-adipocytes (3T3-L1 cells) were obtained from ATCC (Manassas, Virginia, USA, or via ECACC, Porton Down, UK) and were cultured in 60-mm dishes as described (Dodds *et al.* 1995), except that differentiation was induced by adjusting the culture medium to contain 0.25 µM dexamethasone, 0.5 mM isobutyl methyl xanthine, 1.7 µM insulin and 1 µM rosiglitazone for 48 h, after which the cells were maintained in medium supplemented with insulin and rosiglitazone only. After 5 days in the latter medium, when 80–100% of them contained visible fat droplets, the cells were used for incorporation studies.

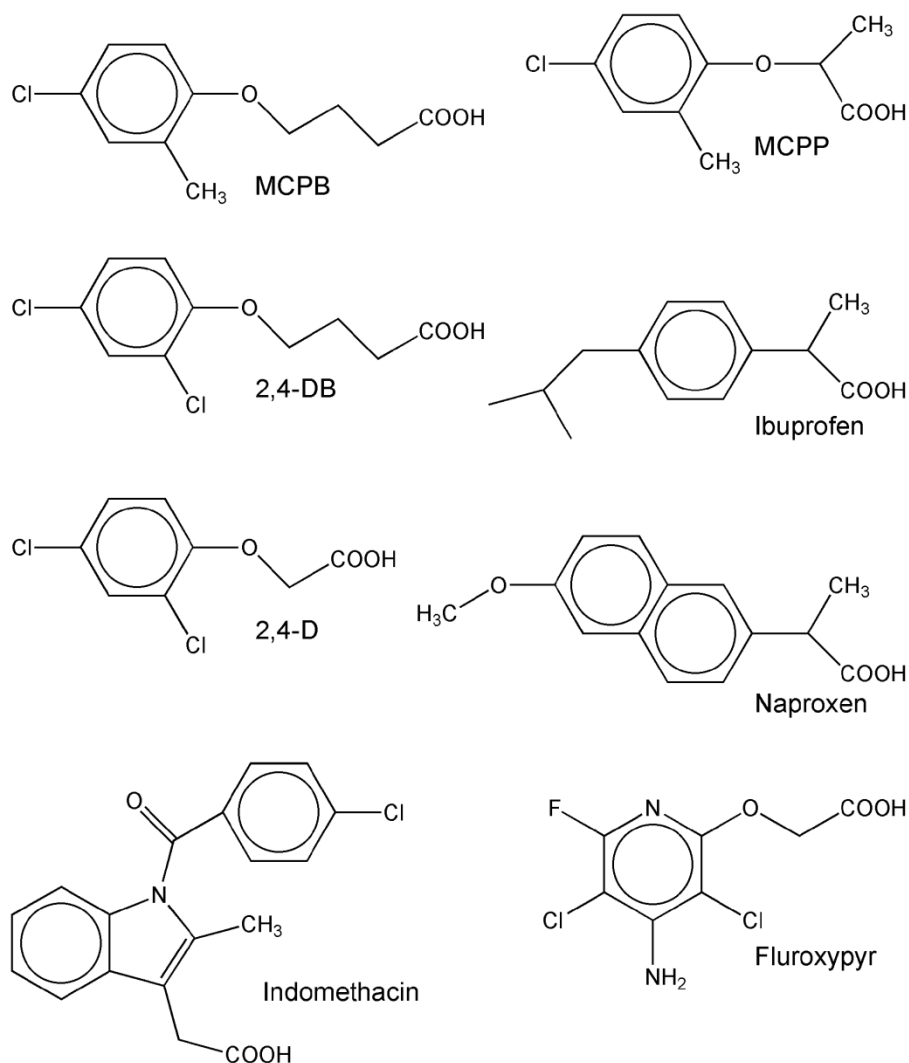


Figure 1. Structures of the xenobiotic acids used. For abbreviations, see the Materials and methods.

Incubation of cultured cells with xenobiotic acids

To commence the incubations under standard conditions, the medium of each 60-mm Petri dish of cultured cells was replaced with 2 ml standard DMEM medium containing insulin and rosiglitazone and 5 mM ^{14}C -xenobiotic acid of known specific radioactivity. The cells were cultured for up to 2 days at 37°C in 5% CO_2 . Then incubations were terminated by removal of the medium and washing of the cells four times in 1% (w/v) BSA in PBS. Immediately afterwards, the cells were scraped from the dish in 1 ml cold methanol, followed by a second 1 ml methanol and then 0.5 ml water. To the combined scrapings was added 1 ml chloroform and the mixture was stored at -20°C for at least 12 h. The phases were then separated by the addition of 1 ml chloroform and 1.3 ml water. The lipids were recovered in the lower, chloroform, phase. In parallel experiments, this method gave recoveries of radioactive lipid equivalent to those obtained using a more elaborate extraction protocol previously described (Dodds *et al.* 1995).

Analysis of products

Samples (1.0 ml) of the chloroform phase were carefully evaporated to dryness in a stream of dry N_2 gas. The lipid extracts were redissolved in a minimum volume of chloroform-methanol (1:2, v/v) and applied to individual lanes of a 19-lane silica gel TLC plate; washings were applied to the same lanes.

Radioactive samples were applied to alternate lanes of the plate and non-radioactive standards (authentic xenobiotic lipid standards when available and appropriate natural lipid standards) were applied to the intervening lanes. The plates were developed for 5 cm beyond the origin in chloroform-methanol-acetic acid-water (60:40:9:3, by volume) to separate phospholipids while carrying neutral lipids at the solvent front. After drying the plate completely, it was developed a second time, for the full length of the plate, in chloroform-methanol-acetic acid (100:2.5:5, by volume) which is less polar, does not cause the phospholipids to migrate and separates the neutral lipids. This double development allowed both neutral lipids and polar lipids to be resolved in a single dimension of a TLC plate and is a development of the method of Brindley and Bowley (1975). Individual lanes of the plate were scanned for radioactivity as described below. The plates were also visualized by fluorescent quenching under ultraviolet light at 254 nm and after exposure to iodine vapour.

On occasion, radioactive bands co-migrating with authentic xenobiotic TAG were scraped from the TLC plates and subjected to lipolysis. The silica gel was extracted three times in 2 ml chloroform-methanol (1:2, v/v), the extracts combined and divided equally between three tubes. Solvent was removed in a stream of N₂ and 1.2 ml 0.75 M Tris (pH 7.4), 0.167 M CaCl₂, 15 mM sodium taurocholate were added to each replicate. The lipids were emulsified by brief sonication using a sonicating water bath, the reaction initiated by the addition of pancreatic lipase (100 µl containing 1000 units) and incubated at 37°C. Samples (200 µl) were removed after 0.5, 5, 10, 20 and 40 min of incubation and added to 200 µl 6 M HCl to terminate the reaction. Lipids were extracted three times with 3 ml diethyl ether. As described above, the combined extract was evaporated to dryness, applied to a silica-gel TLC plate, and developed in chloroform-methanol-acetic acid (100:2.5:5, by volume). The plates were visualized also as described above and scanned for radioactivity as described below.

Phospholipid determination

Samples of the lower phase (500 µl) from the chloroform/methanol extraction of the incubated 3T3-L1 adipocytes were taken and made up to 1 ml with chloroform. To this, 2 ml thiocyanate reagent (0.1 M iron(III) chloride, 0.4 M ammonium thiocyanate) was added and mixed thoroughly for 1 min. The phases were separated by centrifugation in a bench centrifuge and the aqueous, thiocyanate, phase removed by aspiration. The absorbance of the chloroform phase was measured at 488 nm. Appropriate blanks were measured and a fresh calibration curve using 0–75 µg phosphatidylcholine (PC) was constructed on each occasion. Results were expressed as µg of PC equivalent (Stewart 1980).

Determination of radioactivity

Samples containing organic solvent were evaporated to dryness and mixed with 5 ml EcoLite scintillation fluid. The extracts were counted for 10 min in a LKB-Wallac 1211 liquid scintillation counter and the results corrected for quench using an external standard ratio method.

TLC plates were scanned for radioactivity using a Bioscan System 200 Imaging Scanner (Washington, DC, USA) using a 6-mm high-efficiency slit. The percentage of total radioactivity detected, associated with individual bands, was determined using the Win-Scan software, version 1.6 (Lab Logic, Sheffield, UK) provided. The data from a whole plate were viewed as a single (two-dimensional) representation using Win-Scan 2D, version 1.6 (Lab Logic).

Results

Differentiation of confluent 3T3-L1 fibroblast-like cells in culture to adipocytes was initiated by including a mixture of dexamethasone, isobutyl methyl xanthine, insulin and rosiglitazone in the culture medium. After 48 h, the cells were maintained in medium supplemented only with insulin and rosiglitazone. After a further 5 days, the presence of visible fat droplets in the cells indicated successful differentiation to adipocytes. The inclusion of 1 µM rosiglitazone in the culture medium resulted in an increased rate of differentiation to adipocytes, as judged by the appearance of lipid droplets (80–100 compared with 50–80% in its absence). Therefore rosiglitazone was routinely included in the differentiation medium.

The ¹⁴C xenobiotic acids 2,4-DB, ibuprofen and MCPB (and, to a lesser extent, MCPP) were all readily incorporated into the lipid fraction extracted from the 3T3-L1 adipocytes. The rate of incorporation had already reduced from first order with respect to time of incubation by 2 h (figure 2), and slowed more rapidly

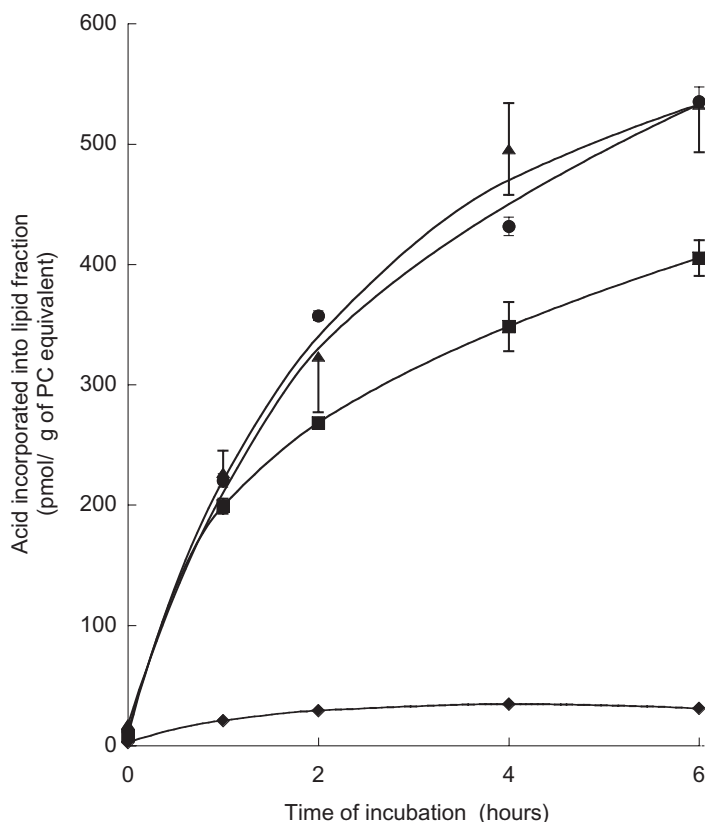


Figure 2. Effect of the duration of incubation on the incorporation of xenobiotic acids by 3T3-L1 adipocytes into lipids. Differentiated 3T3-L1 adipocytes were cultured with 0.5 mM radioactive acids as described in the Materials and methods. After the indicated times, the cultures were washed, the cells extracted and the radioactive content of the lipid phase determined. The acids shown are MCPB (■) ($0.188 \text{ Ci mol}^{-1}$), 2,4-DB (●) ($0.485 \text{ Ci mol}^{-1}$), MCPP (◆) (0.40 Ci mol^{-1}) and ibuprofen (▲) ($0.165 \text{ Ci mol}^{-1}$). All determinations were performed in duplicate and error bars represent the mean $\pm$ SEM from three independent experiments. Error bars are not shown where they are smaller than the symbol.

between 2 and 6 h. The incorporation of MCPB appears to have slowed earlier than the others but the 2 h figure is close enough to first order to justify its use as a standard condition. The rates are expressed relative to the phospholipid content of the culture dish but qualitatively similar results were obtained if the incorporations were expressed per incubation dish, indicating that the dishes all contained similar numbers of cells. At 6 h, the percentage of the acid originally supplied in the medium and incorporated into the lipid fraction were $0.32 \pm 0.011\%$ (2,4-DB, mean $\pm$ standard error from three independent experiments), $0.23 \pm 0.011\%$ (MCPB), $0.19 \pm 0.006\%$ (ibuprofen) and $0.02 \pm 0.001\%$ (MCPP).

The effect of changing the concentration of xenobiotic acid upon its incorporation into extractable lipids is displayed graphically (figure 3). Of the eight acids used, the highest incorporation was seen with ibuprofen: in the 2 h incubation at 1 mM ibuprofen, over 500 pmol was incorporated into lipid product per μg PC equivalent. The corresponding incorporations for 2,4-DB and for

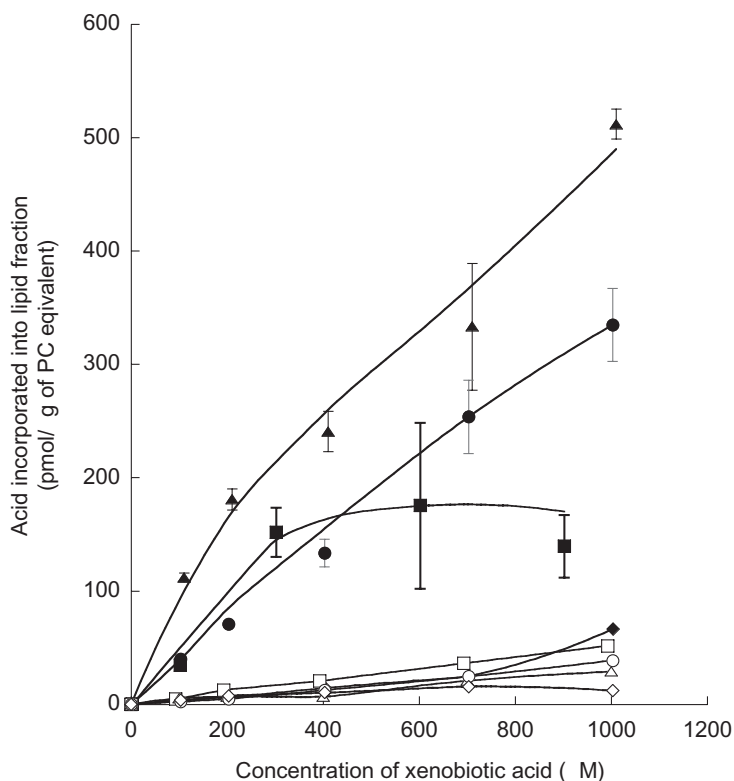


Figure 3. Effect of varying the concentration of the xenobiotic acids on their incorporation into lipids by 3T3-L1 adipocytes. The experiments were carried out as described for figure 1, except that all incubations were terminated after 2 h and that the indicated concentrations of the acids were used. The acids were MCPB (■) (specific radioactivities ranged from 43 Ci mol^{-1} at the lowest concentration to 0.11 Ci mol^{-1} at the highest), 2,4-DB (●) ($2.35\text{--}0.24 \text{ Ci mol}^{-1}$), MCPP (◆) ($1.92\text{--}0.20 \text{ Ci mol}^{-1}$), ibuprofen (▲) ($0.74\text{--}0.08 \text{ Ci mol}^{-1}$), indomethacin (□) ($1.16\text{--}0.11 \text{ Ci mol}^{-1}$), 2,4-D (○) ($1.00\text{--}0.10 \text{ Ci mol}^{-1}$), naproxen (Δ) ($0.63\text{--}0.06 \text{ Ci mol}^{-1}$) and fluroxypyr (◇) ($1.00\text{--}0.01 \text{ Ci mol}^{-1}$). All determinations were performed in duplicate and error bars represent the mean $\pm$ SEM from three independent experiments, except for indomethacin, naproxen and fluroxypyr, where only one experiment was carried out. Error bars are not shown where they are smaller than the symbol.

MCPB were about 60 and about 30%, respectively, of the figure for ibuprofen. These three acids might be regarded as 'high incorporators', of which only MCPB appears to have reached a saturating concentration at about $300 \mu\text{M}$, while 2,4-DB and ibuprofen show no tendency to reach a plateau even at 1 mM . The remaining five acids, all of which gave incorporations of less than 10% of the ibuprofen figure, showed similar non-saturating behaviour up to 1 mM acid. It should be noted that ibuprofen and MCPP were both supplied as racemic mixtures but that only the *R*-isomer has been reported to form CoA thioesters: the effective concentration of these two acids may be half that shown on the graphs (Mayer 1996, Knight 1998).

The chloroform phase of the lipid extracts was subjected to TLC and radioactive peaks were quantified by radio-scanning. Typical radio-scans for MCPB, 2,4-DB, MCPP and ibuprofen are shown as figure 4a–d. In all these cases, the major peak co-migrates with the appropriate synthetic xenobiotic TAG standard.

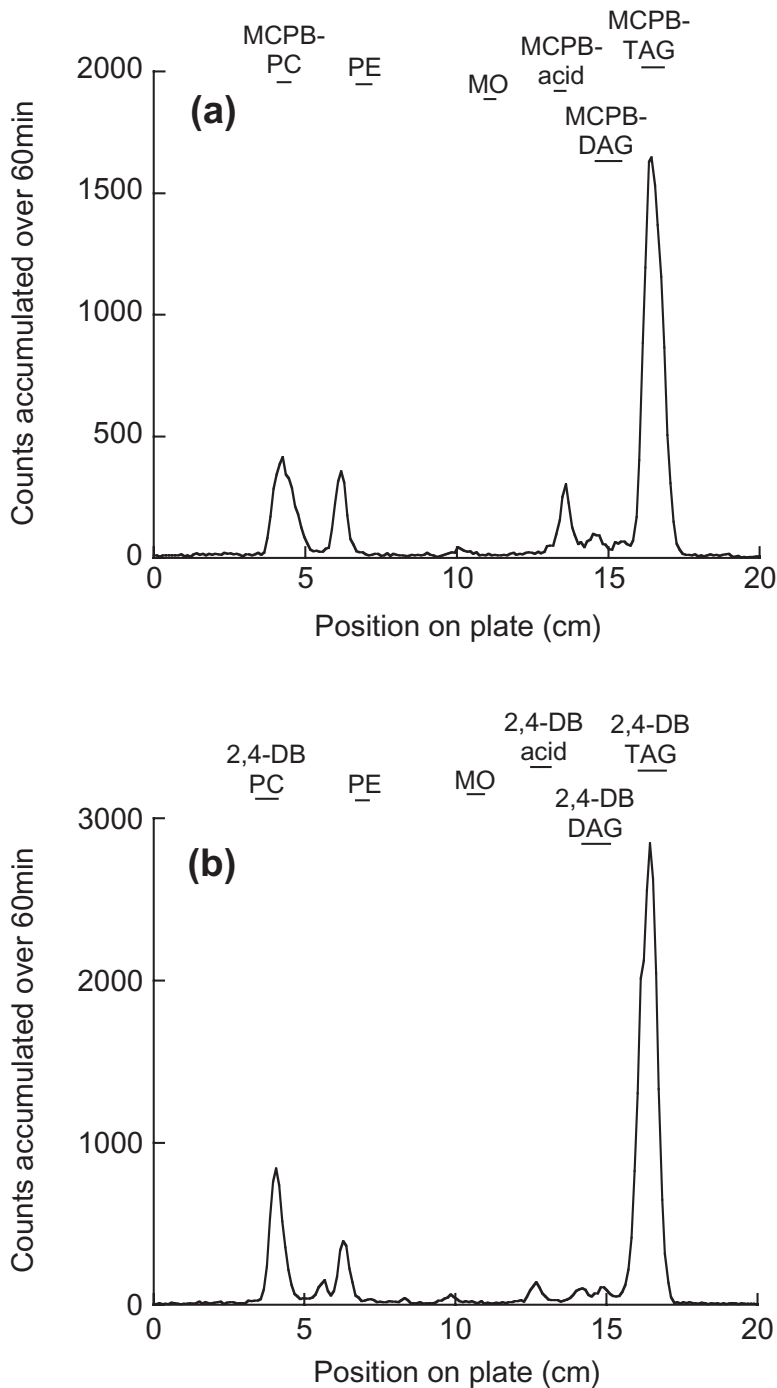


Figure 4. Thin-layer chromatograms of lipid products formed from xenobiotic acids by 3T3-L1 adipocytes. Samples of the lipid extract from cells incubated with xenobiotic acid, as described in figure 3, were analysed by TLC and the plates were scanned for 60 min per lane as described in the Materials and methods. The resultant traces for MCPB (a), 2,4-DB (b), MCPP (c) and ibuprofen (d) are shown. The positions of the authentic xenobiotic-lipid standards and of 'natural' phosphatidylethanolamine (PE) and mono-oleoylglycerol (MO) are shown by bars at the top of each trace. In all cases the origin is at 3.5 cm and the solvent front at 19.5 cm.

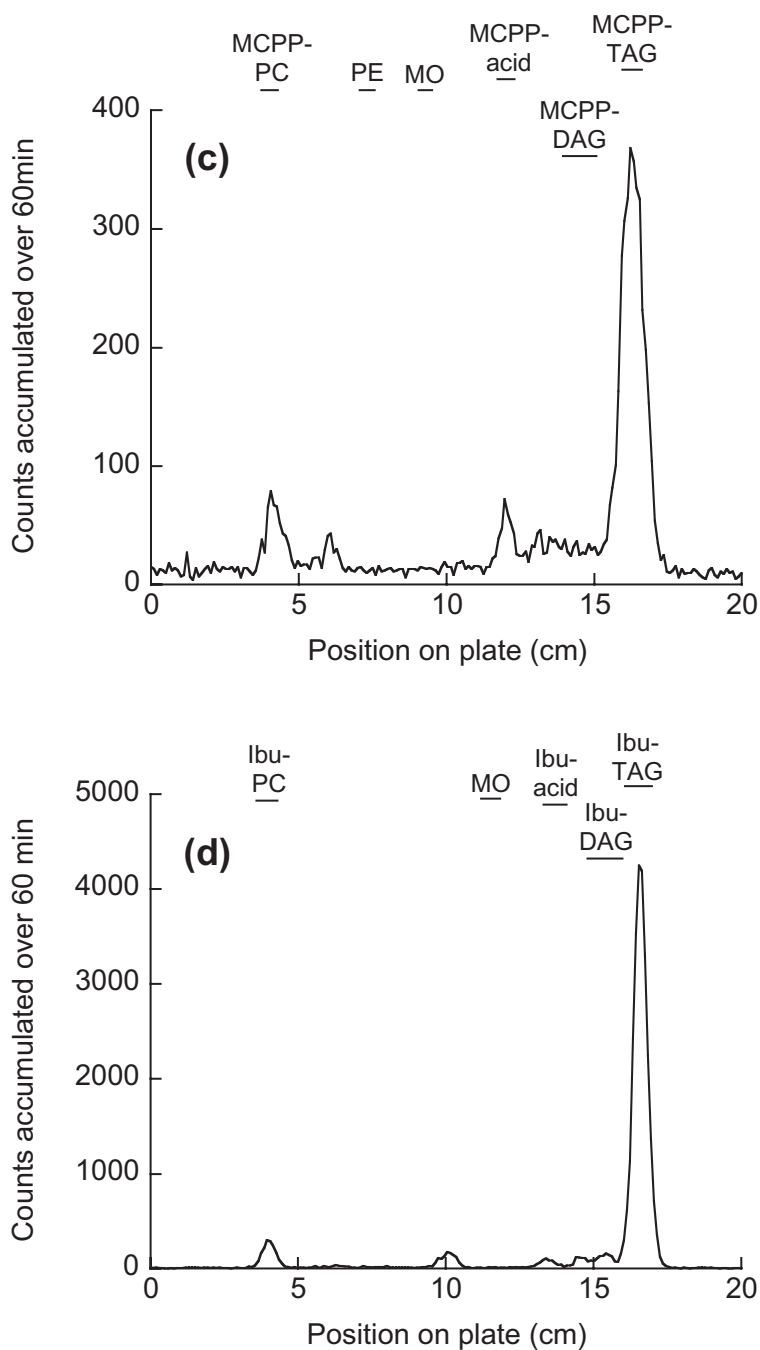


Figure 4. Continued.

Another peak can be identified as the unchanged non-esterified acid. Two peaks are found close to the origin in the 'phospholipid area' of the plates: one co-migrated with the appropriate synthetic xenobiotic phosphatidylcholine (PC) standard; the second migrated slightly less far than authentic (non-xenobiotic)

phosphatidylethanolamine (PE). There were no xenobiotic PE standards available. Some scans showed evidence for minor components which co-migrated with the xenobiotic diacylglycerol standard or close to the position of the monoacylglycerol, mono-oleoylglycerol.

Radio-scans of extracts of incubations which used the other acids (not shown) revealed that 2,4-D radioactivity appeared in a small TAG peak as well as the peak for non-esterified 2,4-D and the others only showed peaks for the non-esterified acids. No further analyses were conducted in any of these cases.

Table 1a–d summarizes the effect of concentration of acid in the incubations on the proportion of product associated with the different acids. With all four acids and at all concentrations, the major product was the xenobiotic TAG varying from 91% at 1 mM ibuprofen to 34% at 2 μ M MCPB. The general trends, observable for MCPB, 2,4-DB and ibuprofen, are a fall in the proportion (if not the total amount) present as non-esterified acid as the concentration increases, accompanied by a fall in the proportion of PC and an increase in the TAG. The total incorporation for MCPB was smaller than for the other three xenobiotic substrates.

In order to confirm the identification of the xenobiotic TAGs, the bands were scraped from the TLC plates and the lipid eluted. They were then subjected to lipolysis by pancreatic lipase and the products of the digestion re-analysed by TLC. After 5 min of incubation with the lipase, the MCPB, 2,4-D and MCPB scans (figure 5a–c) all showed almost complete conversion of the original radioactive product to the non-esterified acid. Minor peaks could be detected which migrated in positions consistent with the corresponding monoacylglycerols but these never accounted for more than 11% of the total product: after 40 min incubation, the only detectable product was the acid. Digestion of the product from the ibuprofen incubations gave different results. After 5 min digesting (figure 5d) with pancreatic lipase, the radioactivity identified as ibuprofenyl-TAG was reduced to 9% of the total radioactivity but the non-esterified ibuprofen peak only accounted for a further 8%. The two remaining peaks, one which co-migrated with standard 1-acyl-2-ibuprofenyl glycerol and one in a position consistent with it being mono-ibuprofenyl glycerol, accounted for 17 and 65% respectively. As the reaction progressed the proportion of TAG and diacylglycerol fell to 2 and 0%, respectively, at 40 min; the non-esterified acid and the monoacylglycerol increased to 14 and 84%, respectively.

Discussion

The formation of lipophilic metabolites of xenobiotic acids called xenobiotic triacylglycerols (TAG) has been recognized since 1978, when 4-benzoyloxybenzoic acid was shown to be incorporated into a TAG by rat liver slices (Fears *et al.* 1978). Since then, at least 40 other acids have been shown to form similar conjugates in a variety of systems ranging from whole animals to subcellular preparations (Dodds 1991, 1995). Few attempts have been made to compare xenobiotic acids under a set of standard conditions with the notable exception being Fears's original paper when 15 acids (five giving positive results) were compared using a rat liver slice system (Fears *et al.* 1978). Moorhouse *et al.* (1991) compared five acids with the natural fatty acid oleic acid using isolated rat hepatocytes. The sensitivity of both these approaches was compromised by the rapid metabolism of the xenobiotic acid by active detoxication systems of the liver

Table 1. The composition of the xenobiotic lipids formed by 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated with the indicated concentrations of ^{14}C -xenobiotic acid as described under figure 3. The products were analysed by TLC, the plates scanned and the percentage content of individual components determined as described in the 'Materials and methods'. The results are presented as mean percentages of the total radioactivity detected on each lane of the TLC plate $\pm$ SEM from three independent experiments. The percentages corresponding to the xenobiotic triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), phosphatidylcholine (PC) and the lipid tentatively identified as the xenobiotic phosphatidylethanolamine (PE) as well as the non-esterified precursor acid are shown

(a) MCPB

Lipid product	MCPB incorporated into lipid component (% of total radioactivity)				
	2.3 μM	102 μM	302 μM	602 μM	902 μM
TAG	34.2 $\pm$ 0.9	40.2 $\pm$ 0.9	50.4 $\pm$ 1.7	61.3 $\pm$ 0.5	62.5 $\pm$ 1.0
DAG	4.7 $\pm$ 0.3	2.8 $\pm$ 0.2	4.3 $\pm$ 0.3	3.9 $\pm$ 0.3	5.9 $\pm$ 0.5
MAG	1.0 $\pm$ 0.1	0.7 $\pm$ 0.2	1.2 $\pm$ 0.3	0.9 $\pm$ 0.1	1.5 $\pm$ 0.2
PC	26.9 $\pm$ 1.9	33.9 $\pm$ 1.8	25.5 $\pm$ 1.7	18.5 $\pm$ 0.9	17.0 $\pm$ 0.7
PE	10.2 $\pm$ 1.0	11.2 $\pm$ 0.3	9.4 $\pm$ 0.3	7.9 $\pm$ 0.5	7.5 $\pm$ 0.4
Acid	23.0 $\pm$ 3.8	11.2 $\pm$ 0.4	9.2 $\pm$ 1.3	7.6 $\pm$ 1.0	5.7 $\pm$ 0.2

(b) 2,4-D

Lipid product	2,4-DB incorporated into lipid component (% of total radioactivity)				
	103 μM	203 μM	403 μM	703 μM	1003 μM
TAG	59.7 $\pm$ 1.9	66.9 $\pm$ 0.7	68.8 $\pm$ 1.1	71.5 $\pm$ 2.3	70.0 $\pm$ 1.3
DAG	3.2 $\pm$ 0.1	3.0 $\pm$ 0.2	3.6 $\pm$ 0.1	4.6 $\pm$ 0.3	7.1 $\pm$ 0.6
MAG	1.2 $\pm$ 0.3	0.6 $\pm$ 0.2	1.0 $\pm$ 0.1	1.7 $\pm$ 0.2	0.8 $\pm$ 0.3
PC	22.2 $\pm$ 1.1	18.0 $\pm$ 0.9	15.3 $\pm$ 0.7	13.7 $\pm$ 1.2	12.6 $\pm$ 0.3
PE	7.6 $\pm$ 0.2	7.2 $\pm$ 0.5	6.4 $\pm$ 0.3	6.1 $\pm$ 0.7	4.9 $\pm$ 0.4
NE	6.1 $\pm$ 1.27	3.5 $\pm$ 0.7	3.2 $\pm$ 0.7	4.9 $\pm$ 0.6	2.8 $\pm$ 0.9

(c) MCPP

Lipid product	MCPP incorporated into lipid component (% of total radioactivity)				
	100 μM	200 μM	400 μM	700 μM	1000 μM
TAG	76.0 $\pm$ 2.4	73.8 $\pm$ 2.9	71.7 $\pm$ 1.5	77.1 $\pm$ 2.7	78.6 $\pm$ 2.3
DAG	10.8 $\pm$ 1.2	10.7 $\pm$ 2.3	12.0 $\pm$ 0.5	6.9 $\pm$ 1.4	7.8 $\pm$ 3.7
MAG	0.8 $\pm$ 0.5	0.7 $\pm$ 0.7	0.4 $\pm$ 0.4	0.6 $\pm$ 0.5	0.8 $\pm$ 0.8
PC	4.6 $\pm$ 0.6	4.9 $\pm$ 1.0	7.1 $\pm$ 0.7	9.1 $\pm$ 3.0	7.4 $\pm$ 0.8
PE	2.4 $\pm$ 0.2	2.2 $\pm$ 0.2	2.7 $\pm$ 0.1	2.9 $\pm$ 0.8	2.1 $\pm$ 0.3
Acid	5.7 $\pm$ 1.3	7.5 $\pm$ 2.4	6.1 $\pm$ 0.5	3.5 $\pm$ 0.0	4.1 $\pm$ 1.0

(d) Ibuprofen

Lipid product	Ibuprofen incorporated into lipid component (% of total radioactivity)				
	111 μM	211 μM	411 μM	711 μM	1011 μM
TAG	85.0 $\pm$ 0.8	88.7 $\pm$ 0.6	88.3 $\pm$ 0.3	89.8 $\pm$ 0.3	90.6 $\pm$ 0.7
DAG	5.1 $\pm$ 0.2	4.4 $\pm$ 0.2	5.1 $\pm$ 0.3	6.3 $\pm$ 0.2	5.7 $\pm$ 0.4
MAG	2.9 $\pm$ 0.4	2.1 $\pm$ 0.3	2.7 $\pm$ 0.5	1.1 $\pm$ 0.2	1.0 $\pm$ 0.2
PC	5.2 $\pm$ 0.0	3.8 $\pm$ 0.1	2.8 $\pm$ 0.2	2.2 $\pm$ 0.2	2.0 $\pm$ 0.2
PE	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	0.1 $\pm$ 0.1	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1
Acid	1.3 $\pm$ 0.4	0.5 $\pm$ 0.1	1.0 $\pm$ 0.1	0.2 $\pm$ 0.1	0.6 $\pm$ 0.2

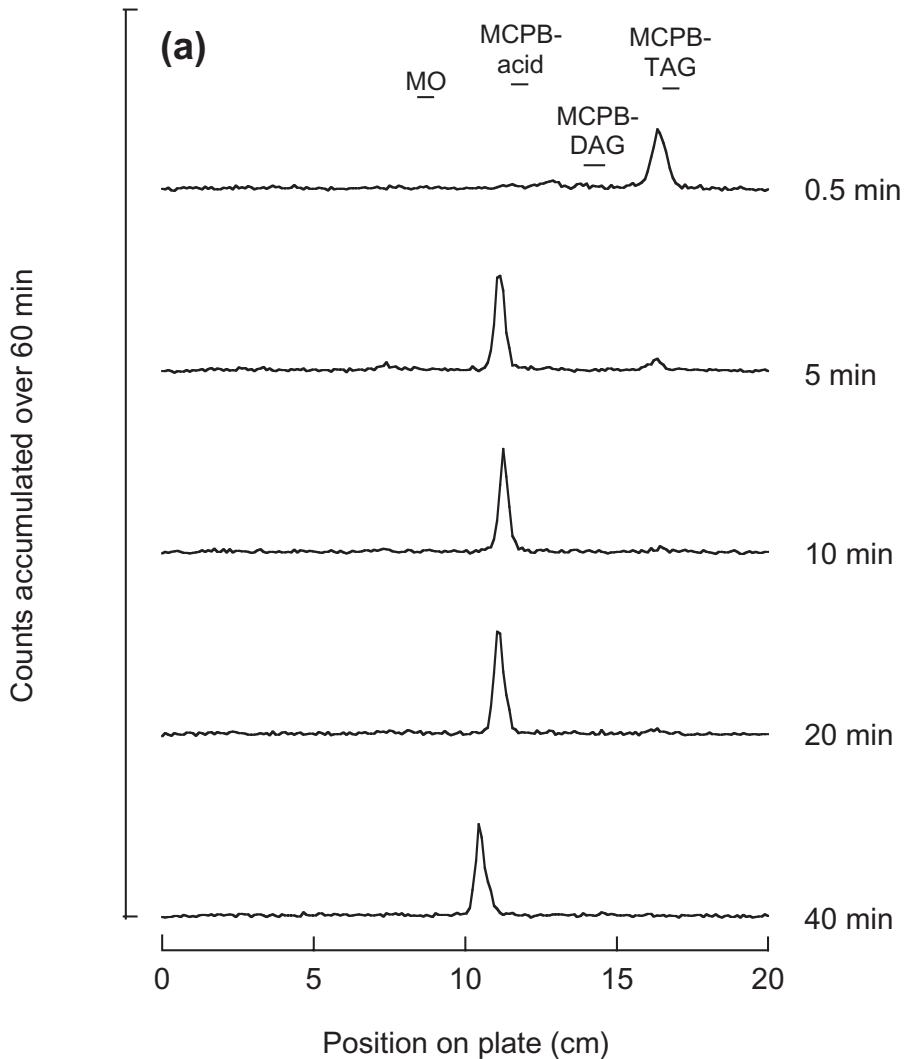


Figure 5. Digestion of xenobiotic triacylglycerols by pancreatic lipase. The triacylglycerol product from TLC plates (figure 4) was extracted, subjected to digestion with pancreatic lipase for the indicated time and the products subjected to further TLC analysis and radioscanning as described in the Materials and methods. The scans for a single xenobiotic acid [MCPB (a), 2,4-DB (b), MCPP (c) and ibuprofen (d)] are superimposed on the same axes and the entire vertical scale represents 3000 (MCPB), 3500 (2,4-DB), 1000 (MCPP) or 3500 (ibuprofen) counts accumulated over 60 min. The positions of standards, the origin and the solvent front are indicated as described in figure 4.

to form other, non-lipid, products (Murray 2000). The Fears approach (Fears *et al.* 1978) also used ^{14}C -glycerol as the source of radioactive label and so would have had a background activity of radioactive TAG synthesis from natural fatty acids in the preparation and relied upon chromatographic separation of the xenobiotic TAG from natural TAG.

We have previously used cultured 3T3-L1 adipocytes to study the incorporation of the non-steroidal anti-inflammatory drug fenbufen into lipids (Dodds *et al.* 1995), a process simplified by the lack of detoxication metabolites produced by

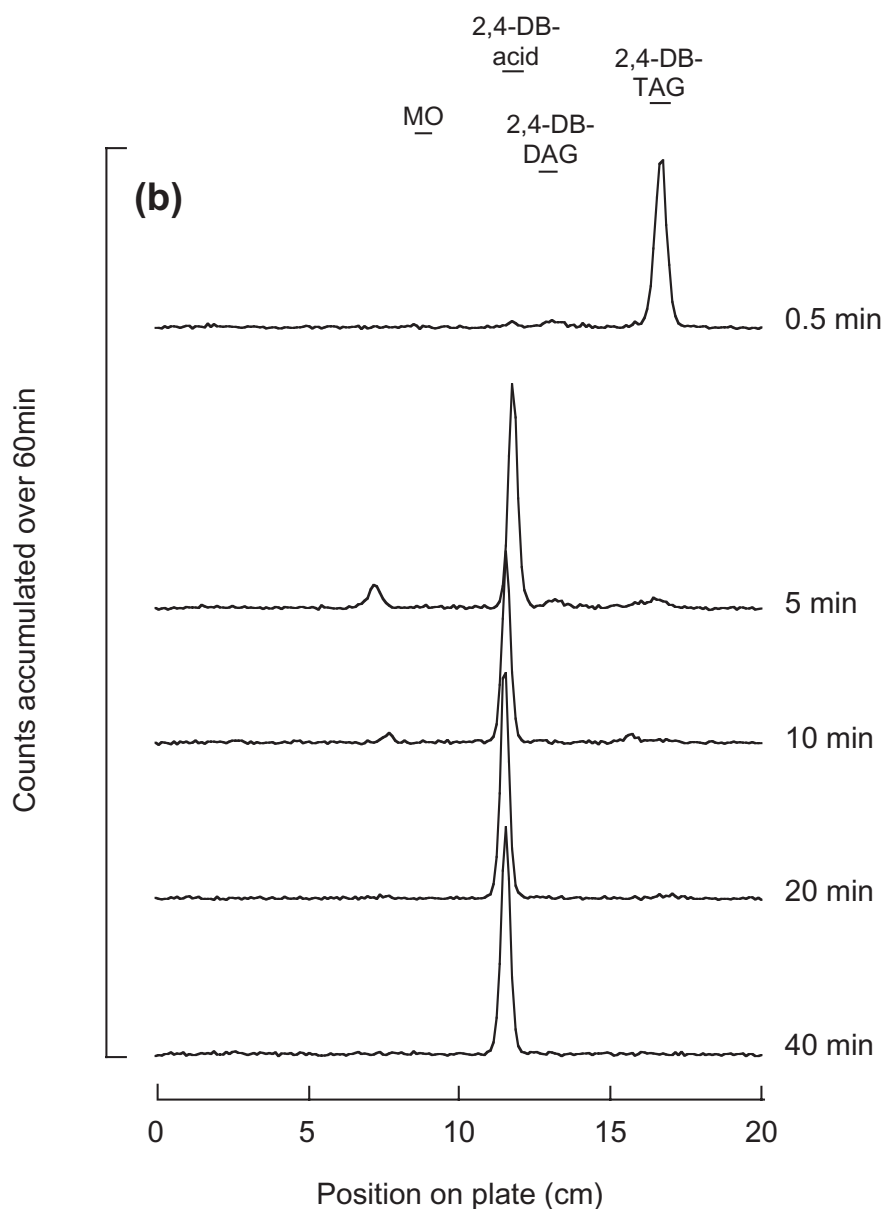


Figure 5. Continued.

this cell type. Here we have extended the use of the method to eight further xenobiotic carboxylic acids. The method is only slightly modified from that published before (Dodds *et al.* 1995) with the only major change being the inclusion of the thiazolidinedione PPAR γ_2 agonist, rosiglitazone, at 1 μ M in the differentiation medium. This modification was introduced in response to reports that it provided an improvement in the reliability of the conversion of the fibroblast-like precursor cells into adipocytes (Kim *et al.* 2000), a finding which we can now confirm.

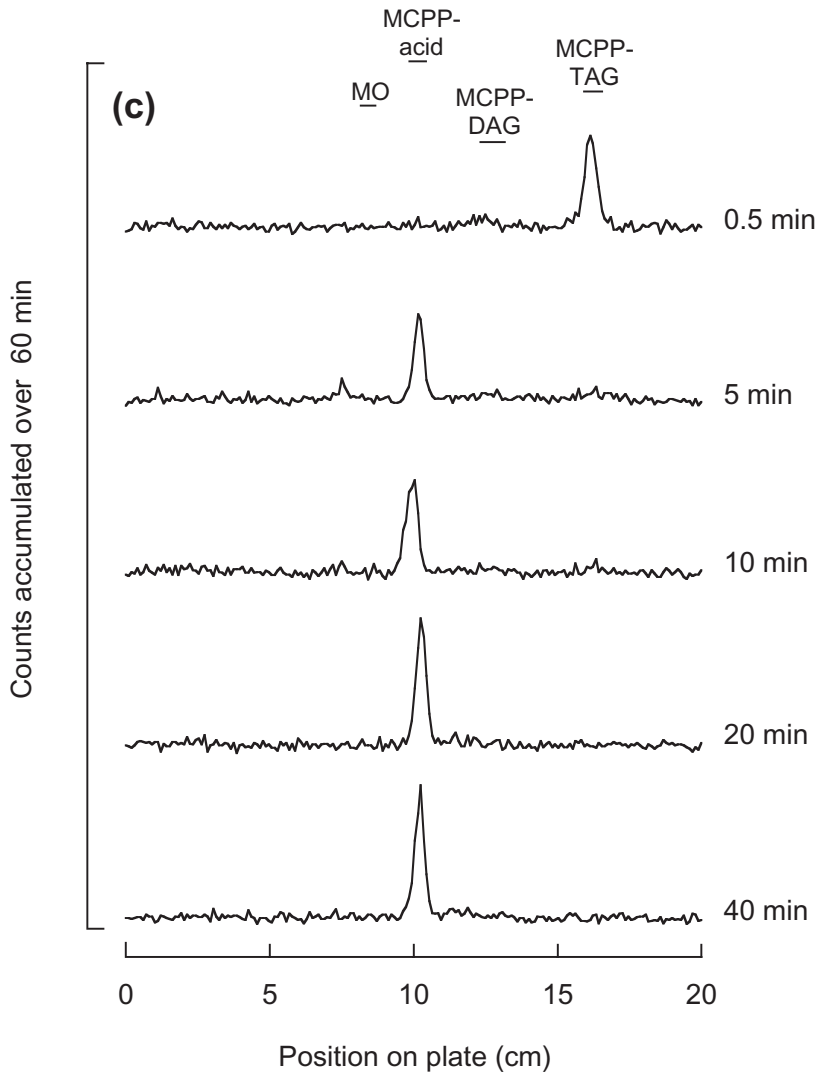


Figure 5. Continued.

A problem with studies of this sort is the expression of results in a standard form which takes account of the number of cells present in each culture dish. Determining cell number requires the release of the cells from the culture dish, followed by counting before the lipid extraction can begin. The delay in beginning the extraction compromises the results because the process of lipolysis can be shown already to have started (P. F. Dodds and R. A. Coleman, unpublished observations). Protein determination is not feasible because of the use of foetal bovine serum in the incubation medium and DNA determination requires a different extraction protocol, which is not compatible with the simultaneous recovery of lipid products. Membrane phospholipids, however, are extracted into the lower phase in the standard lipid extraction protocol and a sample of that phase can be used to measure the phospholipid content of the culture dish. This method makes assumptions about the membrane content of all cells

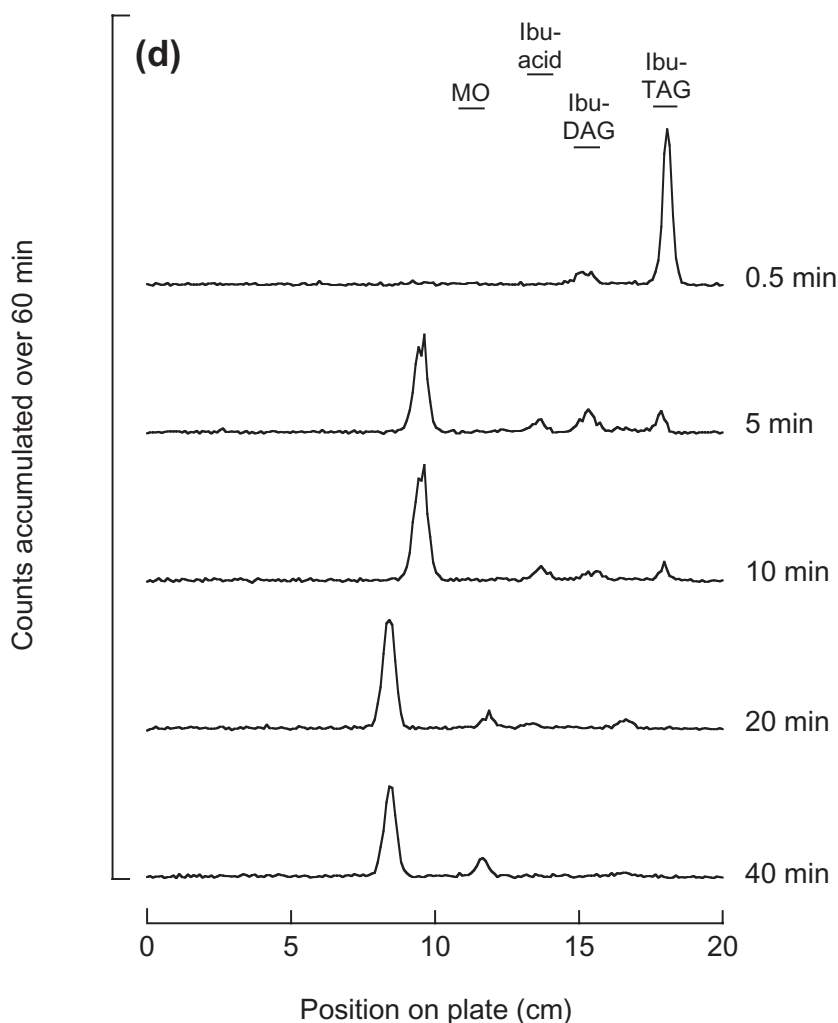


Figure 5. Continued.

being similar but the results were satisfactory because there was very little difference, in relative terms, between results expressed 'per dish' and those expressed as a function of phospholipid contents of the same dishes.

Three of the acids used (MCPB, 2,4-DB and ibuprofen) were taken up rapidly by the 3T3-L1 cells and converted into lipid conjugates. MCPB was taken up and converted at a lower rate; of the remaining four acids, only 2,4-D showed any evidence of incorporation into a lipid product. The low amount of radioactivity recovered as the non-esterified acid from the cells suggests that uptake from the medium was not rate limiting but reached an equilibrium which could be maintained whether or not the imported acid was further metabolized. The rate of uptake would, therefore, be determined by a subsequent acyl-CoA synthase or acyltransferase catalysed reaction. When there was no incorporation into lipids, the amount recovered in the cells reflected only the establishment of an equilibrium between the intracellular concentration of acid and the concentration of xenobiotic acid provided. The data here are insufficient to distinguish between

uptake by passive diffusion or using an active or facilitated mechanism perhaps involving fatty acid transport proteins (Abumrad *et al.* 1984, Martin *et al.* 2000).

Ibuprofen has been shown before to be incorporated into lipids in animal systems, including human volunteers (Fears *et al.* 1978, Williams *et al.* 1986, Moorhouse *et al.* 1990, Johnson *et al.* 1995), forming TAG and diacylglycerol. 2,4-DB had previously been shown to form elongated forms in alfalfa, soybean and cocklebur (Linscott *et al.* 1968, Wathana and Corbin 1972) but the authors did not investigate possible conjugation of the elongated acid to form complex lipids. To the best of our knowledge, this is the first report of either MCPB or MCPB being incorporated into lipids.

The lipid products were identified primarily by their behaviour on TLC, with the confidence in the identification being greatly enhanced by the availability of authentic standards for the xenobiotic TAGs, diacylglycerols and PCs. The identity of the TAG products was further confirmed by their rapid degradation in the presence of the TAG lipase, pancreatic lipase. In most cases, the radioactive product of the degradation was the non-esterified acid with only a little evidence for radioactivity being associated with the di- and mono-acylglycerol intermediates in the lipolytic process, under the conditions used. Given the known specificity of pancreatic lipase for the *sn*-1 and then the *sn*-3 positions (Carey *et al.* 1983, Small 1991), these results suggest that MCPB, 2,4-DB and MCPB are esterified primarily to one of the peripheral positions and that little is associated with the *sn*-2, central, position of the TAG, although chemical isomerization (acyl-migration) of the acids of di- and mono-acylglycerol between the three positions might also account for the results (Christie 1982). Furthermore, ester bonds with these three acids would appear to be relatively susceptible to lipolysis compared with results obtained (albeit under different conditions) with xenobiotic TAGs containing 3-phenoxybenzoic acid (Haselden *et al.* 1998). Ibuprofenyl-TAG, on the other hand, was digested primarily to a monoacylglycerol analogue suggesting that the acid was incorporated into the *sn*-2 position of the TAG. Similar resistance to complete lipolysis had been observed before (Moorhouse *et al.* 1991) and suggests that a different acyltransferase is responsible for the formation of the glycerol-ibuprofen ester bond, presumably *sn*-2-monoacylglycerol-phosphate acyltransferase. Less vigorous conditions for lipolysis may have revealed more structural information but all the results are consistent with the major products being xenobiotic analogues of natural TAGs.

The four most active acids also formed radioactive products that behaved like phospholipids on TLC. In all cases, one of these products migrated to exactly the same position as the corresponding synthetic xenobiotic phosphatidylcholine standard. This identification was supported by preliminary experiments in which the supposed xenobiotic phosphatidylcholine was incubated with phospholipase A₂ to generate the radioactive xenobiotic acid or with phospholipase C to generate the corresponding diacylglycerol (L. Potter, A. Prokopi, S. Vickery and P. F. Dodds, unpublished results). A second product, which migrated further and was visible when MCPB, 2,4-DB or MCPB was the precursor, was tentatively identified as a phosphatidylethanolamine. Experience has shown that lipids bearing aromatic acids interact more strongly with the silica stationary phase and, hence, migrate less far than the natural, aliphatic, phospholipids. In the absence of a genuine xenobiotic phosphatidylethanolamine standard, it can be seen that the second phospholipid peak has migrated slightly less far than the

natural phosphatidylethanolamine standard as expected. In the case of 2,4-DB only, a third minor product was also found in this vicinity.

There is much less published evidence for the formation of xenobiotic phospholipids. Reports exist of acids, which closely resemble natural fatty acids but are modified only in the ω -position (Quistad *et al.* 1978a-d, Tulliez 1979, LeBlanc *et al.* 1982, Reske *et al.* 1984, Schmitz *et al.* 1984, Reske 1985), or in some other minor way (Okuyama *et al.* 1969, Quistad *et al.* 1976, Soltysiac *et al.* 1984, Skrede *et al.* 1989), being incorporated into phospholipids. The evidence presented for the formation of phospholipids from tetradecylglycidic acid (Tutwiler *et al.* 1981) and 5-tetradecyloxy-2-furan carboxylic acid (Parker *et al.* 1977) is weak. We have preliminary evidence that 3-phenoxybenzoic acid and ibuprofen (Haselden *et al.* 1991) can form phospholipids in isolated rat hepatocytes and have presented evidence that fenbufen also forms phospholipids (Dodds *et al.* 1995). The problem with obtaining unequivocal proof by mass spectroscopy that the product is indeed, say, a xenobiotic phosphatidylcholine, is in obtaining a sample that is completely free from contaminating natural phosphatidylcholine which is present in the extracts in much greater quantity. Nevertheless, the evidence for their formation is beginning to accumulate. No assessment of the likely toxic consequences of such formation has yet been made but the possibility of disruption of membrane function cannot be discounted.

Given the complexity of the multi-step process involved in uptake of an acid by the cells and its incorporation into a triacylglycerol, it is difficult at this stage to draw any firm conclusions about structure/activity relationships. It can be seen that those acids with the longest aliphatic chain between the carboxylic acid group and the aromatic moiety show the highest activity (MCPB, 2,4-DB), whereas those with shorter chains or more complex ring structures (2,4-D, naproxen, indomethacin, fluroxypyr) show the lowest — or no — activity. Ibuprofen and MCPP are both 2-substituted propanoic acids but their rates of incorporation are very different; it might be presumed that the higher activity of the former results from its more hydrophobic and less constrained 'tail'.

In conclusion, if the xenobiotic acid under test is available in radioactive form, it is a simple procedure to test its activity with cellular lipid synthetic pathways using cultured 3T3-L1 adipocytes. The procedure simply requires that the acid is included in the incubation medium for a period and that the lipid extract from the cells are subjected to TLC. Identification of products is greatly facilitated if authentic xenobiotic lipid standards are available but the method for synthesizing the standards is available (Bergelson 1980). The method could be modified to allow assessment of non-radioactive acids, which will require the use of another lipid precursor in a radio-labelled form and will rely upon careful chromatographic separation of xenobiotic from natural products. The effects of hormones and other compounds upon the turnover of these xenobiotic lipids in adipose tissue could be assessed by minor adaptations of the protocol. The method benefits from the absence of detoxication products in adipose tissue but, by the same token, cannot be considered a model of whole body metabolism of the compound. *In vivo*, the rate of accumulation of xenobiotic lipids in adipose tissue will depend, amongst other things, upon the hepatic metabolism of the compound. The method does provide a simple assessment of foreign compounds which should precede studies on bioaccumulation and the potential toxicity of xenobiotic diacylglycerol and phospholipid analogues.

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Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into *Fasciola hepatica*

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1. Triclabendazole (TCBZ) is an halogenated trematocidal benzimidazole compound extensively used in veterinary medicine. It is active against immature and adult stages of the liver fluke *Fasciola hepatica*.

2. Free and conjugated TCBZ metabolites have been identified in the bile of treated sheep.

3. The experimental aims were to characterize the *in vitro* patterns of TCBZ biotransformation both in the animal host (sheep liver microsomes) and target parasite (*F. hepatica* microsomal preparation); and to compare the *ex vivo* diffusion of TCBZ parent drug and its oxidized metabolites (TCBZ sulphoxide [TCBZSO], TCBZ sulphone [TCBZSO₂], and TCBZ-hydroxy derivatives) into *F. hepatica*. Additionally, the octanol–water partition coefficients for TCBZ and all its metabolites were estimated as an indicator of the relationship between drug lipophilicity and diffusion into the target parasite.

4. Drug/metabolites concentrations were quantified by HPLC after sample clean up and a solvent-mediated chemical extraction.

5. Sheep liver microsomes metabolized TCBZ into its sulphoxide and sulphone metabolites after 30 min of incubation. The rate of TCBZ sulphoxidation in the liver was significantly greater ($p < 0.01$) than that observed for the sulphonation of TCBZSO.

6. The trematode parasite oxidized TCBZ into its sulphoxide metabolite after 60 min of incubation at a metabolic rate of $0.09 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$.

7. TCBZ and all its oxidized metabolic products were recovered from *F. hepatica* as early as 15 min after their *ex vivo* incubation in a Krebs's Ringer Tris buffer. However, the diffusion of the hydroxy-derivatives into the fluke was lower than that observed for TCBZ, TCBZSO and TCBZSO₂. There was a high correlation ($r = 0.82$) between drug lipophilicity (expressed as octanol–water partition coefficients) and drug availability measured within the parasite.

8. Unlike the uptake pattern previously observed for albendazole, the parent TCBZ and its sulphoxide and sulphone metabolites showed a similar ability to penetrate into the trematode parasite.

9. Understanding the relationship between TCBZ metabolism, the relative pharmacological potency of its metabolic products and their ability to reach the target parasite may be critical to optimize its flukicidal activity, particularly when TCBZ resistant flukes have been already isolated in the field.

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Introduction

Fascioliasis, caused by the trematode liver fluke *Fasciola hepatica*, is the cause of considerable loss in sheep and cattle production systems all over the world (Courtney and Roberson 1995). Human fascioliasis occurs as an accidental zoonotic disease in Africa, Western Europe and Latin America (Mas-Coma *et al.* 1999). The main strategy for the effective control of fascioliasis is based on chemotherapy. Most of the available flukicidal compounds, at their recommended therapeutic doses, have good activity against the mature stage of the liver fluke, but are not sufficiently effective against the pathogenic immature stages located in the liver parenchyma. Interestingly, triclabendazole (6-chloro-5(2-3-dichlorophenoxy)-2-methyl thio-benzimidazole) (TCBZ), an halogenated benzimidazole thiol derivative, shows excellent efficacy against both the mature and immature stages of the liver fluke in sheep and cattle, which is a differential feature compared to other available trematodicidal drugs (Boray *et al.* 1983).

Flukicidal drugs can reach the liver fluke either by oral ingestion of blood or by transtegumental diffusion. The high absorption surface of the trematode tegument may have a major role in drug diffusion from the surrounding medium. The entry of a drug into the parasite may mainly depend on the diffusion surface, the concentration gradient across the membrane, the pH/pK relationship and the lipophilicity of the molecule (Mottier *et al.* 2003). TCBZ is the most potent flukicidal compound, even though its *ex vivo* transtegumental diffusion into adult *F. hepatica* was shown to be significantly lower than that observed for albendazole (Alvarez *et al.* 2004). Although TCBZ mode of action remains unknown, its strong binding to various types of protein structures other than microtubules (the site of action for nematodicidal benzimidazole compounds), may suggest a multiplicity of targets for the trematodicidal activity of this drug (Bennett and Köhler 1987).

The methylcarbamate benzimidazole (albendazole, fenbendazole, etc.) anthelmintics are extensively metabolized in all mammalian species studied (Gottschall *et al.* 1990, Lanusse and Prichard 1993). There is only limited information available describing the plasma pharmacokinetic behaviour of TCBZ in ruminant species (Alvinerie and Galtier 1986, Hennessy *et al.* 1987, Bogan *et al.* 1988). TCBZ parent drug was not detected in plasma after its oral administration to sheep, indicating it was completely removed from portal blood by the liver following absorption (Hennessy *et al.* 1987). TCBZ is oxidized to form the sulphoxide and sulphone metabolites, triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone (TCBZSO₂), respectively. Hydroxylation of TCBZ occurs at the 4' position of the dichlorophenoxy ring, forming the corresponding hydroxylated metabolites, hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZSO (OH-TCBZSO) and hydroxy-TCBZSO₂ (OH-TCBZSO₂) (figure 1). Extremely low concentrations of TCBZ were recovered in bile. TCBZSO, TCBZSO₂ and the hydroxy derivatives were the major biliary metabolites identified in sheep bile (Hennessy *et al.* 1987). TCBZ biliary metabolites were recovered in their unconjugated and conjugated forms. The major metabolite found in bile was conjugated OH-TCBZSO and contributed to almost half of the total conjugated metabolites. Hydroxylated TCBZ metabolites have not been detected in plasma, with TCBZSO and TCBZSO₂ being the only metabolites found in the blood-stream. TCBZ metabolites are strongly bound to plasma proteins (≥90%) which could account for their long residence time in the animal body (Hennessy *et al.*

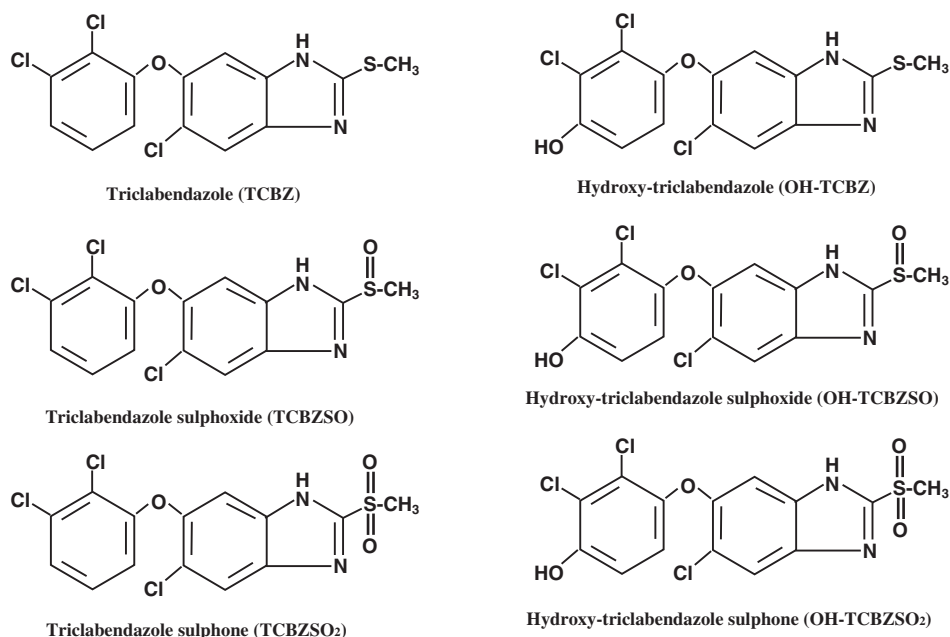


Figure 1. Chemical structures of the halogenated trematocidal benzimidazole thiol molecules assayed.

1987). One of the purposes of the work reported herein was to provide detailed information on the biotransformation pathways involved on the formation of the TCBZ sulfo- and hydroxy- metabolites in the host liver and within its main target parasite.

To gain further insight on the relationship between the pharmacological behaviour and the flukicidal activity of TCBZ, the goals of the work reported here were: (1) to characterize *in vitro* the patterns of TCBZ biotransformation both in the animal host (sheep liver microsomes) and target parasite (*F. hepatica* microsomal preparation) and (2) to compare the *ex vivo* diffusion of TCBZ parent drug and its oxidized metabolites into *F. hepatica*. The data obtained from the transtegumental diffusion kinetic studies were correlated with molecular lipophilicity, estimated by lipid-to-water partition coefficients (PC), the most frequently used parameter for defining the lipophilic character of a given drug molecule.

Materials and Methods

Chemicals

Reference standards (97–99% pure) of TCBZ and its metabolites were provided by Novartis Animal Health (Basel, Switzerland). The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), tris[hydroxymethyl]aminomethane hydrochloride (Tris), methimazole (MTZ) and piperonyl butoxide (PB) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). The solvents used for the chemical extraction and chromatographic analysis were high-performance liquid chromatography (HPLC) grade (Baker, Inc., Phillipsburg, USA). Buffer salts (NaHCO₃, Na₂HPO₄ and CH₃COONH₄) were purchased from Baker.

Collection of parasite material

Eight parasite-free Corriedale weaned lambs (males, 8–10 months old) were infected with 200 metacercariae of *F. hepatica* (provided by Instituto DILAVE, 'Miguel C. Rubino', Montevideo,

Uruguay), given in a gelatine capsule by the oral route. Sixteen weeks after infection, the animals were stunned and exsanguinated immediately, according to internationally accepted animal welfare guidelines (AVMA 2001). Adult specimens of *F. hepatica* were recovered from the liver, common bile ducts and the gall-bladder of each lamb. The parasite specimens were rinsed extensively with saline solution (NaCl 0.9%) (37°C) to remove bile and/or adhering materials.

TCBZ biotransformation by sheep liver microsomes and F. hepatica microsomal fraction

Microsome preparation. Two healthy (uninfected) Corriedale lambs (males, 8–10 months old) were sacrificed to be used as a source of liver material for preparation of microsomes. The microsomal fraction from pooled *F. hepatica* specimens was also prepared. Briefly, samples (approximately 2 × 2 × 2 cm) of liver and parasite specimens (1 g) were rinsed with cold KCl (1.15%) and then transported to the laboratory kept in flasks filled with phosphate buffer (0.1 M, pH 7.3) at 4°C. All subsequent operations were performed between 0 and 4°C. Liver samples were cut into small pieces with scissors and washed several times with the phosphate buffer (to remove haemoglobin). Then, tissue samples and parasite material were homogenized in the above phosphate buffer using a Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, DE, USA), centrifuged at 10 000g for 20 min and the resulting supernatant was further centrifuged at 100 000g for 60 min. The pellet (microsomal preparation) was suspended in phosphate buffer and stored at –70°C until used for incubation assays. An aliquot of the microsomal preparation was used to determine protein content using bovine serum albumin as a control standard (Smith *et al.* 1985).

Enzyme assays. Metabolic activity was assessed by the amount of TCBZSO and TCBZSO₂ formed in the presence of NADPH. A typical reaction mixture contained 250 µl NADPH solution prepared in phosphate buffer (0.1 M, pH 7.3), 100 µl tissue preparation (0.5 mg microsomal protein) and 15 nmol TCBZ (sheep liver and parasite microsomes) or TCBZSO (liver microsomes) dissolved in 10 µl methanol. The incubation mixture was adjusted to a final volume of 500 µl with phosphate buffer (0.1 M, pH 7.3). The initial concentration of the incubated substrates (TCBZ and TCBZSO) was 30 µM. Incubations (30 min for sheep liver and 60 min for parasite microsomes) were carried out at 37°C in glass vials in an oscillating water bath under aerobic conditions. The drug substrates were also incubated, under the same conditions, either with inactivated (boiled) microsomes or without NADPH. These incubations were used as controls for possible non-enzymatic drug conversion. All reactions were stopped by the addition of 200 µl ice-cold acetonitrile and stored at –20°C until analysis. The liver microsomal oxidation of both incubated substrates was linear up to 30 min. The maximal oxidation rates of TCBZ and TCBZSO were obtained using 1 µmol (sheep liver microsomes) and 0.5 µmol (*F. hepatica* microsomes) NADPH (table 1).

Inactivation of the FMO system was performed by heating the diluted microsomal preparation (2 min at 50°C) without NADPH, which was immediately chilled in ice and followed by pre-incubation (5 min at 37°C) with 100 µM of the FMO substrate methimazole (MTZ) (Dixit and Roche 1984). Then, the incubated substrate (TCBZ or TCBZSO) was added and the reaction started with the addition of NADPH. MTZ was dissolved in 25 µl distilled water.

The oxidation of TCBZ and TCBZSO was also studied in the presence of 100 µM piperonyl butoxide (PB), a well known cytochrome P450 inhibitor. Incubation mixtures containing PB were pre-incubated for 5 min at 37°C without NADPH followed by the addition of TCBZ or TCBZSO. The reaction started with the addition of NADPH. The inhibitor (PB) was dissolved in 10 µl methanol and parallel control tubes contained the same volume of the solvent. Both MTZ and PB were

Table 1. Comparative sulphoxidation of triclabendazole by sheep liver and *Fasciola hepatica* microsomal fractions: effect of the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) concentration on the rate of triclabendazole sulphoxide (TCBZSO) formation after 60 min of incubation.

NADPH (µmol ml ⁻¹)	TCBZSO rate of formation (nmol min ⁻¹ mg ⁻¹)	
	Sheep liver microsomes	<i>F. hepatica</i> microsomes
0	0.006	0.002
0.125	0.140	0.020
0.250	0.240	0.040
0.500	0.350	0.100
1.000	0.380	0.100
2.000	0.370	0.100

Data are derived from six (sheep liver microsomes) and five (*F. hepatica* microsomal fraction) determinations.

also incubated in the absence of the substrates under the same conditions to ensure that their presence in the incubation mixture did not interfere with the chromatographic determination of TCBZ and its metabolites. All the incubated microsomal mixtures have the same volume (20 μ l) of methanol.

Drug diffusion assays

The collected flukes were maintained for 2 h before starting the incubation process in a Krebs–Ringer Tris (KRT) buffer (pH 7.4) at 37°C (McCracken and Lipkowitz 1990). Two fluke specimens (approximately 0.2 g) were incubated at 37°C in the KRT buffer (2 ml), containing either TCBZ, TCBZSO, TCBZSO₂, OH-TCBZ, OH-TCBZSO or OH-TCBZSO₂ at a final concentration of 5 nmol ml⁻¹. This is a pharmacologically relevant concentration obtained from previously reported work where TCBZ concentrations in bile and plasma were measured after conventional treatments in ruminants (Hennessy *et al.* 1987). Times of incubations were 15, 45, 60, 90 and 180 min. There were four replicate incubation assays for each drug at each incubation time. Blank samples containing parasite material and incubation medium without drug, and drug-spiked medium without parasite material were incubated during the same time intervals. Once the incubation time elapsed, the flukes were rinsed thoroughly with saline solution, blotted on coarse filter paper and stored at -20°C until their preparation for HPLC analysis to measure drug concentrations. The parasite material was processed within 2–4 h after the incubation assays.

Sample preparation, extraction and analytical procedures

HPLC analysis for TCBZ and its metabolites was carried out as described (Mottier *et al.* 2004).

Enzyme assays. The internal standard (IS) mebendazole (MBZ) (5 nmol dissolved in 20 μ l methanol) was added to inactivated incubation mixtures. Spiked samples (500 μ l), fortified with TCBZ and its metabolites, were mixed with 200 μ l cold acetonitrile followed by the addition of the IS. Experimental and fortified samples were mixed with 1.5 ml ethyl acetate and shaken on a mechanical shaker for 5 min. This extraction clean-up step was repeated once and the combined ethyl acetate extracts were evaporated using an Automatic Environmental Speed Vac System (Savant, Holbrook, USA). The dry residue was re-dissolved in 300 μ l mobile phase and 50 μ l were injected into the HPLC system (Shimadzu Corporation, Kyoto, Japan). Validation of the analytical procedures for extraction and quantification of TCBZ and its metabolites was performed before starting the analysis of the experimental samples from the incubation assays. Known amounts of each analyte (1–60 nmol ml⁻¹) were added to aliquots of boiled (inactivated) microsomal preparations, extracted and analysed by HPLC (triplicate determinations) to obtain calibration curves and percentages of recovery. Calibration curves were analysed using the least squares linear regression analysis (Instat 3.00, Graph Pad Software, Inc., San Diego, CA, USA) of HPLC peak area ratios of analytes/IS and nominal concentrations of spiked samples. Correlation coefficients (*r*) for the different analytes ranged between 0.995 and 0.999. A lack-of-fit test was also carried out to confirm the linearity of the regression line of each analyte. The concentrations in the experimental samples were determined following interpolation using the standard curves. Absolute recoveries were established by comparison of the detector responses (peak areas) obtained for spiked microsomal samples and those of direct standards prepared in mobile phase. Drug/metabolite absolute recoveries were 93–99% (OH-TCBZSO₂), 96–99% (OH-TCBZSO), 91–99% (TCZSO₂), 74–87% (OH-TCBZ), 84–98% (TCBZSO) and 89–92% (TCBZ). Inter-assay precision coefficients of variation (CVs) were <15% and the limits of quantification (nmol ml⁻¹) were 0.07 (OH-TCBZSO₂), 0.18 (OH-TCBZSO), 0.39 (TCZSO₂), 0.32 (OH-TCBZ), 0.37 (TCBZSO) and 0.38 (TCBZ).

Drug diffusion assays. Drug concentrations are expressed as nmol/100 mg trematode protein⁻¹. The determination of parasite protein concentrations was carried out according to the methodology described by Smith *et al.* (1985). Protein concentration in *F. hepatica* (*n* = 5) was 183.7 $\pm$ 20 mg g⁻¹ trematode parasite. Drug-free *F. hepatica* material (0.1 g) was spiked with each target molecule (TCBZ, TCBZSO, TCBZSO₂, OH-TCBZ, OH-TCBZSO, OH-TCBZSO₂) to achieve the following final concentrations: 0.27, 0.54, 1.36, 2.72, 5.44, 10.89, 16.33 nmol/100 mg protein⁻¹, and with MBZ (IS) (10 μ l, stock solution of 500 μ M). Validation of the analytical procedures for extraction and quantification of TCBZ and its metabolites from trematode material was carried out as described (Mottier *et al.* 2004).

Octanol–water partition coefficients (PC). The octanol–water PC (log *P*) was used as an indicator of lipid solubility of the drug/metabolites used in the current experiments. The methodology used to calculate this parameter was adapted from Péhourcq *et al.* (2000).

Analysis of the data

The data for the enzyme assays are expressed as mean ($\pm$ SD) of six determinations (three incubations with the liver microsomal preparation obtained from each animal). Metabolic rates of the

products formed are expressed in $\text{nmol min}^{-1} \text{mg}^{-1}$ of microsomal protein. Statistical comparisons were carried out using analysis of variance (ANOVA) (Instat 3.0 Software, Graph Pad Software). The Tukey's range test was used to indicate the order of significance when a significant F was obtained. A value of $p < 0.05$ was considered statistically significant. Statistical comparison between the oxidation rates obtained for TCBZ and TCBZSO in the liver microsomal fraction was performed using the Mann-Whitney U -test. The same non-parametric test was used for the statistical comparison between TCBZSO and TCBZSO₂ rates of formation following TCBZ incubation with sheep liver microsomes.

Once the concentration values (expressed as $\text{nmol } 100 \text{ mg protein}^{-1}$) for each compound within the target parasites were determined for each individual incubation assay, the area under the concentration-time curve (AUC) was calculated by the trapezoidal rule method (Gibaldi and Perrier 1982), using the PkSolution 2.0 program (Summit Research Services, Ashland, OH, USA). The AUC ($\text{nmol min } 100 \text{ mg protein}^{-1}$) was considered as an indicator of the total drug availability within the trematode parasite. The individual concentrations and AUCs are presented as mean $\pm$ SD (four replicates). The individual concentrations, AUC and log P values obtained for each molecule assayed were also compared by ANOVA.

Results

Both the sheep liver and *F. hepatica* microsomal fractions were capable of oxidizing TCBZ into its sulpho-metabolites. A negligible sulphoxidation activity was observed in the absence of NADPH, whereas the production of TCBZSO was related to the NADPH concentration added to the incubation mixture. The relationship between the required NADPH concentrations and the rates of TCBZ sulphoxidation in both liver and trematode parasite microsomal fractions is shown in table 1. Also, there was no measurable oxidation activity in control incubations with inactivated (boiled) microsomes (data not shown).

Although both TCBZSO and TCBZSO₂ were produced after 30 min incubation of TCBZ with sheep liver microsomes, the rate of production of the sulphoxide metabolite was significantly higher ($p < 0.001$). TCBZSO₂ was the major metabolite formed following TCBZSO incubation with sheep liver microsomes. Only trace amounts of OH-TCBZSO were recovered in sheep liver microsomal incubations after incubations with TCBZ and TCBZSO. The rates ($\text{nmol min}^{-1} \text{mg}^{-1}$) of OH-TCBZSO production by sheep liver microsomes ranged between 0.006–0.009 (TCBZ incubations) and 0.01–0.02 (TCBZSO incubations).

The rate of TCBZ sulphoxidation by sheep liver microsomes was $0.38 \pm 0.06 \text{ nmol TCBZSO formed per min}^{-1} \text{mg}^{-1}$ microsomal protein. Sheep liver microsomes were also able to metabolize TCBZSO into the sulphone metabolite (TCBZSO₂) at a rate of $0.12 \pm 0.01 \text{ nmol min}^{-1} \text{mg}^{-1}$. Thus, the rate of TCBZ sulphoxidation was significantly higher ($p < 0.01$) compared with that observed for the sulphonation of TCBZSO. On the other hand, the mean rate of TCBZ conversion into TCBZSO₂ by sheep liver microsomes was $0.028 \pm 0.005 \text{ nmol min}^{-1} \text{mg}^{-1}$. Inhibition of FMO in the presence of MTZ reduced TCBZSO production (62%, $p < 0.01$) following TCBZ incubation with sheep liver microsomes (figure 2). Similarly, inhibition of the cytochrome P450 system by PB decreased (51%, $p < 0.05$) the sulphoxidation of TCBZ to TCBZSO. Formation of the sulphone metabolite was not observed when either the FMO or cytochrome P450 were inhibited (data not shown). Inhibition of liver microsomal FMO also inhibited (47%, $p < 0.001$) the sulphonation of TCBZSO (figure 3). Similarly, a significant ($p < 0.001$) reduction (69%) in the rate of TCBZSO₂ production was observed after TCBZSO incubation in the presence of PB, a cytochrome P450 inhibitor.

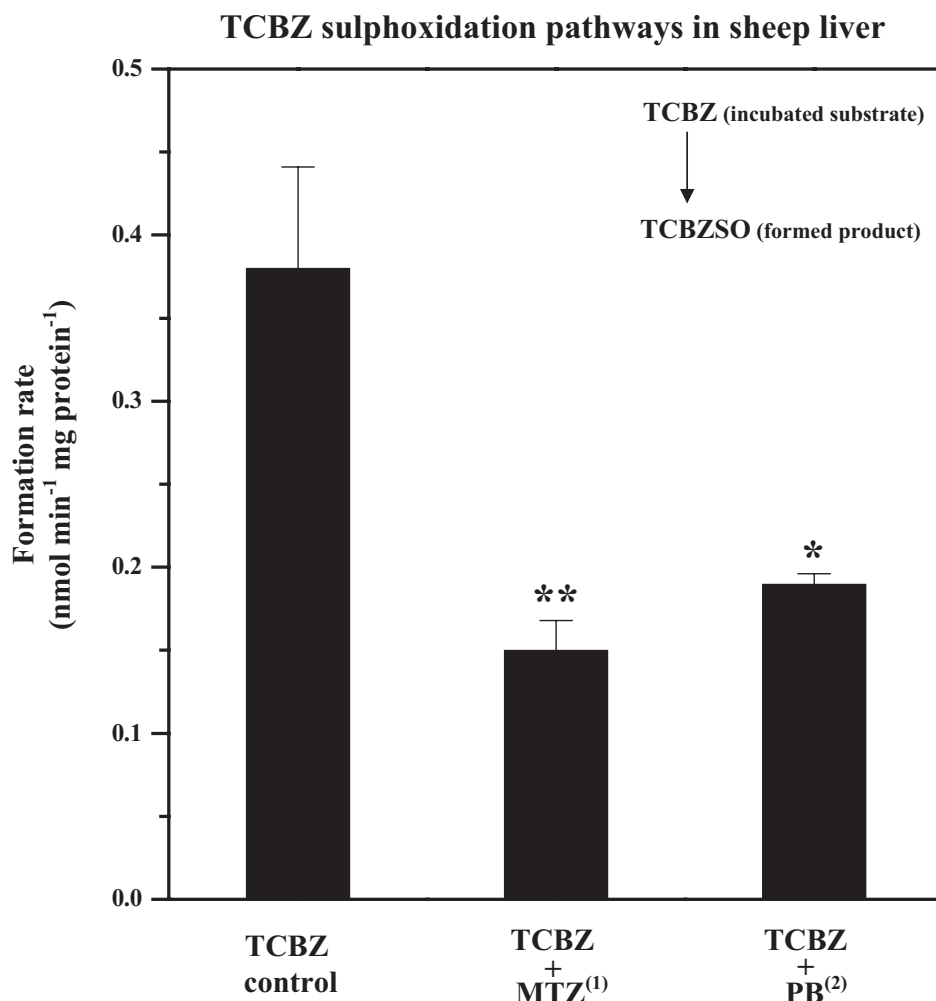


Figure 2. *In vitro* biotransformation of triclabendazole (TCBZ) by sheep liver microsomes. Effects of FMO inhibition (methimazole, MTZ) and piperonyl butoxide (PB)-mediated inhibition of the cytochrome P450 system on the sulphoxidation of TCBZ. Data (nmol min⁻¹ mg⁻¹ microsomal protein) are the mean ($\pm$ SD) of six determinations. Values are significantly different from control incubations at * $p < 0.05$ and ** $p < 0.01$. ⁽¹⁾Inactivation of the FMO system was carried out by heat pre-treatment (2 min at 50°C) of the microsomal preparation followed by pre-incubation with MTZ (100 μ M) (see the Materials and methods). The initial TCBZ concentration was 30 μ M. ⁽²⁾Inhibition of cytochrome P450 was carried out with 100 μ M PB and the initial TCBZ concentration was 30 μ M.

Representative chromatograms of TCBZ and TCBZSO analysis by HPLC obtained after incubation of TCBZ with *F. hepatica* microsomes are shown in figure 4. There was not a measurable conversion of TCBZ to TCBZSO in the inactivated (boiled) parasite microsomal sample (figure 4a). Conversely, TCBZ was oxidized to TCBZSO after 60 min incubation in presence of NADPH (figure 4b). The rate of TCBZ sulphoxidation by the parasite microsomal fraction was 0.09 ± 0.009 nmol TCBZSO formed min⁻¹ mg⁻¹ microsomal protein. Only trace amounts of TCBZSO₂ were recovered in some parasite microsomal incubations.

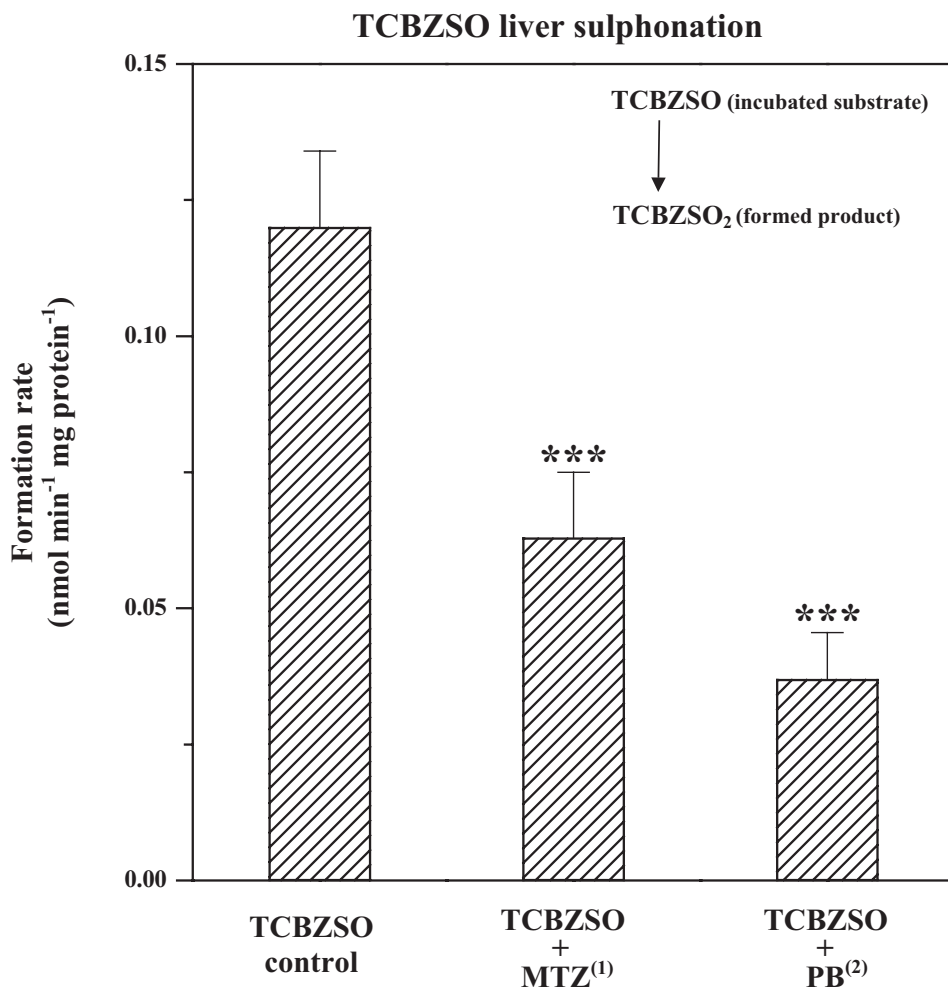


Figure 3. *In vitro* metabolism of triclabendazole sulfoxide (TCBZSO) by sheep liver microsomes. Effects of FMO inhibition (methimazole, MTZ) and piperonyl butoxide (PB)-mediated inhibition of the cytochrome P450 system on the sulphonation of TCBZSO. Data (nmol min⁻¹ mg⁻¹ microsomal protein) are the mean ($\pm$ SD) of six determinations. Values are significantly different from control incubations at *** $p < 0.001$. ⁽¹⁾Inactivation of the FMO system was carried out by heat pre-treatment (2 min at 50°C) of the microsomal preparation followed by pre-incubation with MTZ (100 μ M) (see the Materials and methods). The initial triclabendazole (TCBZ) concentration was 30 μ M. ⁽²⁾Inhibition of the cytochrome P450 was carried out with 100 μ M PB and the initial TCBZ concentration was 30 μ M.

TCBZ and all its metabolic products were recovered from *F. hepatica* after their *ex vivo* incubation as early as 15 min post-incubation, demonstrating a fast diffusion of TCBZ-related molecules from the medium to the trematode parasite tissues. The concentrations (mean $\pm$ SD) of TCBZ, TCBZSO, TCBZSO₂, OH-TCBZ, OH-TCBZSO and OH-TCBZSO₂ measured in *F. hepatica* at different times post-incubation in a KRT medium are shown in table 2. The amount of TCBZ and TCBZSO recovered over time did not significantly increase throughout the incubation period. Only slight differences on the concentration of TCBZSO₂, OH-TCBZ, OH-TCBZSO and OH-TCBZSO₂

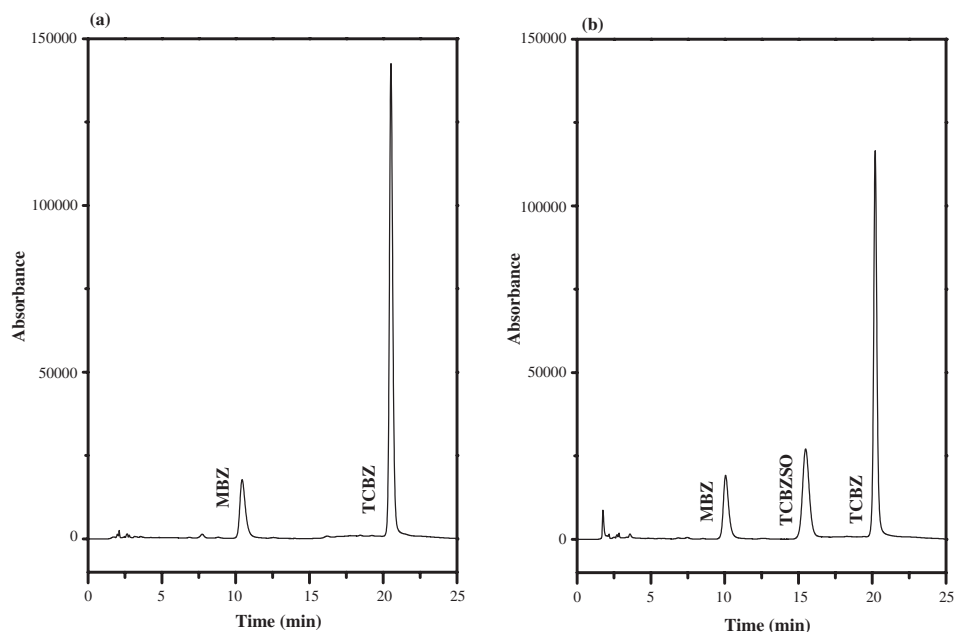


Figure 4. Oxidative metabolism of triclabendazole (TCBZ) in *Fasciola hepatica*. Chromatograms obtained from the HPLC analysis of the *F. hepatica* microsomal fraction incubated with TCBZ over 60 min: (a) inactivated (boiled) microsomes and (b) microsomes incubated in the presence of NADPH (0.5 μ mol), where the formation of TCBZ sulphoxide (TCBZSO) was observed. Mebendazole (MBZ) was used as the internal standard.

Table 2. Mean concentrations (nmol 100 mg protein⁻¹) ($n=4$, $\pm$ SD) of triclabendazole (TCBZ) and metabolites measured in *Fasciola hepatica* at different incubation times under *ex vivo* conditions. The area under the concentration versus time curve (AUC) (nmol min 100 mg protein⁻¹) ($n=4$, mean $\pm$ SD) shown for each drug molecule is the total availability measured within the parasite after 180 min of incubation.

Incubation time (min)	TCBZ	TCBZSO	TCBZSO ₂	OH-TCBZ	OH-TCBZSO	OH-TCBZSO ₂
15	5.9 $\pm$ 1.5 ^a	4.0 $\pm$ 1.2 ^a	4.7 $\pm$ 0.6 ^a	2.8 $\pm$ 1.9 ^a	3.0 $\pm$ 0.5 ^a	2.6 $\pm$ 1.0 ^a
45	9.1 $\pm$ 2.2 ^a	6.5 $\pm$ 1.6 ^a	7.0 $\pm$ 1.1 ^{ab}	6.5 $\pm$ 3.1 ^{ab}	7.3 $\pm$ 1.1 ^b	5.7 $\pm$ 2.2 ^{ac}
90	12.8 $\pm$ 4.9 ^a	11.6 $\pm$ 3.9 ^a	13.2 $\pm$ 4.0 ^{bc}	9.9 $\pm$ 3.5 ^b	8.6 $\pm$ 0.8 ^b	8.2 $\pm$ 0.7 ^{bc}
120	8.8 $\pm$ 4.6 ^a	11.9 $\pm$ 5.6 ^a	12.6 $\pm$ 2.7 ^{bc}	9.3 $\pm$ 1.5 ^{ab}	5.0 $\pm$ 1.9 ^{ab}	9.9 $\pm$ 3.6 ^{bc}
180	10.5 $\pm$ 5.7 ^a	13.4 $\pm$ 8.1 ^a	14.0 $\pm$ 3.4 ^c	3.4 $\pm$ 2.6 ^a	6.0 $\pm$ 2.3 ^{ab}	6.7 $\pm$ 1.9 ^{ac}
AUC	1665 $\pm$ 228 ^a	1708 $\pm$ 106 ^a	1852 $\pm$ 256 ^a	1198 $\pm$ 106 ^b	1100 $\pm$ 90 ^b	1222 $\pm$ 178 ^b

^{a-c} Means not having a common superscript within a column are significantly different at $p < 0.05$, except for the AUCs, where the statistical differences are within the row.

were observed between different incubation times. Drug diffusion into the parasite occurred as a slowly accumulative process up to 90 min of incubation, when drug concentrations measured in the parasite became statistically higher compared with those measured during the first 15 min of incubation. After 90 min of incubation, the concentration of TCBZSO₂ measured inside the fluke remained stable (table 2). Conversely, the concentration of the hydroxy-metabolites recovered inside the parasite tended to decrease. The concentration values measured in the parasite for the different drug molecules at each incubation time did not show

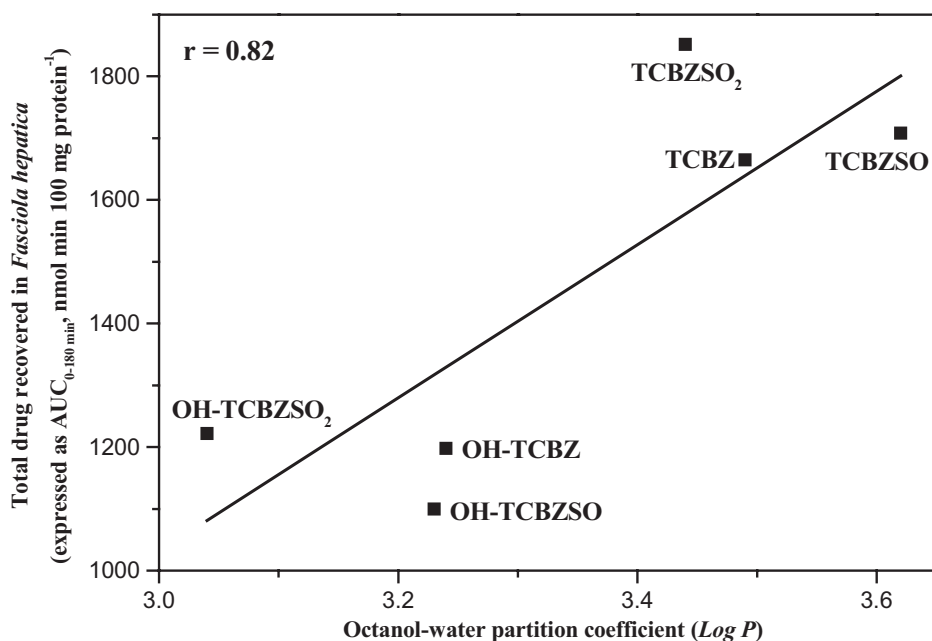


Figure 5. Correlation between total drug recovered in *Fasciola hepatica* ($n=4$), expressed as area under the concentration versus time curves (AUC) after 180 min of incubation, and the octanol-water partition coefficients (log P) estimated for triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZSO), triclabendazole sulphone (TCBZSO₂), hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZSO (OH-TCBZSO) and hydroxy-TCBZSO₂ (OH-TCBZSO₂).

statistical significance. However, the level of TCBZ measured after 15 min of incubation was significantly higher than that observed for OH-TCBZSO₂.

The results obtained from the transtegumental diffusion kinetic studies were correlated with the lipid-to-water PC of the anthelmintic drugs assayed. The PC values obtained were 3.62 (TCBZSO), 3.49 (TCBZ), 3.42 (TCBZSO₂), 3.24 (OH-TCBZ), 3.23 (OH-TCBZSO) and 3.03 (OH-TCBZSO₂). The correlation between drug diffusion over 180 min of incubation (measured as total drug amount recovered in the liver flukes expressed as AUCs) and drug lipophilicity (measured as octanol-water PC) is shown in figure 5. There was a high correlation ($r=0.82$) between drug diffusion and drug lipophilicity. Despite the TCBZ, TCBZSO and TCBZSO₂ log P values tending to be higher than those obtained for the hydroxy-derivatives, these differences did not reach statistical significance.

In spite of the lack of statistical significance between the log P values (PC), there was a clear tendency showing an enhanced concentration profile within the parasite for the most lipophilic compounds (TCBZ, TCBZSO, TCBZSO₂). Significantly lower AUC_{0-180 min} values were obtained for the hydroxy-derivatives, OH-TCBZ (1198 nmol min 100 mg protein⁻¹), OH-TCBZSO (1100 nmol min 100 mg protein⁻¹), OH-TCBZSO₂ (1222 nmol min 100 mg protein⁻¹), compared with those observed for TCBZ (1665 nmol min 100 mg protein⁻¹), TCBZSO (1708 nmol min 100 mg protein⁻¹) and TCBZSO₂ (1852 nmol min 100 mg protein⁻¹). There were no statistically significant differences between the total amount of TCBZ, TCBZSO and TCBZSO₂ recovered in

the fluke parasite. Similar concentrations of OH-TCBZ, OH-TCBZSO and OH-TCBZSO₂ were recovered in the trematode parasite.

Discussion

The benzimidazole anthelmintics require extensive hepatic oxidative metabolism to achieve sufficient polarity for excretion (Hennessy *et al.* 1993). Despite the plasma pharmacokinetic profile of TCBZ having been described in many species, including cattle, sheep, goats, horses, ponies, donkeys, pigs and humans (Hennessy *et al.* 1987, Bogan *et al.* 1988) the involvement of the cytochrome P450 and FMO systems on the liver biotransformation of TCBZ is still unknown. The implication of these enzymatic systems on the sulphoxidation of albendazole in sheep (Galtier *et al.* 1986), pigs (Souhaili El Amri *et al.* 1987), cattle (Lanusse *et al.* 1993), rats (Moroni *et al.* 1995) and humans (Rawden *et al.* 2000) has been reported. It has been demonstrated that FMO is primarily involved in albendazole hepatic sulphoxidation in sheep (Galtier *et al.* 1986, Lanusse *et al.* 1993) and cattle (Lanusse *et al.* 1993). Recent work from our group reported that FMO-mediated sulphoxidation accounted for 60% of the albendazole sulphoxide production, whilst cytochrome P450 contributed 40% of the albendazole sulphoxide formation in both sheep and cattle liver microsomes (Virkel *et al.* 2004). Similarly, FMO was estimated to be the main enzymatic system involved of total fenbendazole liver sulphoxidation in both species (about 80%). The involvement of both enzyme systems in TCBZ sulphoxidation and TCBZSO sulphonation was shown in the current work after FMO inactivation and/or pre-incubation of microsomes with PB. FMO was inactivated by heat pre-treatment of the microsomal preparation followed by MTZ pre-incubation (Dixit and Roche 1984). Heat treatment inactivates the FMO system without affecting CYP-mediated metabolism (McManus *et al.* 1987), although some loss of activity of CYP2A6 and CYP2C9 isoenzymes has been reported (Grothusen *et al.* 1996). On the other hand, equivalent albendazole sulphoxidation rates were observed in heat-treated compared with MTZ-treated liver microsomes obtained from humans (Rawden *et al.* 2000), sheep and cattle (Virkel *et al.* 2004). Thus, heat treatment of the microsomal preparation followed by MTZ pre-incubation seem to be an adequate strategy to assess the relative contribution of FMO and cytochrome P450 to TCBZ and TCBZSO hepatic oxidation. The relative involvement of both enzymatic systems may be estimated following the assumption that inactivation of FMO leaves the cytochrome P450 system able to metabolize TCBZ or TCBZSO. Thus, the FMO-mediated sulphoxidation accounted for about 62% of TCBZSO and about 48% of TCBZSO₂ formation, whilst cytochrome P450 contributed 38% (TCBZSO) and 52% (TCBZSO₂) of the oxidized metabolites. Additionally, the greater ability of the sheep liver for TCBZ sulphoxidation rather than TCBZSO sulphonation was demonstrated in the current work. Despite this, the liver capacity to produce the sulphone metabolite from TCBZ parent drug appeared to be greater than that shown for albendazole in a previous work carried out under similar experimental conditions (Virkel *et al.* 2004).

Helminth parasites have an apparent lack of cytochrome P450 as a detoxification route and oxidation pathways in *Ascaris lumbricoides* (nematode) and *Moniezia expansa* (cestode) occur exclusively by the FMO system (Precious and Barret 1989), which requires NADH or NADPH as cofactors (Douch and Buchanan

1979). It has been previously shown that *F. hepatica* has significantly higher oxidative activity than nematode and cestode parasites. Adult liver flukes have the ability to oxidize albendazole to albendazole sulphoxide and albendazole sulphone (Solana *et al.* 2001). On the other hand, the sulphone metabolite has been identified in *F. hepatica* incubated with TCBZSO over 3–12 h (Robinson *et al.* 2004). The work reported here permitted the characterization of the metabolism of TCBZ within the target parasite. The fluke microsomal fraction was able to generate TCBZSO (main product) and TCBZSO₂ from TCBZ parent drug. TCBZSO production by the microsomal fraction of *F. hepatica* was about 25% that measured for liver microsomes when the concentration of NADPH was optimal (1 $\mu\text{mol ml}^{-1}$) (table 1). Although further studies should be conducted to elucidate the metabolic pathways (i.e. enzymatic systems) involved in the oxidation of TCBZ in *F. hepatica*, this set of preliminary results on parasite metabolism are pharmacologically relevant.

F. hepatica infects a variety of hosts such as sheep, cattle, goats, deer, buffaloes, horses, camelids, pigs and humans (Coles 1986). Adult flukes are located in the common bile ducts and the gall-bladder. A large number of experiments have shown that different chemical substances, including anthelmintic drugs, are mainly taken up from the site of parasite location through the external surface, as opposed to oral ingestion. This has been shown in *Haemonchus contortus* (Rothwell and Sangster 1997, Alvarez *et al.* 2000), *Ascaris suum* (Ho *et al.* 1990, Alvarez *et al.* 2001), *Moniezia* spp. (Alvarez *et al.* 1999, Mottier *et al.* 2003), *F. hepatica* (Fetterer and Rew 1984, Alvarez *et al.* 2000, 2001) and *Onchocerca ochengi* (Cross *et al.* 1998) among other helminth parasites. It has been shown that under *in vitro* conditions (Bennett and Köhler 1987), TCBZ can penetrate into liver flukes even when the oral route had been closed off by ligation, which demonstrates the relevance of drug entry through the external surface even in an hematophagous parasite. On the other hand, the large quantities of free (pharmacologically active) TCBZ-related molecules present in bile certainly provide substantial chemical contact with liver-dwelling *F. hepatica* (Hennessey *et al.* 1987).

In contrast to the patterns of diffusion observed for albendazole and its sulphoxide metabolite (albendazole sulphoxide) into *F. hepatica* (Alvarez *et al.* 2001), the amount of TCBZ and its sulphoxide derivative (TCBZSO) recovered over time did not increase during the incubation period. It could be that 180 min of incubation time was not long enough, since more than 180 min were necessary to demonstrate TCBZ accumulation in flukes under *in vitro* conditions (Bennett and Köhler 1987). Considering the experimental evidence shown here, where the ability of the fluke to oxidize TCBZ into its sulphoxide and sulphone metabolites was demonstrated, it could be hypothesized that the lack of an accumulative process may be due to the capability of the liver fluke to metabolize the drug. Such a finding may agree with the fact that concentrations of the hydroxy-derivatives measured inside the liver flukes decreased after 90 min of incubation. However, the parent drug added to the incubation medium was the predominant analyte recovered within *F. hepatica*. The capability of the parasite to biotransform TCBZ observed in the metabolism assays, was not evident in the drug diffusion assays. It is likely that TCBZ concentrations higher than that used in the current drug diffusion assays (5 nmol ml^{-1}) are necessary to chromatographically detect TCBZ metabolic products. In fact, the presence of TCBZSO₂ in flukes incubated with TCBZSO concentrations as high as 140 nmol ml^{-1} has recently been reported

(Robinson *et al.* 2004). Alternatively, other TCBZ-related end products than those identified until now could have been generated by the liver flukes without being detected under the current analytical conditions.

When the transtegumental diffusion of all the molecules assayed was compared at each incubation time, the access of the hydroxylated derivatives to the parasite tended to be lower than that of TCBZ, TCBZSO and TCBZSO₂, despite the difference did not reach statistical significance. However, estimation of the total drug amount recovered from the parasite as AUC demonstrated that TCBZ parent drug and its sulpho- metabolites (TCBZSO and TCBZSO₂) have a significantly higher ability to penetrate into *F. hepatica* compared with the hydroxylated metabolites. The accumulated data show that the main route of acquisition of broad-spectrum anthelmintics by target parasites appears to be by passive diffusion through their external surface. The rate of penetration of a drug will mainly depend on the intrinsic lipid-to-water partition coefficient of the molecule (Mottier *et al.* 2003), pH/p*K* relationship, molecular size, concentration gradient and the surface area of contact between drug and parasite. Lipid solubility is a major factor determining drug penetration across the nematode cuticle (Alvarez *et al.* 2000, 2001) as well as through the tegument of cestodes (Alvarez *et al.* 1999, Mottier *et al.* 2003) and trematodes (Fetterer and Rew 1984, Alvarez *et al.* 2000, 2001). This correlates with the ability of the drug to reach therapeutically relevant concentrations within the target parasite. TCBZ, TCBZSO and TCBZSO₂ are more lipophilic (higher octanol–water PC) and, therefore more soluble in the lipoidal surface membranes of the helminth parasites, than are their respective polar hydroxy-metabolites (lower octanol–water PC). Such a phenomenon accounted for the greater availability of the non-hydroxylated molecules inside the incubated trematode parasite. This was confirmed by the high correlation observed between transtegumental diffusion of TCBZ-related molecules into *F. hepatica* and drug lipophilicity (expressed as log *P*) (figure 5).

In contrast to the differences observed on the diffusion of albendazole and albendazole sulfoxide into *F. hepatica* (Alvarez *et al.* 2001), the results reported here indicated that TCBZ parent drug and its sulpho- metabolites have a similar ability to penetrate through the tegument of the liver flukes. This finding may correlate with the octanol–water PC values obtained for these molecules. While albendazole and albendazole sulfoxide log *P* values were markedly different (3.83 versus 1.24, respectively), the PC values obtained for TCBZ, TCBZSO and TCBZSO₂ were similar. The high parasite diffusion capacity observed for the sulfoxide and sulphone metabolites may be pivotal for TCBZ action, since the parent compound is short lived, and both TCBZSO and TCBZSO₂ are the main unconjugated analytes recovered in the bloodstream and bile of treated animals (Hennessy *et al.* 1987). Consequently, flukicidal activity has been mainly related to TCBZSO systemic availability, although the sulphone metabolite seems to have some flukicidal activity (41% efficacy on 28-day-old liver flukes) (Büscher *et al.* 1999). On the other hand, the sulfoxide metabolite was found to exert a delayed effect on the motility of the trematode parasite (Bennett and Köhler 1987). In conclusion, the pharmacological activity of TCBZ against liver flukes may depend on the irreversible impairment of essential cellular functions induce by an additive effect of these three molecules.

Knowledge of the patterns of drug/metabolites diffusion into different target parasites, together with the available pharmacokinetic/dynamic information may

substantially contribute to elucidate the mechanism of drug penetration and the pharmacological activity of most anthelmintics. Understanding the relationship among TCBZ metabolism, the relative potency of its metabolic products and their ability to reach the target parasite, may be critical to optimize its flukicidal activity, particularly when TCBZ resistant flukes have already been recognized.

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Renal elimination of a novel and potent $\alpha_v\beta_3$ integrin antagonist in animals

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1. Compound A (3-{2-oxo-3-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)propyl]-imidazolidin-1-yl}-3(S)-(6-methoxy-pyridin-3-yl)propionic acid), a hydrophilic zwitterion, is a potent and selective $\alpha_v\beta_3$ integrin antagonist currently under clinical development for the treatment of osteoporosis. The mechanism of renal excretion of compound A was investigated using a combination of *in vivo* and *in vitro* approaches.

2. In rats, renal excretion of compound A involved tubular secretion; ratios between renal clearance, corrected for unbound fraction in plasma ($CL_{r,u}$) and glomerular filtration rate (GFR) were greater than unity (2–5). The tubular secretion of compound A was saturable at high plasma levels ($> 26 \mu\text{M}$), and was inhibited significantly, although modestly (about twofold) by relatively high plasma concentrations of the organic anion PAH (160 μM) and the cation cimetidine (about 400 μM), but not by the P-gp inhibitor quinidine (about 50 μM). However, compound A (about 100 μM) had a minimal effect on CL_r /GFRs for cimetidine and PAH.

3. In rhesus monkeys, renal elimination of compound A also involved tubular secretion, with a $CL_{r,u}$ /GFR ratio of about 30. The renal secretion of compound A was not affected by either cimetidine (about 120 μM) or PAH (about 80 μM). Similarly, compound A (about 40 μM) had a minimal effect on the renal tubular secretion of both cimetidine and PAH.

4. At the doses studied, neither rat nor monkey plasma protein binding of compound A, cimetidine or PAH was affected in the presence of each other.

5. *In vitro* transport studies showed that compound A was not a substrate for P-gp in the Caco-2, human *MDR1* and mouse *mdr1a* transfected LLC-PK1 cell lines. In an uptake study using rOAT1 and rOAT3 transfected HEK cell lines, compound A was shown to be a substrate for rat OAT3 ($K_m = 15 \mu\text{M}$), but not rat OAT1.

6. The results suggest that the tubular secretion of compound A is not mediated by P-gp, but rather is mediated, at least in part, via the organic anion transporter OAT3, the renal transporter shown to be capable of transporting both the organic anion PAH and the organic cation cimetidine. Although there is a possibility for pharmacokinetic interactions between compound A and substrates or inhibitors of OAT3, at the renal excretion level, the magnitude of interaction would likely be modest in humans at clinically relevant doses.

Introduction

Compound A, 3-{2-oxo-3-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)propyl]-imidazolidin-1-yl}-3(S)-(6-methoxy-pyridin-3-yl)propionic acid (figure 1), has been under clinical development for treatment of osteoporosis. *In vitro*, it is a potent and selective antagonist of the $\alpha_v\beta_3$ integrin receptor (Rodan *et al.* 2002), which is highly expressed in osteoclasts. Consistent with the known role of $\alpha_v\beta_3$ receptor in the bone resorption processes (Hartman and Duggan 2000, Miller *et al.*

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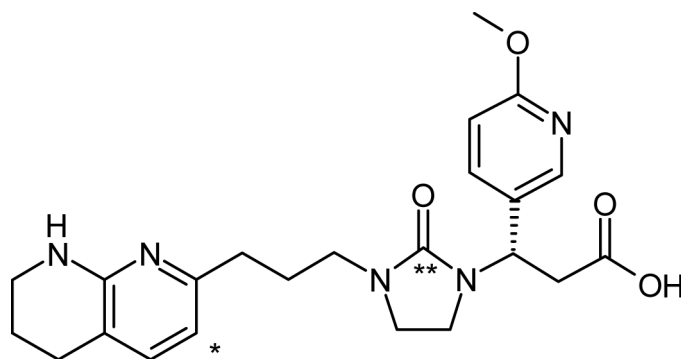


Figure 1. Chemical structure of compound A. ***Positions of the [^3H] and [^{14}C] labels, respectively.

2000), compound A has also been shown to increase significantly bone mineral density in growing male rats and in 6-month-old ovariectomized rats (Rodan *et al.* 2002, Hutchinson *et al.* 2003), and recently in humans (Murphy *et al.* 2004). Early pharmacokinetic studies in preclinical animal species (rats, dogs and rhesus monkeys) revealed that compound A was well absorbed orally, and eliminated primarily as unchanged drug in urine and bile (Prueksaritanont *et al.* 2004, Cui *et al.* 2004). Detailed analysis of renal clearance and plasma protein binding data suggested that the renal elimination of compound A involved tubular secretion in all the preclinical species examined. Based on these data and together with additional *in vitro* studies, it was suggested that compound A would also exhibit good oral absorption and substantial renal elimination in humans (Prueksaritanont *et al.* 2004, Cui *et al.* 2004). Recently, this has been confirmed; more than 80% of the dose was excreted as unchanged drug in urine following oral administration of compound A to healthy volunteers (unpublished data).

Compound A contains both the basic tetrahydronaphthylidene moiety (pK_a about 8) and the acidic carboxylic (pK_a about 3) functional group, and thus is a zwitterion at physiological pH. To date, limited information is available regarding the mechanism for active tubular secretion of zwitterionic compounds and their interactions with known renal transporters as compared with the literature on renal handling for acidic and basic drugs. Organic cations and anions are known to be actively transported into urine via the organic cation transporter systems and the organic anion transporter systems, respectively (Pritchard and Miller 1993, Dresser *et al.* 2001). Among the transporters identified, the organic cation transporters OCT1 and OCT2 and the organic anion transporters OAT1 and OAT3 have been shown to be localized in the basolateral membrane of renal proximal tubules and functionally active in transporting several organic cations and anions, respectively (Dresser *et al.* 2001, Burckhardt and Burckhardt 2003). In addition, the multidrug resistance protein P-glycoprotein (P-gp) has also been shown to be involved in the tubular secretion of ionic compounds, via the luminal membrane of proximal tubules (Dresser *et al.* 2001, Masereeuw and Russel 2001). Although few studies have shown that zwitterions are substrates of P-gp (Yamaguchi *et al.* 2000, Prueksaritanont *et al.* 2002), limited information is available regarding the role of P-gp in the renal elimination of zwitterions.

In order to provide an insight into the mechanism of renal excretion of compound A, and to help anticipate a possible drug-drug interaction at the level

of renal elimination during clinical development of this compound, we investigated the interaction potential between compound A, a zwitterion, and the prototypical anion PAH, or the cation cimetidine in preclinical species, rats and rhesus monkeys, using an *in vivo* clearance approach. In addition, the role of P-gp in the renal excretion of compound A was examined *in vivo* in rats in the presence of quinidine, a known P-gp inhibitor. Furthermore, using an *in vitro* approach, susceptibility of compound A as a substrate for P-gp and rat OAT1 and rat OAT3 also was examined.

Materials and methods

Materials

Compound A and ^3H - or ^{14}C -compound A (figure 1) were synthesized at Merck Research Laboratories (West Point, PA, USA). ^{14}C -inulin, ^3H -vinblastine (VBL), *p*-amino hippuric acid (PAH), cimetidine and quinidine were obtained from Sigma Chemical Co. (St Louis, MO, USA). ^3H -PAH was obtained from NEN (Boston, MA, USA), and ^3H -cimetidine, ^{14}C -benzyl penicillin were obtained from Amersham (Piscataway, NJ, USA). All radiolabelled materials used had radiochemical purities of > 98%. Solvents used for analysis were of analytical or HPLC grade (Fisher Scientific, Pittsburgh, PA, USA). The human intestinal cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Opti-MEM supplemented with 10% foetal calf serum, non-essential amino acids and Glutamax, and were used at passages 20–50. Human *MDR1* transfectants L-MDR1, mouse *mdr1a* transfectants L-mdr1a, and their parenteral cell line LLC-PK1 pig kidney epithelial cells were kindly provided by Dr A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, the Netherlands) and used under a license agreement. The cells were cultured in medium 199 (Life Technologies, Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine, penicillin (50 units ml⁻¹), streptomycin (50 µg ml⁻¹), and 10% foetal calf serum. The drug-sensitive human epidermoid carcinoma cell line KB-3-1 (parent cell line) and its vinblastine (VBL)-selected MDR variant KB-V1 (P-gp-overexpressing cell line) were kindly provided by Dr Michael M. Gottesman (National Cancer Institute) and used under a licensing agreement. Both cell lines were maintained in Dulbecco's modified Eagle's medium (D-MEM, high glucose, GIBCO BRL, Gaithersburg, MD, USA) containing 10% FCS (Bio*Whittaker, Walkersville, MD, USA), 50 units ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin (GIBCO BRL, Gaithersburg, MD, USA), and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO₂/95% air. KB-V1 cells were grown in the continuous presence of VBL (1 µg ml⁻¹).

pEF6 plasmids for expression of rat OAT1 and OAT3 were supplied from the laboratory of Dr R. Kim (Vanderbilt University, Nashville, TN, USA). The plasmids were sequenced and differences from the published rat accession number AB004559 (OAT1) and AB017446 (OAT3) were corrected before use. HEK293 cells were obtained from Merck-Frosst (Montreal, Canada) and were routinely maintained in D-MEM with 10% FCS. One day before transfection of the HEK cells, 24-well plates were treated 30 min at 37°C with 0.5 ml matrigel diluted 500-fold in cold D-MEM. The plates were then rinsed with D-MEM and HEK cells were plated at 100 000 cells/well. The next day the cells were transfected with control, OAT1 or OAT3 plasmids (0.8–1.0 µg DNA/well in 24-well plates) in Opti-MEM containing 2.5% FCS using lipofectamine 2000 in accordance with the manufacturer's procedure.

Animals

Male and female Sprague–Dawley rats (290–320 g), with an indwelling cannula implanted in the right jugular vein and left carotid artery for blood sampling and drug administration, respectively, were used. The surgery was performed as described (Lin *et al.* 1988) under light pentobarbital anaesthesia 1 day before the experiment. The animals were housed individually in metabolism cages for urine collection, and unrestrained during the experiment.

Male rhesus monkeys ($n = 4$, 4–6 kg), with a vascular port in the iliac artery for blood sampling and a urinary catheter cannulated for urine sampling, were used. The animals sat on a chair during the study for 7 h.

In vivo studies

All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

Rat studies. To characterize the renal elimination process of compound A in rats, three steady-state plasma levels of compound A were targeted using three different dosing regimens, each comprising a loading i.v. bolus dose and followed immediately by a maintenance i.v. constant infusion. Each rat was given three consecutive pairs of i.v. bolus doses at 2-h intervals and constant i.v. infusions for 2 h, for the total study period of 6 h. The respective loading and maintenance doses of compound A were 3, 12 and 50 mg kg⁻¹ i.v. bolus, and 30, 120 and 500 µg min⁻¹ i.v. infusion. In studies to examine effects of inhibitors on renal clearance (CL_r) of compound A, compound A was given by an i.v. bolus of 4 mg kg⁻¹ followed by a constant infusion at 40 µg min⁻¹ for a total of 6 h. After 3 h post-compound A, inhibitors (cimetidine, PAH and quinidine) were given by i.v. bolus and followed immediately by i.v. infusion for 3 h. Two different doses of cimetidine were studied; 40 and 200 mg kg⁻¹ i.v. bolus followed by 120 and 700 µg min⁻¹, respectively. The i.v. bolus doses for PAH and quinidine were 2 and 25 mg kg⁻¹, respectively, and the infusion rates for PAH and quinidine were 180 and 280 µg min⁻¹, respectively. In the studies investigating the effect of compound A on CL_r of cimetidine or PAH, compound A was given at 40 mg kg⁻¹ i.v. bolus followed by a 400 µg min⁻¹ constant infusion after 3 h of the initiation of either cimetidine or PAH administration (30 mg kg⁻¹ i.v. bolus + 20 µg min⁻¹ and 0.9 mg kg⁻¹ i.v. bolus + 0.05 µg min⁻¹, respectively, for over 6 h). For all studies, ¹⁴C-inulin was given over the 6-h study period by an i.v. bolus of 5 µCi kg⁻¹ followed by a constant infusion at a rate of 0.09 µCi min⁻¹, for GFR measurement. Blood samples were taken in series for every 20 min up to 6 h. Urine samples were collected during 3-h intervals with or without inhibitors, as previously described (Lin *et al.* 1988). All samples were kept frozen until analysis by LC/MS/MS or HPLC.

Monkey studies. Compound A was given to monkeys by a constant intravenous infusion of 85 µg min⁻¹ for 3 h, following an i.v. bolus dose of 0.4 mg kg⁻¹. To study the effect of cimetidine and PAH on the renal excretion of compound A, cimetidine or PAH was given concomitantly with compound A to the same animals (following a 10-day wash-out period) at 2250 or 1500 µg min⁻¹, for 3 h, following an i.v. bolus of 24 or 15 mg kg⁻¹, respectively. In the study to investigate the effect of compound A on CL_r of cimetidine or PAH, compound A was given (at 4 mg kg⁻¹ i.v. bolus followed by a 850 µg min⁻¹ constant infusion for 3 h) simultaneously with either cimetidine or PAH at 3.5 or 1.2 mg kg⁻¹ i.v. bolus followed by a constant infusion of 150 or 100 µg min⁻¹, respectively, for over 3 h. For all studies, ¹⁴C-inulin was also given for over the 3-h study period by an i.v. bolus of 1.5 µCi kg⁻¹ followed by a constant infusion at a rate of 0.22 µCi min⁻¹, for GFR measurement. Blood samples were taken in series at predose, 40, 80, 120, 150, 180, 195, 225, 270, 330, 420 and 1440 min after dosing. Urine samples were collected during 0–7- and 7–24-h intervals. All samples were kept frozen until analysis by LC/MS/MS or HPLC.

Plasma protein binding

The binding of compound A to rat or monkey plasma proteins was determined *in vitro* and/or *ex vivo* using an ultrafiltration method, as described (Prueksaritanont *et al.* 2004). *In vitro*, plasma samples from control rats (pooled from three different rats) were spiked with compound A to obtained final concentrations of 5 and 150 µM, and plasma protein binding was determined in triplicate. *Ex vivo*, plasma samples used for protein binding determination were collected during the study before and after the addition of inhibitors from animals used in the above experiments described under 'Rat studies' or 'Monkey studies'. Due to limited sample volumes, determination of protein binding *ex vivo* was done in duplicate using pooled plasma samples (rats — five different animals/treatment), or individual plasma samples (monkeys). Following incubation of plasma samples containing compound A at 37°C for 15 min, an aliquot of plasma (3 × 1 ml) was transferred immediately to a centrifuge tube (Amicon Co., Danvers, MA, USA). The tube was centrifuged at 1500g for 15–30 min at 37°C to obtain about 0.2 ml filtrate. The filtrate and original plasma samples were analysed for compound A by HPLC. The unbound fraction was estimated directly from the ratio of the intact drug in the filtrate to that in the original plasma samples before centrifugation.

Plasma protein binding of PAH and cimetidine was also carried out *ex vivo* using ultrafiltration method as described above. Preliminary studies indicated that neither compound was bound to the ultrafiltrate device.

In vitro transport/uptake studies

In vitro P-gp studies were performed in two transport models; Caco-2 cells and L-MDR1 or L-mdr1a transfected LLC-PK cells. In Caco-2 cells, studies to determine whether compound A is a substrate for P-gp were conducted as described (Prueksaritanont *et al.* 2002) using ³H- compound A concentrations of 1 and 10 µM in the presence or absence of 10 µM cyclosporine A, a known inhibitor of P-gp. In L-MDR1, L-mdr1a, and LLC-PK1 cells, the directional transport studies of compound A (1 µM) also was carried out as described previously (Prueksaritanont *et al.* 2002), except that a 5-h incubation was used in this experiment. Aliquots from the two compartments were analysed for compound A using a HPLC method described below.

Drug uptake studies by rat OAT1 and OAT3 were performed 48–72 h following the transfections of control, rOAT1 or rOAT3 plasmids to HEK293 cells. The plated cells were rinsed once with Hepes

buffered Hank's balanced salt solution (HBSS) and 0.5 ml HBSS containing $0.1 \mu\text{Ci ml}^{-1}$ radiolabelled drugs (^3H -PAH, ^3H -cimetidine, ^{14}C -benzyl penicillin, and ^{14}C -compound A) at the indicated concentrations. Drug uptake studies were performed for 20 min at 37°C , after which the dosing solution was collected and the cells were washed four times with cold HBSS. The cells were then lysed by the addition of $50 \mu\text{l}$ 100 mM NaOH for 35 min. The cell lysate was then neutralized with $50 \mu\text{l}$ 100 mM HCl, $300 \mu\text{l}$ HBSS were added and the samples were collected. Radioactivity in the cell lysate was then measured by scintillation counting on a Beckman 6500 scintillation counter (Paolo Alto, CA, USA). Uptake experiments were also conducted for ^{14}C -compound A over a concentration range of $6\text{--}200 \mu\text{M}$ in the absence and presence of probenecid ($100 \mu\text{M}$). For K_m determinations, the differences in uptake obtained in the absence and presence of probenecid at corresponding concentrations were used. A preliminary study showed that uptake of compound A by rOAT3 transfected HEK cells was inhibited by probenecid ($100 \mu\text{M}$), a prototypical inhibitor of rOAT3, to the same level of that by the vector-transfected HEK cells ($0.14 \pm 0.03\%$ versus $0.13 \pm 0.02\%$).

Analytical procedures

Concentrations of compound A, quinidine and cimetidine in plasma and urine, as well as PAH in plasma were analysed using an LC/MS/MS method similar to that previously described for compound A (Prueksaritanont *et al.* 2004). In brief, aliquots of acidified plasma or urine samples were extracted using a solid phase extraction method using a Waters OASIS[®] 96-well plate (Waters Corporation, Milford, MA, USA) and 70% acetonitrile/water as an eluent. Chromatography was effected with an Aqua Sep C₈ column ($2.1 \text{ mm} \times 50 \text{ cm}$, $5 \mu\text{m}$) using a mobile phase consisting of 30%, 90:10 acetonitrile: H₂O, and 70%, 10: 90 methanol: H₂O, both containing 0.1% formic acid and 10 mM ammonium acetate, at a flow rate of 0.35 ml min^{-1} . The analytes are quantitated by LC-MS/MS in the positive ionization SRM mode (m/z 440 $\rightarrow$ 261 for compound A, m/z 325 $\rightarrow$ 172 for quinidine, m/z 253 $\rightarrow$ 95 for cimetidine, and m/z 195 $\rightarrow$ 120 for PAH) using a SCIEX API 3000 mass spectrometer (Concord, Ontario, Canada) interfaced with a SCIEX Turbo IonSpray source to a Perkin Elmer Series 200 liquid chromatography system.

HPLC methods with fluorescence detection described previously for compound A (Prueksaritanont *et al.* 2004) and cimetidine (Lin *et al.* 1988), as well as an HPLC method with ultraviolet light detection (Prueksaritanont *et al.* 1984) were also used in this study for plasma protein binding experiments and for the determination of urine concentrations of respective analytes.

Data analysis

Plasma clearance (CL_p) of compound A was calculated as the infusion rate divided by the average plasma concentration obtained at steady-state (C_{pss}). The CL_r was estimated by dividing urinary excretion rate with C_{pss} . The glomerular filtration rate (GFR) was obtained directly from the CL_r of inulin. The unbound renal clearance ($\text{CL}_{r,u}$) was calculated by dividing CL_r by the unbound fraction in plasma.

The kinetic parameters for compound A uptake by rOAT3 were estimated according to the following equation:

$$v = V_{\text{max}} \times S / (K_m + S),$$

where v is the rOAT3-mediated uptake rate ($\text{pmol}/20 \text{ min}/\text{well}$), evaluated by subtracting the uptake obtained in the presence of probenecid from that without probenecid, S is the concentration of compound A (μM), K_m is the Michaelis constant (μM), and V_{max} is the maximum uptake rate ($\text{pmol}/20 \text{ min}/\text{well}$), respectively. Fitting was performed by the non-linear least-squares method using a MULTI program (Yamaoka *et al.* 1981) with Damping Gauss Newton Method algorithm.

Statistical analysis

Statistical analysis was performed by Student's pair t -test; $p < 0.05$ was considered significant.

Results

Rat studies

Dose-dependent studies. In rats, plasma levels of compound A reached steady-states within 1 h infusion under the present study protocol (figure 2). The steady-state plasma levels appeared to increase linearly from 6 to $26 \mu\text{M}$ with the infusion rate increasing from 30 to $120 \mu\text{g min}^{-1}$ (table 1). However, at a $500 \mu\text{g min}^{-1}$ infusion rate, plasma levels of compound A increased slightly more than dose proportional (table 1). Values for CL_p and CL_r of compound A were

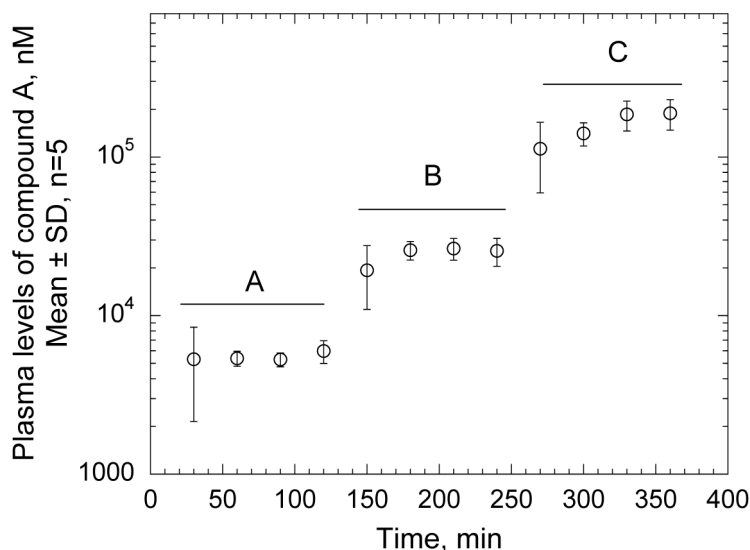


Figure 2. Plasma concentration–time profiles of compound A in rats following 3 mg kg⁻¹ i.v. bolus followed by 30 µg min⁻¹ i.v. infusion (A), 12 mg kg⁻¹ i.v. bolus followed by 120 µg min⁻¹ i.v. infusion (B), 50 mg kg⁻¹ i.v. bolus followed by 500 µg min⁻¹ i.v. infusion (C). Results are the mean ± SD, *n* = 5.

Table 1. Pharmacokinetic parameter of compound A in rats.

Infusion rate (µg min ⁻¹)	<i>C</i> _{pss} (µM)	CL _p (ml min ⁻¹)	CL _r (ml min ⁻¹)	GFR* (ml min ⁻¹)	CL _r /GFR	CL _{r,u} **/GFR
30	6.0 ± 0.5	12.0 ± 1.5	4.1 ± 0.8	3.6 ± 0.4	1.3 ± 0.1	5.2 ± 0.4
120	25.9 ± 3.2	10.7 ± 1.4	3.4 ± 1.2	2.6 ± 0.5	1.3 ± 0.2	5.2 ± 0.8
500	184.5 ± 43.0	6.5 ± 1.6***	1.6 ± 0.5***	3.4 ± 0.7	0.5 ± 0.1***	2.0 ± 0.4***

Values are mean ± SD, *n* = 4.

*GFR was estimated based on the CL_r of inulin in the same experiment (see the Materials and Methods).

**Calculation was made based on *f*_u of 0.26 obtained previously (Prueksaritanont *et al.* 2004).

***Significantly different (*p* < 0.05) from the first dose level.

relatively constant over the dose range of 30 and 120 µg min⁻¹, and were comparable with those observed previously in this species (Prueksaritanont *et al.* 2004). However, both CL_p and CL_r were significantly decreased (about twofold) following the 500 µg min⁻¹ infusion rate (table 1). For all the studied doses, GFRs were comparable (table 1). Based on the unbound fraction of 0.26 (Prueksaritanont *et al.* 2004), ratios for CL_{r,u}/GFR of compound A were greater than unity; the ratios were about 5 for the 30 and 120 µg min⁻¹ dosing rates, but decreased to about 2 at the 500 µg min⁻¹ dose (table 1).

Effects of cimetidine, PAH and quinidine on renal elimination of compound A. The study was carried out under linear kinetic conditions following a 4 mg kg⁻¹ i.v. bolus dose and a 40 µg min⁻¹ i.v. constant infusion rate of compound A to rats. During the first 3-h infusion of compound A alone, the pharmacokinetic parameters of compound A and GFRs for all treatments were comparable with each other (table 2). Co-administration with cimetidine at 700 µg min⁻¹ decreased

Table 2. Pharmacokinetic parameters of compound A in rats.

Without Inhibitor (0–3 h)					With Inhibitor (3–6 h)					
Substrate	CL _p (ml min ⁻¹)	CL _r (ml min ⁻¹)	GFR (ml min ⁻¹)	CL _r /GFR	Inhibitor	Dose (μ g min ⁻¹)	CL _p (ml min ⁻¹)	CL _r (ml min ⁻¹)	GFR (ml min ⁻¹)	CL _r /GFR
Compound A	16.3 $\pm$ 3.8	5.8 $\pm$ 1.6	3.8 $\pm$ 0.4	1.7 $\pm$ 0.3	Cimetidine	700	10.0 $\pm$ 2.8*	1.4 $\pm$ 0.7*	2.3 $\pm$ 1.0	0.7 $\pm$ 0.1*
Compound A	16.7 $\pm$ 3.2	5.1 $\pm$ 1.0	2.5 $\pm$ 0.9	2.7 $\pm$ 0.7	Cimetidine	120	13.4 $\pm$ 4.9	4.2 $\pm$ 1.5	2.4 $\pm$ 0.7	1.7 $\pm$ 0.4
Compound A	15.6 $\pm$ 7.2	3.1 $\pm$ 1.3	2.5 $\pm$ 0.3	1.2 $\pm$ 0.5	PAH	180	11.9 $\pm$ 2.9	1.9 $\pm$ 1.0	2.5 $\pm$ 0.5	0.8 $\pm$ 0.4*
Compound A	13.8 $\pm$ 1.9	3.7 $\pm$ 1.8	3.3 $\pm$ 0.6	1.6 $\pm$ 0.8	Quinidine	280	3.6 $\pm$ 0.7*	0.2 $\pm$ 0.1*	0.2 $\pm$ 0.1*	1.8 $\pm$ 1.7
Cimetidine	7.3 $\pm$ 2.3	10.6 $\pm$ 2.1	3.6 $\pm$ 0.4	2.9 $\pm$ 0.8	Compound A	400	10.7 $\pm$ 3.0	7.3 $\pm$ 3.9	3.9 $\pm$ 0.5	2.1 $\pm$ 0.9
PAH	8.8 $\pm$ 1.8	7.9 $\pm$ 2.0	3.7 $\pm$ 0.8	2.1 $\pm$ 0.2	Compound A	400	7.4 $\pm$ 1.0	6.3 $\pm$ 1.0	3.6 $\pm$ 0.5	1.7 $\pm$ 0.1

Results are the mean $\pm$ SD, $n = 4-5$.

*Significantly different ($p < 0.05$) from values obtained without inhibitors.

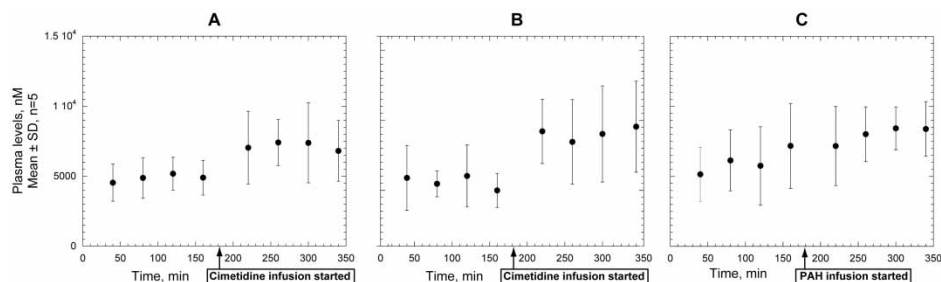


Figure 3. Plasma concentration–time profiles of compound A in rats following $40 \mu\text{g min}^{-1}$ i.v. infusion of compound A for 6 h, with a 3-h i.v. infusion of $120 \mu\text{g min}^{-1}$ cimetidine (A), $700 \mu\text{g min}^{-1}$ cimetidine (B), or $180 \mu\text{g min}^{-1}$ PAH (C). The arrow indicates the time the infusion of cimetidine or PAH was started. Results are the mean $\pm$ SD, $n = 5$.

CL_p and CL_r of compound A by about 40 and 75%, respectively (table 2). However, at $120 \mu\text{g min}^{-1}$ cimetidine, only a slight decrease in CL_r of compound A was observed (table 2). For both doses of cimetidine, GFR was not significantly affected. Consequently, CL_r of compound A corrected for GFR was significantly decreased only after co-infusion with cimetidine at $700 \mu\text{g min}^{-1}$ (table 2). Plasma levels of compound A following co-infusion of compound A with cimetidine showed slight increases (< 2 -fold), when compared with those obtained after 3 h of administration of compound A alone (figure 3A, B). At the 120 and $700 \mu\text{g min}^{-1}$ doses, steady-state plasma concentrations of cimetidine were approximately 50 and $400 \mu\text{M}$, respectively.

With PAH co-infusion ($180 \mu\text{g min}^{-1}$), the CL_p , CL_r and CL_r/GFR of compound A were slightly decreased by PAH, but only the decrease in CL_r/GFR was statistically significant (table 2). Plasma levels of compound A were minimally affected by PAH co-infusion (figure 3C). Quinidine at the dose used in this study ($280 \mu\text{g min}^{-1}$) appeared to elicit pharmacological activity in rats; marked decreases in GFR was observed (table 1). Pharmacokinetic parameters of compound A, CL_p and CL_r also were markedly decreased during co-infusion with quinidine. The degree of reduction in CL_r was comparable with that for GFR, and as a result, CL_r/GFR was unaffected (table 2). At the inhibitor doses studied, plasma concentrations of PAH and quinidine were 160 and $50 \mu\text{M}$, respectively.

Effects of compound A on renal elimination of cimetidine. Effects of compound A on renal transport of cimetidine were also examined by co-infusion of compound A with cimetidine following the infusion of cimetidine alone for 3 h. During the entire experimental period, the GFRs were relatively constant (table 2). In rats, cimetidine was eliminated primarily by renal elimination; values for CL_r and CL_p of cimetidine were comparable with each other (table 2). As expected based on literature data (Boom *et al.* 1994), the renal elimination of cimetidine, which is a low protein binding compound (Ji *et al.* 2002), clearly involved renal tubular secretion in this species, as evident by the CL_r/GFR ratio of > 2 (table 2). Compound A did not affect CL_r and CL_p of cimetidine and GFR. At the dose studied, steady-state plasma concentrations of compound A were about $100 \mu\text{M}$ over the 3-h infusion,

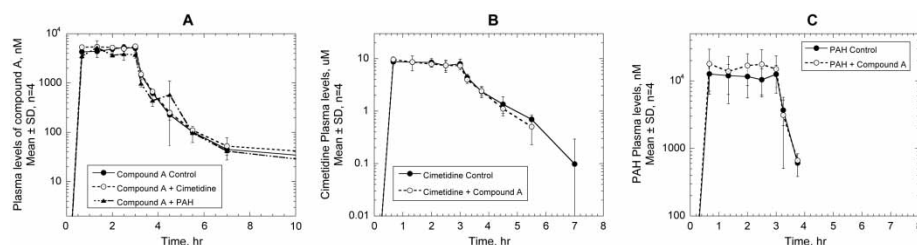


Figure 4. Plasma concentration–time profiles of compound A following a 3-h concomitant i.v. infusion of $85 \mu\text{g min}^{-1}$ compound A with $2250 \mu\text{g min}^{-1}$ cimetidine or $1500 \mu\text{g min}^{-1}$ PAH (A), of cimetidine following a 3-h concomitant i.v. infusion of $150 \mu\text{g min}^{-1}$ cimetidine with $850 \mu\text{g min}^{-1}$ compound A for 3 h (B), and of PAH following a 3-h concomitant i.v. infusion of $100 \mu\text{g min}^{-1}$ PAH with $850 \mu\text{g min}^{-1}$ compound A (C) in rhesus monkeys. Results are the mean $\pm$ SD, $n = 4$.

Table 3. Pharmacokinetic parameters of compound A in rhesus monkeys.

Substrate	Inhibitor	CL_p ($\text{ml min}^{-1} \text{kg}^{-1}$)	CL_r ($\text{ml min}^{-1} \text{kg}^{-1}$)	GFR ($\text{ml min}^{-1} \text{kg}^{-1}$)	CL_r/GFR	$\text{CL}_{r,u}^*/\text{GFR}$
Compound A	none	7.2 ± 1.0	6.2 ± 2.0	3.3 ± 0.6	1.9 ± 0.6	33 ± 12
	cimetidine	6.4 ± 0.6	6.6 ± 1.8	3.0 ± 0.5	2.2 ± 0.4	
	PAH	8.7 ± 1.4	7.4 ± 2.5	2.9 ± 0.3	2.5 ± 0.7	
Cimetidine	none	13.5 ± 1.9	12.1 ± 1.9	3.1 ± 0.6	4.1 ± 1.0	
	compound A	13.5 ± 2.0	13.3 ± 1.8	3.2 ± 0.2	4.2 ± 0.4	
PAH	none	9.5 ± 3.9	11.6 ± 2.8	3.2 ± 0.3	3.6 ± 0.7	
	compound A	8.1 ± 5.0	10.0 ± 5.5	3.3 ± 0.7	3.0 ± 1.6	

Values are the mean $\pm$ SD, $n = 4$.

*Calculation was made based on $f_u = 0.06$ obtained previously (Prueksaritanont *et al.* 2004).

Effects of compound A on renal elimination of PAH. In this experiment, GFRs obtained during the infusion with PAH alone or co-infusion of PAH with compound A also were relatively constant and comparable with each other (table 2). Under the present protocol, the CL_r/GFR for PAH, a low protein binding compound (Reyes *et al.* 1998, Ji *et al.* 2002), in rats was about 2 (table 2), suggesting net tubular secretion of PAH. Co-infusion with compound A did not affect the net renal secretion of PAH, as measured by CL_r/GFR . CL_p of PAH also was not affected by compound A (table 2).

Monkey studies.

Effects of cimetidine and PAH on renal elimination of compound A. Under the study protocol, plasma levels of compound A reached steady-state of approximately $4\text{--}5 \mu\text{M}$ at about 1 h after dosing (figure 4A). As was observed previously (Prueksaritanont *et al.* 2004), compound A is a moderately cleared compound in monkeys, with CL_p of $7 \text{ ml min}^{-1} \text{kg}^{-1}$, and CL_r constituted about 90% of CL_p (table 3). In control animals (without co-infusion with cimetidine or PAH), the CL_r/GFR s of compound A were about 2. Similar to rats, the results suggested net tubular secretion of compound A, with $\text{CL}_{r,u}/\text{GFR}$ s of > 30 (table 3), based on the unbound fraction of 0.06 reported earlier (Prueksaritanont *et al.* 2004). Unlike in rats, neither cimetidine nor PAH had an effect on CL_p , CL_r or plasma levels of compound A in monkeys (table 3 and figure 4A). GFRs also were not affected by either compound. At the inhibitor doses studied, steady-state plasma levels of cimetidine and PAH were about 80 and $120 \mu\text{M}$, respectively.

Table 4. Unbound fractions of compound A, cimetidine or PAH in plasma from rats and monkeys in the presence of inhibitors.

	Plasma source	Substrate	Without inhibitor unbound fraction	With inhibitor	
				inhibitor	unbound fraction
Rat	<i>In vitro</i>	compound A	0.26 ± 0.01 ^a 0.27 ± 0.02 ^b		
Rat	<i>ex vivo</i> ^c	compound A	0.20	cimetidine	0.27
			0.18	PAH	0.28
			0.22	quinidine	0.24
		PAH	0.92	compound A	0.97
		cimetidine	0.82	compound A	0.81
Monkey	<i>ex vivo</i> ^d	compound A	0.06 ± 0.01	cimetidine	0.07 ± 0.01
				PAH	0.07 ± 0.00
		PAH	0.27 ± 0.02	compound A	0.28 ± 0.02
		cimetidine	0.72 ± 0.10	compound A	0.73 ± 0.07

^{a,b}Values are the mean ± SD of triplicate determination obtained at 5 and 150 µM compound A, respectively.

^cPlasma samples used for plasma protein binding determination (in duplicate) were pooled from five different rats receiving the same treatment.

^dPlasma samples from individual monkeys ($n=4$ /treatment) were used for plasma protein binding determination (in duplicate). Values are mean ± SD, $n=4$.

Effects of compound A on renal elimination of cimetidine and PAH. In control animals, both cimetidine and PAH were eliminated primarily by renal excretion; for both compounds, CL_r values approximated their respective CL_p values (table 3). As was observed in rats, their renal elimination involved net tubular secretion, as evident by CL_r/GFR ratio of > 2 (table 3) before corrected for free fraction (table 4; also see below). Effects of compound A on renal excretion of cimetidine and PAH were studied following a constant infusion rate of 850 µg min⁻¹ compound A. Under this dosing protocol, the steady-state plasma concentration of compound A was about 40 µM, and there were minimal changes in CL_r , CL_p or plasma levels of either cimetidine or PAH (table 3 and figure 4B, C). Values for GFR also were comparable in animals with or without co-infusion with compound A (table 3).

Plasma protein binding studies

In the present study, *in vitro* rat plasma protein binding of compound A showed concentration-independency over a wide range of plasma concentrations (5–150 µM) (table 4); the unbound fraction was 26–27%, similar to that observed previously (Prueksaritanont *et al.* 2004). Plasma protein binding of compound A determined *ex vivo* from plasma of rats or monkeys dosed with compound A alone ranged from 20–28% in rats and 6–7% in monkeys (table 4), also similar to the respective value observed *in vitro* (Prueksaritanont *et al.* 2004). In both species, co-administration of compound A with cimetidine or PAH had no effect on plasma protein binding of compound A (table 4).

In control plasma (without compound A), plasma protein binding of cimetidine was relatively low (about 70–80% unbound) and similar in both rats and monkeys (table 4). In contrast, plasma protein binding of PAH exhibited significant species differences (about 90% unbound in rats versus about 30% unbound in monkeys). The relatively low protein binding of cimetidine and PAH observed in rats in this

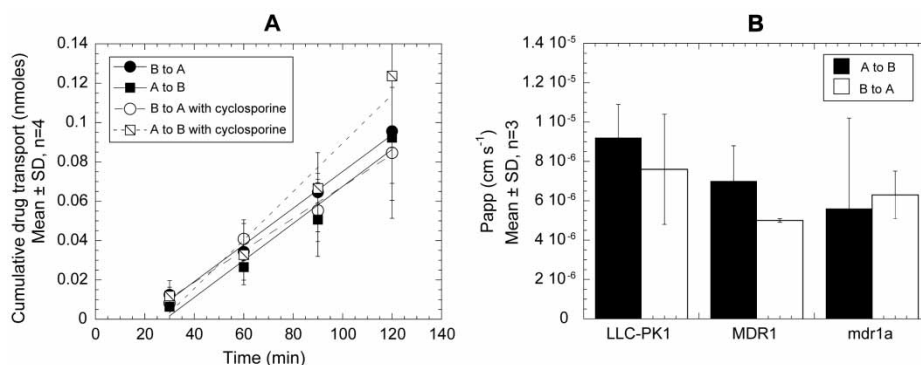


Figure 5. Transcellular transport of compound A across monolayers of Caco-2 in the absence or presence of cyclosporine (A), and apparent permeability of compound A across monolayers of LLC-PK1 and LLC-PK1 transfected with human *MDR1* (LLC-PK1:MDR1) and mouse *mdr1a* (LLC-PK1:mdr1a) (B). Results are the mean $\pm$ SD of triplicate determinations.

study was consistent with literature data (Reyes *et al.* 1998, Ji *et al.* 2002). Plasma protein binding of both PAH and cimetidine in rhesus monkeys has not been reported previously. During treatment with compound A, plasma protein binding of either cimetidine or PAH in both species was not altered relative to control values (table 4).

In vitro transport/uptake studies

Directional transport studies of compound A in Caco-2 cells showed no significant difference in the rate of transport in the apical-to-basolateral (A to B) direction versus the basolateral-to-apical (B to A) direction, in the presence or absence of cyclosporine A, a P-gp inhibitor (figure 5A). For comparison, the positive control VBL, a known P-gp substrate, was run in parallel and showed 20-fold higher transport in the B–A direction than in the A–B direction (data not shown). Under the same conditions, cyclosporine A increased more than 7-fold the A–B transport of vinblastine, and decreased the B–A transport by a factor of 2.

In the control LLC-PK1 cells (without the human *MDR1* or mouse *mdr1a* gene), the transport of compound A also did not show directional transport (figure 5B), the apparent permeability (*Papp*) values for the apical-to-basolateral and basolateral-to-apical directions were 9×10^{-6} and $8 \times 10^{-6} \text{ cm s}^{-1}$, respectively. Similar results also were observed in human *MDR1* (*Papp* = 7×10^{-6} and $5 \times 10^{-6} \text{ cm s}^{-1}$ for the apical-to-basolateral and basolateral-to-apical directions) or mouse *mdr1a* expressing cell lines (*Papp* = $6 \times 10^{-6} \text{ cm s}^{-1}$ for both the apical-to-basolateral and basolateral-to-apical directions). These results support the finding from the Caco-2 cells that compound A is not a P-gp substrate.

Studies with rOAT1 and rOAT3

Initial characterization of PAH and compound A uptake in HEK cells transiently transfected with control plasmid or pEF6 containing rOAT1 and rOAT3 indicated that uptake is complete in 10–20 min. Consequently, all further uptake studies were performed for 20 minutes. A comparison of the uptake of Compound A, PAH, benzyl penicillin, and cimetidine is shown in table 5. Both rOAT1 and rOAT3 transfected cells showed significant PAH uptake, consistent with previous published results (Nagata *et al.* 2002). Also in agreement with

Table 5. Uptake of compound A, PAH, cimetidine and benzyl penicillin by rOAT1 and rOAT3 transfected HEK cell lines.

Substrate	Concentration (μM)	Uptake (%)		
		OAT1	OAT3	Control
Compound A	15	0.13 ± 0.04	$0.33 \pm 0.02^*$	0.13 ± 0.02
PAH	5	$0.71 \pm 0.39^*$	$0.23 \pm 0.14^*$	0.08 ± 0.03
	50	$0.49 \pm 0.14^*$	$0.31 \pm 0.15^*$	0.09 ± 0.04
Cimetidine	5	0.14 ± 0.02	$0.61 \pm 0.30^*$	0.11 ± 0.07
	50	0.11 ± 0.03	$0.34 \pm 0.17^*$	0.11 ± 0.07
Benzyl penicillin	2	0.14 ± 0.05	$1.02 \pm 0.62^*$	0.12 ± 0.05

Data are the mean $\pm$ SD of triplicate determinations.

*Statistically significant difference ($p < 0.05$) from control.

published findings, PAH was a better substrate for rOAT1 than rOAT3; at $5 \mu\text{M}$ concentration, the uptake was about 11- and 3-fold higher in cell transfected with rOAT1 and rOAT3 plasmids, respectively, than in control plasmids (table 5). Unlike PAH, uptake of benzyl penicillin and cimetidine showed preferential uptake in the rOAT3 transfected cells with little or modest uptake in the rOAT1 transfected cells (table 5). These results are consistent with previous reports on differences in substrate specificity for rOAT1 and rOAT3 (Jariyawat *et al.* 1999, Kusuhara *et al.* 1999, Nagata *et al.* 2002). At $15 \mu\text{M}$, Compound A also showed preferential uptake by rOAT3 (about 2.5-fold higher than by control plasmids) with little uptake observed in OAT1 transfected cells (table 5). Figure 6 shows uptake of compound A by rOAT3-transfected HEK cells in the presence and absence of $100 \mu\text{M}$ probenecid. In the presence of probenecid, uptake of compound A exhibited a linear plot whereas in the absence of probenecid, uptake was apparently non-linear, suggesting saturation of the probenecid-sensitive (i.e. rOAT3-mediated) uptake. Indeed, the plot of probenecid-sensitive uptake showed Michaelis–Menten type kinetics and the K_m and $V_{\max}$ were calculated as $15.2 \pm 3.5 \mu\text{M}$ and $274.4 \pm 15.5 \text{ pmol}/20 \text{ min}/\text{well}$, respectively.

Discussion

The present study used a combination of *in vivo* and *in vitro* approaches to characterize the renal elimination of compound A, a hydrophilic zwitterion, and to examine its potential for drug interactions at the level of renal transport. Rats and rhesus monkeys were used as *in vivo* animal models since renal elimination of compound A has been suggested earlier to involve tubular secretion in these species (Prueksaritanont *et al.* 2004), similar to the recent finding in humans (unpublished data). In the current study, the CL_r of compound A accounted for about 25–30% of its CL_p in rats (table 1) and $> 90\%$ of its CL_p in monkeys (table 3), agreeing with previous observations (Prueksaritanont *et al.* 2004). The $\text{CL}_{r,u}/\text{GFR}$ ratios ranged from 2 to 5 in rats and > 30 in monkeys (tables 1 and 3), suggesting that net tubular secretion of compound A comprises a significant portion of the renal clearance in both species. Moreover, the finding in rats that $\text{CL}_{r,u}/\text{GFR}$ s decreased from 5 to 2 with increasing doses is consistent with saturation of tubular secretion of compound A, a key feature of an active transport process.

To elucidate better the role of active transport in renal elimination of compound A, studies were performed to evaluate interactions of compound A

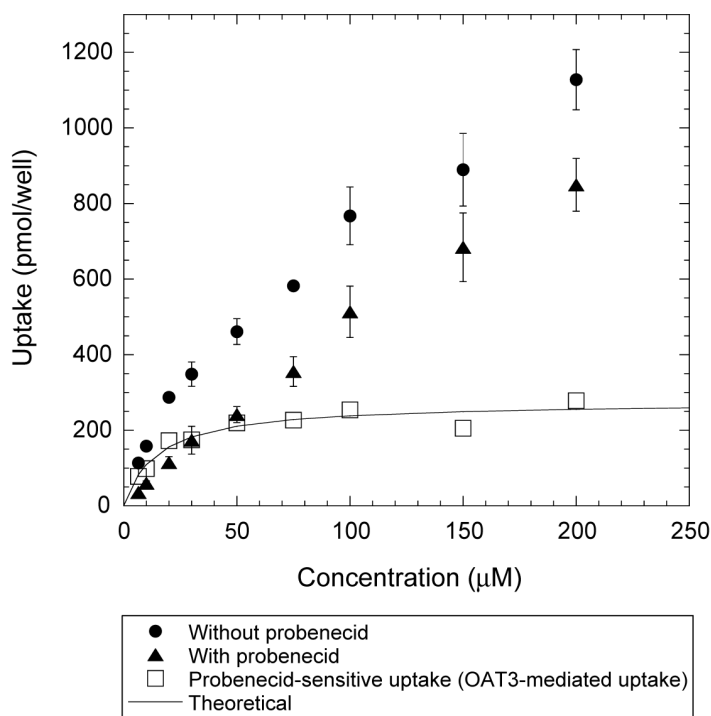


Figure 6. Concentration dependency in the uptake of compound A by rOAT3-transfected HEK cells. Uptake of compound A was determined in the presence (closed triangles) and absence (closed circles) of 100 μ M probenecid. Each point and vertical bar represent the mean and SD from the quadruplicate determinations. Probenecid-sensitive rOAT3-mediated uptake (open squares) was obtained by subtracting the uptake with probenecid from that without probenecid. Solid line represents the calculated values using the kinetic parameters obtained by non-linear least-squares analysis.

with two prototypical substrates for active renal elimination, PAH and cimetidine. In rats, PAH, a model organic anion, inhibited the renal tubular secretion of compound A. This conclusion was made based on the findings that PAH significantly reduced the CL_r/GFR ratio, but had a minimal effect on plasma protein binding of compound A in this species (tables 2 and 4). Considering that PAH is a very good substrate for rat/human organic anion transporter protein OAT1 as well as a substrate for OAT2 and OAT3 *in vitro* (Burckhardt and Burckhardt 2003), the results suggest that compound A may be a substrate for an OAT. Compound A, at about 100 μ M, did not inhibit the renal secretion of PAH, suggesting weak, if any, interactions between compound A and rOAT1, the major renal transporter for PAH. A possibility remains, however, that compound A might be a substrate for a transporter of PAH, provided that this transporter does not contribute significantly to overall renal elimination of PAH in rats. In this regard, PAH is a much poorer substrate for OAT2 and OAT3 than for OAT1.

Similar interaction results were also obtained in rats with the cation, cimetidine, and compound A. Cimetidine, at 400 μ M, did not affect plasma protein binding, while significantly decreased the CL_r/GFR ratio of compound A. Considering that cimetidine inhibited the tubular secretion of compound A only at relatively high plasma concentration (400 μ M), but not 50 μ M, and that the IC_{50} of about 10 μ M has been reported for the inhibition of rat OCT1 and OCT2

transporters by cimetidine (Urakami *et al.* 1998), the results suggest that compound A likely is not a good substrate for rat OCT1 or OCT2. This suggestion is also supported by the *in vivo* finding that quinidine, a known inhibitor of rat OCT1 and OCT2 and P-gp (Arndt *et al.* 2001, Pritchard and Miller 1993), had a minimal effect on renal excretion of compound A. Additionally, compound A, at concentrations as high as 100 μ M, had a minimal effect on the renal elimination of cimetidine, suggesting that compound A did not affect OCT1 and OCT2, the primary renal transporters of cimetidine. Considering that cimetidine has also been characterized as a substrate as well as an inhibitor of rat and human OAT1 and OAT3 *in vitro* (Cha *et al.* 2001, Deguchi *et al.* 2002, Nagata *et al.* 2002, Burckhardt and Burckhardt 2003), the possibility exists that the interaction observed between cimetidine and compound A was via an OAT in this species, in line with the above proposal based on the interaction study with PAH.

Subsequently, the possibility that the above interactions are due to transport of compound A by an OAT uptake systems, but not P-gp efflux, was investigated using *in vitro* systems; rOAT1 and rOAT3 transfected HEK and MDR1 and mdr1a transfected LLC-PK as well as Caco-2 cell lines. The results showed that compound A is a substrate for rat OAT3, further substantiating the aforementioned suggestion derived based on *in vivo* findings. Our finding that compound A is not a substrate for rat OAT1, also is consistent with the *in vivo* results showing no inhibition of PAH elimination at relatively high concentrations of compound A. Additionally, the present finding that compound A, a hydrophilic zwitterions ($\log P=0.2$), is not a P-gp substrate also agrees well with the *in vivo* interaction results with quinidine, a known P-gp inhibitor, and the view that lipophilicity is a common feature for known P-gp substrates, including cationic (Ford and Hait 1990) and zwitterionic compounds (Yamaguchi *et al.* 2000, Prueksaritanont *et al.* 2002).

Given the potential for drug interactions suggested by the results at high drug concentrations in rats, further studies were performed in rhesus monkeys at clinically relevant drug concentrations. The rhesus monkey may serve as a better animal model than the rat for humans, considering that the extent of renal elimination of compound A in the monkey is similar to that in humans and is much more extensive (about 90% of CL_p) than observed in the rat. In this rhesus monkey study, the concentrations of cimetidine and compound A used were about 2–5-fold higher than those observed following their respective therapeutic doses. Under these conditions, our results showed that compound A did not have significant effect on the renal secretion of PAH or cimetidine in this species, similar to the observations in rats. However, cimetidine and PAH did not affect tubular secretion of compound A, in contrast to the results in the rat studies. Considering that the plasma concentrations of cimetidine, PAH or compound A in the present monkey study were about 2–3-fold lower than those studied in rats, it remains possible that the observed differences between the rat and monkey studies could reflect species differences in affinities of the responsible renal transporters, differences in the plasma protein binding (in the case of PAH), as well as the lower drug levels in the rhesus study. Although there has been very limited information available in the literature regarding renal transporters in rhesus monkeys, and species differences in the transporters between rats and monkeys, kinetic differences between different species, including humans, have been reported earlier for organic anion and cation transporters (Khamdang *et al.* 2004, Dresser *et al.* 2000).

To summarize, the present investigation on possible *in vivo* interactions between compound A, a zwitterionic $\alpha_v\beta_3$ integrin antagonist, and the prototypical anion PAH, or the cation cimetidine, revealed modest but significant effects of PAH and cimetidine on the renal tubular secretion of compound A in rats. Compound A is not a substrate for P-gp or OAT1. These modest interactions are mediated, in part, to the interaction with OAT3 transporter since compound A, PAH and cimetidine are all substrates for OAT3. Our results also showed that compound A, at pharmacologically relevant concentrations, had minimal effects on the renal elimination of PAH and cimetidine in both rats and monkeys. Although there is a possibility that compound A might also be a substrate for human OAT3, studies in rhesus monkeys at clinically relevant doses suggest that the pharmacokinetic interactions between compound A and substrates or inhibitors of OAT3, at the renal excretion level, are likely to be modest.

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Characterization and quantification of metabolites of racemic ketoprofen excreted in urine following oral administration to man by ^1H -NMR spectroscopy, directly coupled HPLC-MS and HPLC-NMR, and circular dichroism

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1. The identity of the human metabolites of ketoprofen (2-(3-benzoylphenyl)-propanoic acid) excreted via urine was investigated after a single oral dose of the racemic drug.
2. Drug metabolites were concentrated and partially purified from urine using solid-phase extraction chromatography before separation and identification by directly coupled HPLC-MS and HPLC-NMR.
3. The metabolites identified were the ester glucuronides of the parent drug and its phase I metabolites, 2-[3-(3-hydroxybenzoyl)phenyl]-propanoic acid, 2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid and 2-[3-(hydroxy(phenyl)methyl)phenyl]-propanoic acid, the latter formed by reduction of the ketone group of ketoprofen. In addition, two novel minor metabolites were identified as the ether glucuronides of 2-[3-(3-hydroxybenzoyl)phenyl]-propanoic acid and 2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid. These conjugates were all observed as diastereoisomeric pairs of unequal proportions.
4. Purification of these metabolites by preparative chromatography allowed stereochemistry assignments.
5. Metabolites were quantified by ^1H -NMR spectroscopy after spectral simplification achieved by hydrolysis of the conjugates.

Introduction

Ketoprofen (2-(3-benzoylphenyl)-propanoic acid) is a member of the 2-aryl propionic acid class (generically known as profens) of non-steroidal anti-inflammatory drugs (NSAIDs) with analgesic, anti-inflammatory and antipyretic activities (Julou *et al.* 1976). With the exception of naproxen, profens are used clinically as racemic mixtures, although the individual enantiomers may exhibit different pharmacological, pharmacokinetic and toxicological properties (Foster *et al.* 1989). For the profen class of NSAIDs, pharmacological activity is attributed almost exclusively to the *S*-enantiomer, whereas the *R*-isomer is less active or inactive (Muller *et al.* 1990). Additionally, *in vivo*, the *R*-enantiomer of a profen drug can

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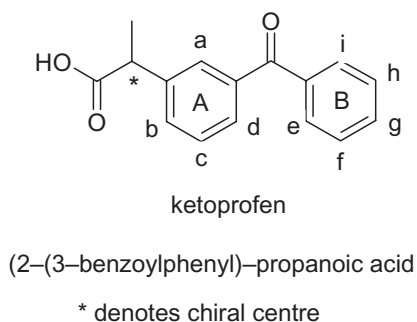


Figure 1. Structure of ketoprofen and the ring-numbering system used.

undergo chiral inversion. This process is usually unidirectional, with some exceptions (Jamali *et al.* 1997), and is both compound and species specific. In humans, ketoprofen exhibits little stereoselectivity in its pharmacokinetics (Jamali and Brocks 1990, Rudy *et al.* 1998) and has been reported to undergo only limited (9–12%) unidirectional chiral inversion (*R*- to *S*-) (Jamali *et al.* 1990, Rudy *et al.* 1998). The chemical structure of ketoprofen and the ring-numbering system used is shown in figure 1.

Ketoprofen undergoes extensive conjugation with D-glucuronic acid and is excreted mainly in urine with a reported renal elimination half-life of 1–3 h (Jamali *et al.* 1990). The principal excretion product is the acyl glucuronide conjugate of the parent drug as a pair of diastereoisomers (Upton *et al.* 1980, Rudy *et al.* 1998). In humans, other metabolites are reported as minor, whereas excretion of unchanged drug is negligible (Upton *et al.* 1980). Metabolites of ketoprofen formed by hydroxylation of the aromatic ring to 2-[3-(3-hydroxybenzoyl)phenyl]-propanoic acid and 2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid, and by reduction of the ketone group to 2-[3-(hydroxy(phenyl)methyl)phenyl]-propanoic acid have been identified in camels and horses (Kurosawa *et al.* 1994, Ryan *et al.* 1994, Alkatheeri *et al.* 1999a, b). These metabolites were identified by gas chromatography-mass spectrometry and ^1H -nuclear magnetic resonance (NMR) spectroscopy, after enzymatic hydrolysis of their glucuronide conjugates. Reports of aromatic hydroxylation of ketoprofen in other animal species and in man have been made, but these failed to determine the position of the -OH group (Populaire *et al.* 1973). Although formation of glucuronide conjugates has been recognized for these metabolites by indirect measurements such as β -glucuronidase hydrolysis (Kurosawa *et al.* 1994), direct evidence of the site of conjugation has not been presented and quantification of the reported metabolites in various species is incomplete (Populaire *et al.* 1973).

Since the successful development of high-performance liquid chromatography-mass spectrometry (HPLC-MS) and high-performance liquid chromatography-NMR, numerous applications have appeared in the literature demonstrating the efficiency in obtaining unambiguous and rapid structural information (Lindon *et al.* 1997, Shockcor *et al.* 2000, Sidelmann *et al.* 2001). In the present study, HPLC-MS and HPLC-NMR, together with a range of techniques such as circular dichroism, have been applied to the investigation of the urinary excretion of ketoprofen metabolites in man after oral administration.

Materials and methods

Chemicals

Analytical-grade methanol and HPLC-grade acetonitrile (ACN) were from BDH Laboratory Supplies (Poole, UK). Ketoprofen tablets (Profénid[®], Rhône-Poulenc Rorer, France) were purchased from a pharmacy. Ammonium formate was obtained from Fisher Scientific (Loughborough, UK) and formic acid from Fisons Scientific (Loughborough, UK). Sodium 3-(trimethylsilyl)-[2,2,3,3-²H₄]-1-propionate (TSP) and *S*-ketoprofen were from Aldrich (Milwaukee, WI, USA). Deuterium oxide (²H₂O) was used for HPLC-NMR and was obtained from Goss Scientific Instruments (UK). HPLC-NMR-grade acetonitrile was purchased from Riedel-de Haën (Seelze, Germany). Methanol-d₄ was from Euriso-Top (Gif-sur-Yvette, France).

Ketoprofen administration and urine collection

Urine from a normal healthy female volunteer (age 25 years, weight approximately 50 kg) was collected over 0–2, 2–4 and 4–6 h after a single oral administration of racemic ketoprofen (200 mg). Samples were acidified to pH 2.5 with 0.01 M HCl and stored at –20°C until further analysis. Control urine was also collected before drug administration.

¹H-NMR spectroscopic analysis of human urine

A 0.8-ml aliquot of each untreated acidified urine sample was placed in a 5-mm NMR tube and 0.08 ml 0.5 mg ml^{–1} TSP in ²H₂O was added to provide the lock signal and chemical shift reference (δ). ¹H-NMR spectra were acquired at 300 K on a Bruker AVANCE600 spectrometer operating at 600.13 MHz. A total of 128 free induction decays (FIDs) were collected into 32 768 computer data points using a spectral width of 8389.3 Hz, giving an acquisition time of 1.95 s with an additional relaxation delay of 1.5 s. Suppression of the water resonance was achieved using the 'noesyprsat' pulse sequence (Nicholson *et al.* 1995). Before Fourier transformation (FT), an exponential apodization function corresponding to a line-broadening factor of 0.3 Hz was applied to the FIDs, which were also zero-filled by a factor of two. The data were manually phase corrected.

Solid-phase extraction chromatography (SPEC) of metabolites from human urine

The samples were combined (160 ml) and subjected to SPEC on a 60-ml (10 g) C₁₈ Mega Bond-Elut solid-phase extraction cartridge (Varian Associates, Walton-on-Thames, UK). The cartridge was conditioned before sample application by washing with methanol (40 ml) followed by 0.01 M HCl (40 ml). After sample application, the cartridge was washed with 0.01 M HCl (50 ml). The retained material was eluted with methanol/0.01 M HCl mixtures starting with 20% and followed by 30, 40, 50, 60 and finally 80% methanol (40 ml each mixture), and six fractions corresponding to each mixture were collected. The methanol in the fractions was evaporated using a rotary evaporator at room temperature and the residual water was removed by freeze-drying. The freeze-dried fractions were stored at –20°C until further manipulation. ¹H-NMR spectroscopy of the SPEC extracts, which were reconstituted in ²H₂O before NMR analysis, showed the presence of ketoprofen glucuronide metabolites in the fractions collected after elution with 50 and 60% methanol.

Chromatography

The HPLC system was comprised of a Hewlett Packard Agilent 1100 pump connected to a Lambda 1010 UV detector operating at 254 nm. Chromatographic data were collected using Bruker HyStar NT 2.2 software.

Analytical chromatography

Chromatography for development of the analytical method was carried out on a Genesis BDS, 250 × 4.6 mm i.d., 4 μm C₁₈ column (Jones Chromatography, Hengoed, UK). The final mobile phase developed for the assay consisted of ACN/25 mM ammonium formate. Ammonium formate was adjusted to pH 5.0 with 10% formic acid. ACN was increased from 18 to 27% in 23 min and further to 43% over 1 min using linear gradients. Elution was finally maintained isocratic at 43% for a further 11 min. Separation was achieved at 26°C at a flow rate of 1 ml min^{–1}.

HPLC-MS of SPEC extracts

HPLC-MS studies were carried out using a Bruker Esquire ion-trap mass spectrometer directly coupled to the HPLC system. Mass spectra were obtained, using Esquire Control 4 software, by electrospray ionization in positive-ion mode scanning a range from 50 to 600 Da. The nebulizer pressure was 15 psi, the drying gas flow 5 l min^{–1} and the drying temperature 300°C. Data were analysed by the Bruker Daltonic EsquireLC data system. Reconstructed ion chromatograms were used to search for the ion fragment at *m/z* 255 (characteristic of the parent compound) to identify all parent-related compounds. Ions of *m/z* 271 and 257 corresponding to hydroxy metabolites were also monitored.

In addition, ion chromatograms were reconstructed to search for the sodium and formate adducts of each metabolite, including the parent fragment.

HPLC-NMR spectroscopy of SPEC extracts

The chromatographic system was connected to a Bruker BPSU-36 collector and the chromatography was controlled using the Bruker HyStar NT 2.2 software. The BPSU-36 collector was connected to the HPLC-NMR flow probe via an inert polyether(ether) ketone capillary. The system was operated in the loop storage mode. The eluting peaks corresponding to ketoprofen metabolites previously detected by HPLC-MS were stored in capillary loops for later off-line NMR study. Selection was based on the retention time and the peak intensity, which changes only slightly when switching from H₂O to ²H₂O. The HPLC-¹H-NMR spectra were acquired at 298 K using a Bruker AVANCE600 instrument equipped with a ¹H flow probe (cell of 4 mm i.d. with a volume of 120 µl) operating at 600.22 MHz. Dual suppression of residual water and acetonitrile signals was achieved by the 'noesyprsat' (Nicholson *et al.* 1995) pulse sequence. The spectra were acquired for between 64 and 3000 transients into 32 768 data points over 12 019.2 Hz spectral width, giving an acquisition time of 1.36 s. An additional relaxation delay of 2.4 s was added between pulse cycles to allow T₁ relaxation. The FIDs were multiplied by a line-broadening function of 1 Hz to improve the signal-to-noise ratio. These data were also zero-filled by a factor of 2 before FT. Spectra were referenced internally to the residual acetonitrile signal at δ2.00.

Preparative chromatography

Reversed-phase preparative chromatography was performed on a Spherisorb ODS-2, 250 × 10 mm i.d., 5 µm column (Hichrom Ltd, Reading, UK). The mobile phase consisted of ACN/25 mM ammonium formate (pH 4.8). Elution was initially isocratic with 15% ACN for 16 min. The content of acetonitrile was then raised to 17% for 5 min and held at 17% for 11 min. It was then increased to 37% over 1 min and maintained isocratic at 37% for a further 13 min. The ultraviolet light detector was operated at 270 nm. Separation was achieved at 26°C with a flow rate of 3 ml min⁻¹ and injection volume of 40 µl (20 mg ml⁻¹ solution). The fractions collected were rotary evaporated at room temperature. The aqueous phase was passed through a BondElut C₁₈ cartridge (1 ml, 100 mg), preconditioned with methanol (2 ml) and then with 0.1 M HCl (2 ml). The retained metabolite was washed with 0.1 M HCl (1 ml) and eluted with acetonitrile, which was then evaporated under a stream of nitrogen gas and residual water was removed by freeze drying. Further purification of other metabolites was performed on the analytical column using the conditions described in the analytical chromatography section and a linear gradient from 18 to 24% acetonitrile for 16 min.

¹H-NMR spectroscopic analysis of purified metabolites

Each purified metabolite was reconstituted in ²H₂O (0.5 ml) and was placed in a 5-mm NMR tube with 0.05 ml 0.5 mg ml⁻¹ TSP/²H₂O to provide the lock signal and chemical shift reference (δ0). ¹H-NMR spectra were acquired in the same manner as the spectra for urine. For one metabolite, the traficante apodization function (Traficante and Nemeth 1987) with a line-broadening factor of 0.6 Hz was applied to the FIDs to enhance resolution. The data were manually phase corrected.

Circular dichroism (CD) for stereochemistry assignments

CD spectra were measured on a Jasco J-720 spectropolarimeter (Great Dunmow, UK) using a 1 cm path-length cell. The spectra were recorded at 20°C in the range 330–190 nm with a scanning speed of 10 nm min⁻¹, step resolution 0.1 nm, band width 1 nm, response 4 s and sensitivity 20 mdeg. Solutions of the purified metabolites at concentrations ranging from 40 to 130 µM were prepared in water. The stereochemistry was established by comparison of the CD spectra with that of *S*-ketoprofen (45 µM in ACN).

Quantification of ketoprofen metabolites

Drug administration (200 mg) and urine collection was repeated as above and the volume was recorded to enable estimation of the overall recovery in urine. Urine (15 ml) from each collection time was loaded onto preconditioned C₁₈ cartridges (6 ml, 1 g) and was eluted with 10% ACN followed by 100% ACN (15 ml each), which were then freeze dried. The 100% ACN-eluted fractions were dissolved in NaOH (2 ml, 0.1 M) to promote hydrolysis of the glucuronide conjugates back to their aglycones and were freeze-dried 4 days later. Methanol-d₄ was used as a solvent for NMR analysis of the three fractions. TSP/methanol-d₄ (0.06 ml 2.5 mg ml⁻¹) was added for chemical shift reference. The experimental parameters as detailed above were used for the acquisition of ¹H-NMR spectra, with the addition of 15 s between pulses, during which time the water presaturation power was switched off, to allow full relaxation without the risk of overheating the probe. The spectra were resolution enhanced by application of the traficante apodization function, corresponding to a line-broadening factor of 0.5 Hz. Relative metabolite concentrations were calculated by comparing peak heights. Fully relaxed ¹H-NMR

spectra were also acquired for the three untreated urines (0.8 ml) and peak areas were used for calculation of the total glucuronide concentration. The error for peak height and peak area measurements was estimated from the NMR spectra of three standard samples containing ketoprofen and ibuprofen of different molar ratios, using the methyl proton resonance integrals. The standard samples also contained a known amount of TSP for estimation of the error for absolute concentration measurements. After NMR analysis of the hydrolysed samples, HPLC-MS analysis confirmed complete hydrolysis of both ether and ester glucuronide conjugates. The ether-to-ester ratio was estimated from HPLC chromatograms of unhydrolysed SPEC fractions eluted with 100% ACN, obtained after repeating SPEC as above.

Results and discussion

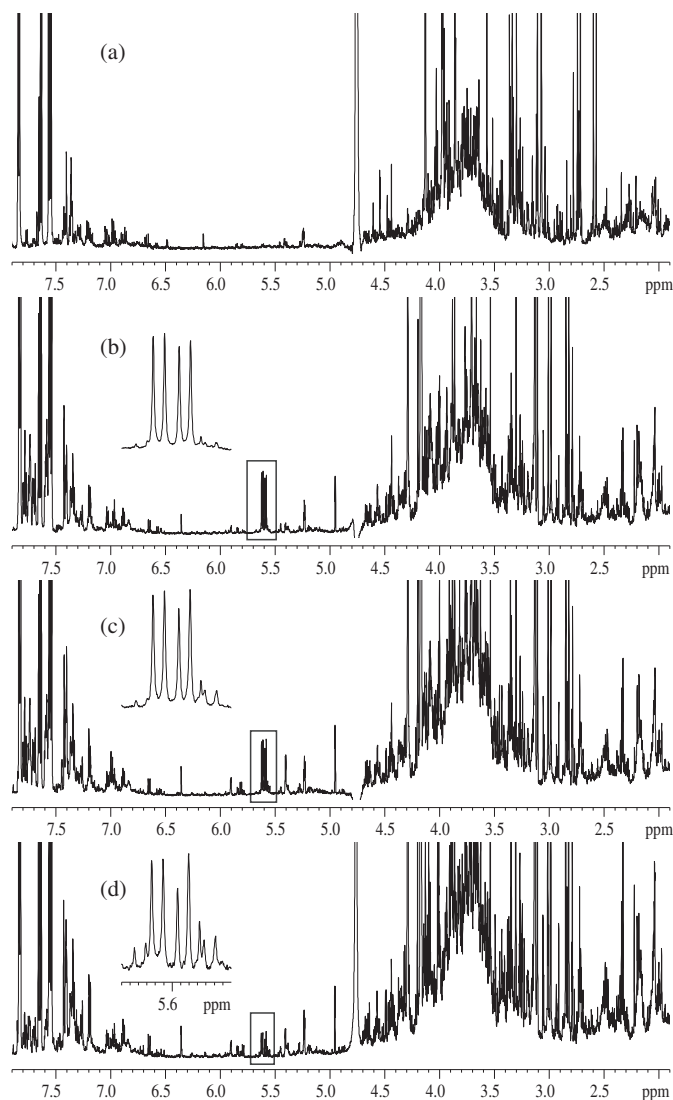
Identification of glucuronide metabolites in urine and SPEC fractions by ^1H -NMR spectroscopy

Inspection of the ^1H -NMR spectra of urine collected after drug administration revealed signals consistent with the presence of acyl glucuronide conjugates. As these resonances were absent from the control urine sample they were presumed to be ketoprofen related (figure 2). Acyl glucuronides are recognized from the appearance of doublets of $J \sim 8\text{ Hz}$ in the region $\delta 5.5\text{--}6.5$ of the ^1H -NMR spectrum, arising from the anomeric proton of the glucuronic acid moiety. The presence of more than one such metabolite was evident from the observation of several doublets at $\delta 5.6$ in the post-dose spectra. SPEC was used to isolate the metabolites from urine where the conjugates were eluted in the 50 and 60% methanolic fractions (the bulk of the interfering endogenous metabolites was eluted in earlier fractions) (Wilson and Nicholson 1987). All fractions were analysed by ^1H -NMR spectroscopy, which showed the presence of several acyl glucuronides in the 50% methanolic eluate fraction, and this was used for the development of the HPLC method. The 60% fraction contained only two glucuronide doublets at $\delta 5.6$. Later HPLC-MS analysis of this sample showed that the two glucuronides were a pair of diastereoisomers of the major ketoprofen metabolite, also present in the 50% methanol-eluted fraction.

Characterization of ketoprofen metabolites by HPLC-MS and HPLC-NMR

The ultraviolet light detected chromatogram of the 50% methanol-eluted SPEC fractions is shown in figure 3(a). As aromatic ring hydroxylation is a common metabolic pathway (such metabolites have been previously reported for ketoprofen in rat, dog, rabbit, camel, horse, man; Populaire *et al.* 1973, Kurosawa *et al.* 1994, Ryan *et al.* 1994, Alkatheeri *et al.* 1999a, b), the masses of these suspected metabolites were monitored in HPLC-MS studies. The resulting reconstructed ion chromatograms are shown in figure 3(b). The metabolites detected were allocated numbers 1–6 and their corresponding structures, as identified by HPLC-NMR, are shown in figure 4. The aglycones were designated with numbers I–IV.

The masses of the peaks numbered 1–4 are identical (m/z 271) and are consistent with addition of a hydroxyl group on the ketoprofen compound, indicating that multiple hydroxylated metabolites are formed (figure 5a–d). The spectra obtained here also suggest the existence of a glucuronide moiety on each of the hydroxylated compounds. The molecular ion peaks (m/z 469, 491) show low intensities. Figure 6(a–d) shows the NMR spectra of the four HPLC peaks, which confirm that the metabolites were indeed glucuronide conjugates. Peaks 1 and 2 were identified to be ether glucuronides based on the ^1H -NMR chemical shifts



2

Figure 2. 600 MHz ^1H -nuclear magnetic resonance spectra of (a) predose urine, (b) 0–2, (c) > 2–4 and (d) > 4–6 h post-dose urine. The appearance of doublets of $J = 8$ Hz in post-dose urine (insets as shown as expansions of regions inside the box) is evidence of the formation of drug β -1-*O*-acyl glucuronides. The majority of nuclear magnetic resonance peaks arise from endogenous metabolites.

(δ 5.1–5.3). The major difference between the spectra of the two metabolites is the appearance of the resonances indicating hydroxylation at different positions on the ring. Peak 1 can be assigned to the ether glucuronide of 2-[3-(4-hydroxybenzoyl)-phenyl]-propanoic acid, whereas peak 2 is that of 2-[3-(3-hydroxybenzoyl)-phenyl]-propanoic acid, based on the chemical shifts and spin-coupling splitting patterns. These two metabolites are reported for the first time. Similarly, peaks 3 and 4 are the ester glucuronides of the same hydroxy aglycones, as evident by the doublet of 8 Hz at the typical chemical shift of δ 5.6 and the similarity of the

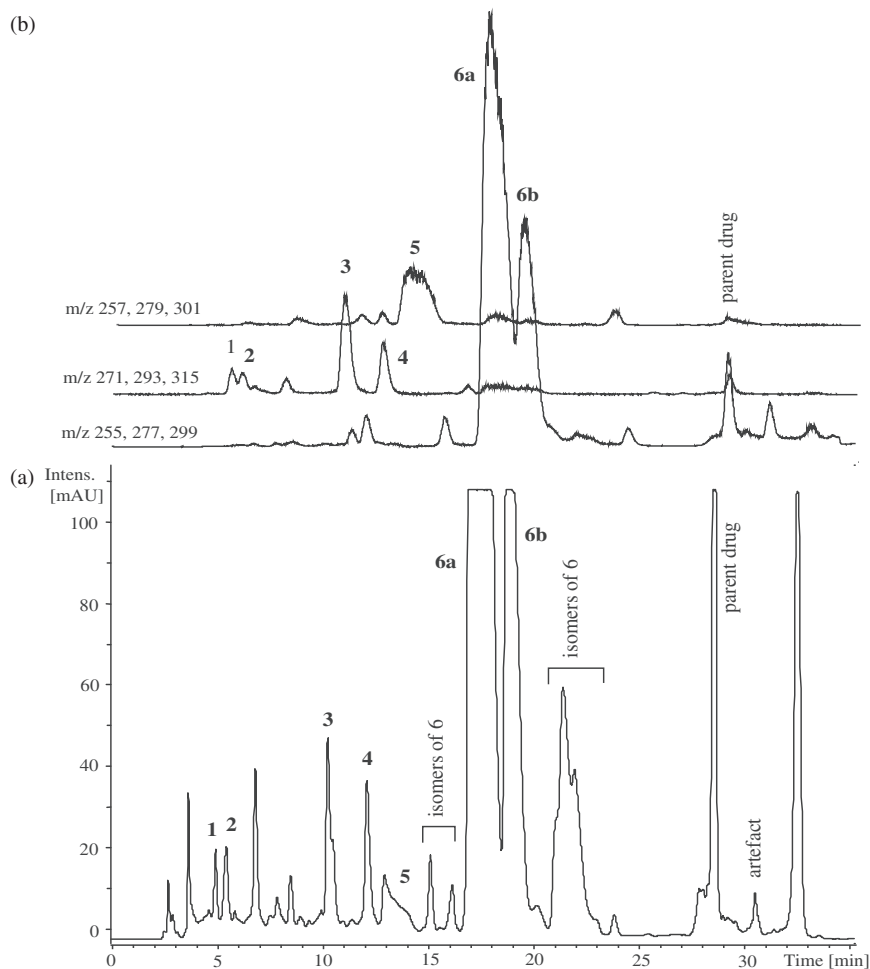


Figure 3. (a) High-performance liquid chromatography-ultraviolet light (254 nm) chromatogram showing metabolites of ketoprofen isolated from urine by solid-phase extraction chromatography after elution with 50% methanol. Separation was achieved on a Genesis BDS, 250 $\times$ 4.6 mm i.d., 4 μ C₁₈ column (Jones Chromatography) with acetonitrile/ammonium formate (pH 5) as the mobile phase, at 1 ml min⁻¹. (b) Reconstructed ion mass chromatogram for m/z 255, 271 and 257 and their sodium and formate adducts.

aromatic resonances with peaks 1 and 2. Peak 3 is hydroxylated at the 4-position of ring B and peak 4 at the 3-position.

Peak 5 matches the mass of a substance formed by reduction of the ketone group of ketoprofen, also containing a glucuronide moiety (figure 5e). The fragment ion at m/z 279 is the sodium adduct of the compound that has lost the glucuronide and the signal at m/z 455 is the molecular ion. Examination of the ¹H-NMR resonances confirms the identity of the compound. The NMR spectrum shows a singlet at δ 5.9 ppm, integrating to one hydrogen. This singlet corresponds to the highly deshielded proton attached to the carbon that links the two aromatic rings. Second, reduction of the ketone group shifts the aromatic protons to lower frequency by about 0.5 ppm. Finally, the doublet seen at δ 5.6 for the anomeric H1' proton is consistent with an ester glucuronide (figure 6e).

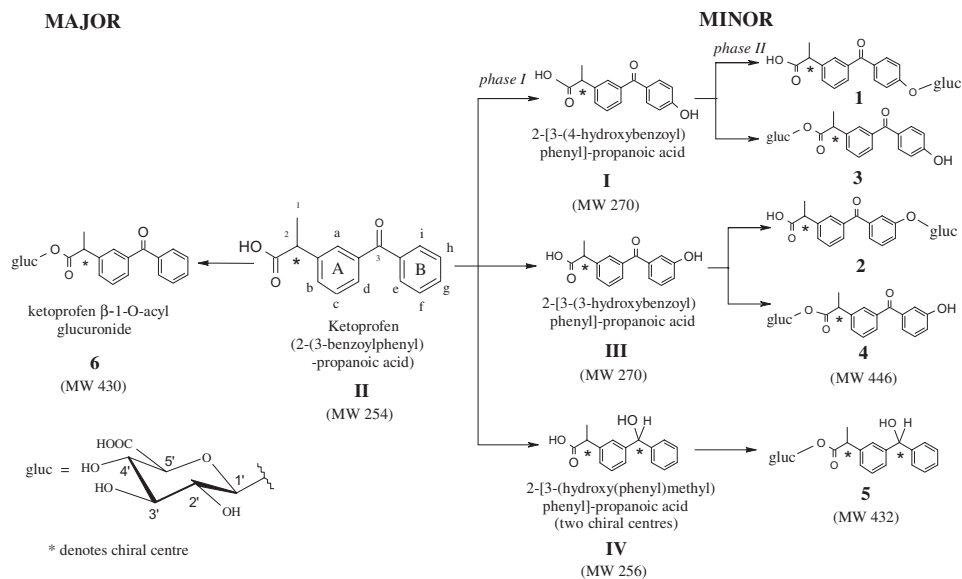


Figure 4. Urinary metabolic pathway of ketoprofen in man.

The mass and ^1H -NMR spectra of peaks 6a and 6b correspond to ester glucuronide metabolites formed by direct conjugation with the parent drug. The mass spectra of the two peaks, shown in figure 5(f), were identical. Neutral loss of the glucuronide moiety (176 Da) gave rise to a signal at m/z 255 (parent fragment), whereas the molecular ion at m/z 453 and 475 is consistent with the sodium and formate adduct of the metabolite. The two peaks seen in the ultraviolet light chromatogram correspond to a pair of diastereoisomers, which were well resolved under the chromatographic conditions employed. The faster eluting peak (retention time, t_R = 18 min) was shown by circular dichroism to be the conjugate of *S*-ketoprofen and the peak at 19.5 min that of *R*-ketoprofen. The ^1H -NMR spectra of the two diastereoisomers, which show minor differences in their chemical shifts, are given in figures 6(f) and (g). The chemical shifts of the metabolites are collected together in tables 1 and 2, which show the assignments for the aglycone and the glucuronic ring protons, respectively.

Although only the diastereoisomers of the major metabolite (peaks 6a, b) were resolved under the analytical chromatography conditions used, all metabolites formed diastereoisomeric pairs, by conjugation of D-glucuronic acid with both *S*- and *R*-isomers. This was observed by ^1H -NMR spectroscopy after purification of the metabolites by preparative HPLC. Purification also permitted stereochemistry assignments by CD. For metabolite 5, creation of a second chiral centre at the reduction site implies that formation of four diastereoisomers is expected. The number of diastereoisomers actually formed was determined by ^1H -NMR spectroscopy of the purified metabolite. Resolution enhancement with the application of the traficante apodization function (Traficante and Nemeth 1987) was necessary before clear observation of four doublets at δ 5.6. The corresponding peak in the ultraviolet light chromatogram (peak 5) appears broad, as it actually consists of four different stereoisomers of the metabolite. Interestingly, the diastereoisomeric pairs were observed in unequal proportions. The metabolites studied by CD

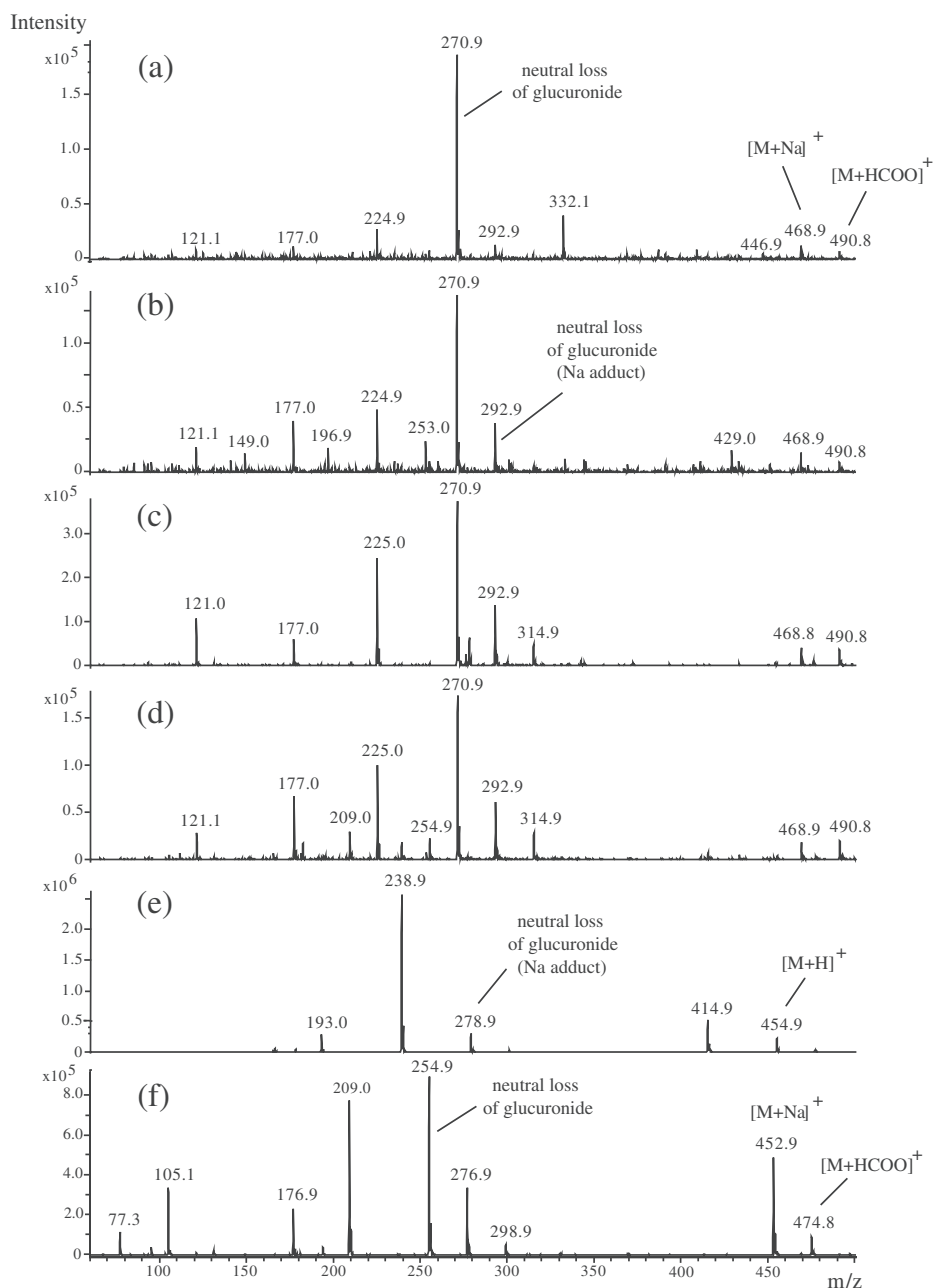


Figure 5. Positive-ion electrospray mass spectra of metabolites 1–6 (a–f, respectively) obtained by high-performance liquid chromatography-mass spectrometry.

revealed the *S*-form to be the one in excess for the ester glucuronides and the *R*- for the ethers. This observation could be an indication of some stereoselectivity in the metabolism or excretion of ketoprofen. Another explanation is that partial degradation of the glucuronides could have occurred in the period between urine collection and analysis; a combination of both mechanisms is also possible. Finally,

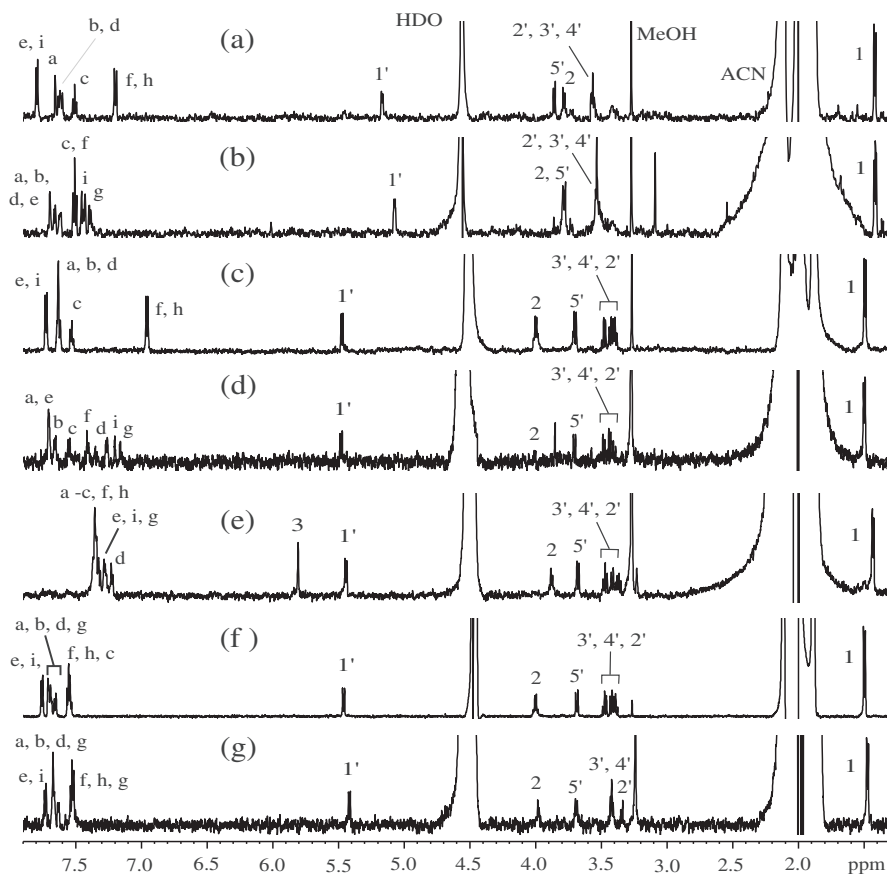


Figure 6. 600 MHz high-performance liquid chromatography- ^1H -nuclear magnetic resonance spectra of the metabolites of ketoprofen: (a–e) metabolites 1–5, respectively, (f) *S*-ketoprofen ester glucuronide and (g) *R*-ketoprofen ester glucuronide. The spectra were obtained using the loop storage mode.

chiral inversion is a possibility and previous reports suggested that there is little or no stereoselectivity for ketoprofen (Jamali and Brocks 1990, Rudy *et al.* 1998) or limited chiral inversion (Jamali *et al.* 1990, Rudy *et al.* 1998) and faster degradation of the major *R*-ketoprofen glucuronide in comparison with the *S*-form (Akira *et al.* 1998). A slight excess of the *S*-ketoprofen ester conjugate has been previously reported and was attributed to chiral inversion (Jamali *et al.* 1990).

The cluster of ultraviolet light peaks eluting after the major metabolite ($t_{\text{R}}=21.5\text{--}23.0\text{ min}$) and the two peaks at $t_{\text{R}}=15$ and 16 min (figure 3a) are positional isomers of the major ketoprofen metabolite, 6, formed by acyl migration. This phenomenon occurs spontaneously at physiological temperature and pH, both *in vivo* and in buffer solution, and has attracted interest because of the ability of the resulting isomers to react with proteins in a covalent manner (Spahn-Langguth and Benet 1992). The degradation of the purified ester glucuronide metabolites of ketoprofen is currently being investigated.

Finally, none of the unconjugated hydroxy metabolites was found, whereas only a small amount of parent drug was observed, consistent with a previous

Table 1. ^1H -NMR chemical shifts δ^1 and multiplicities² of the nuclear magnetic resonance signals of the metabolites of ketoprofen in $^2\text{H}_2\text{O}$: aglycone protons.

Metabolite	Stereo chemistry	CH_3	CH	$\text{CH}(\text{OH})$	Ha	Hb	Hc	Hd	He	Hf	Hg	Hh	Hi
1	<i>S</i>	1.47 d	3.81 q	—	7.41 s	7.66 d	7.54 t	7.65 d	7.85 d	7.25 d	—	7.25 d	7.85 d
	<i>R</i>	1.47 d	3.81 q	—	7.41 s	7.66 d	7.54 t	7.65 d	7.85 d	7.25 d	—	7.25 d	7.85 d
2	<i>S</i>	1.48 d	3.91 ^{3,4} q	—	7.74 s	7.67 d	7.56 t	7.53 d	7.71 d	7.56 t	7.44 d	—	7.46 s
	<i>R</i>	1.49 d	3.91 ^{3,4} q	—	7.75 s	7.67 d	7.56 t	7.53 d	7.71 d	7.56 t	7.44 d	—	7.46 s
3	<i>S</i>	1.55 d	4.07 q	—	7.67 s	7.70 d	7.58 t	7.68 d	7.78 d	7.01 d	—	7.01 d	7.77 d
	<i>R</i>	1.56 d	4.08 q	—	7.70 s	7.69 d	7.57 t	7.67 d	7.79 d	7.02 d	—	7.01 d	7.79 d
4	<i>S</i>	1.57 d	4.09 q	—	7.77 s	7.18 d	7.60 t	7.33 d	7.77 d	7.47 t	7.23 d	—	7.28 s
	<i>R</i>	1.57 d	4.09 q	—	7.77 s	7.18 d	7.60 t	7.33 d	7.77 d	7.47 t	7.23 d	—	7.28 s
5	?^5	1.48 d	3.81 q	5.91 s	7.41 s	7.4 ³	7.3 ³	7.3 d	7.37 d	7.4 ³	7.4 ³	7.4 ³ t	7.37 d
	?^5	1.48 d	3.96 q	5.91 s	7.4 s	7.4 ³	7.3 ³	7.29 d	7.36 d	7.4 ³	7.4 ³	7.4 ³ t	7.36 d
6	<i>S</i>	1.57 d	4.10 q	—	7.77 s	7.73 d	7.60 t	7.78 d	7.82 dd	7.60 t	7.76 t	7.60 t	7.82 dd
	<i>R</i>	1.58 d	4.10 q	—	7.78 s	7.72 d	7.60 t	7.77 d	7.83 dd	7.61 t	7.76 t	7.61 t	7.83 dd

¹ δ relative to TSP at $\delta 0$.²Multiplicities: m, multiplet; s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets.³Cluster of peaks obscures clear observation.⁴Chemical shift was confirmed by 2-D total correlation spectroscopy (TOCSY) and by integration.⁵Molecule has two chiral centres and the stereochemistry has not been defined at either.

Table 2. ^1H -NMR chemical shifts δ^1 and multiplicities² of the nuclear magnetic resonance signals of the metabolites of ketoprofen in $^2\text{H}_2\text{O}$: glucuronide ring protons.

Metabolite	Stereo chemistry	H1'	H2'	H3'	H4'	H5'
1	<i>S</i>	5.29 d	3.63 t	3.66 ^{3,4} t	3.66 ^{3,4} t	3.96 d
	<i>R</i>	5.28 d	3.63 t	3.66 ^{3,4} t	3.66 ^{3,4} t	3.96 d
2	<i>S</i>	5.16 d	3.62	3.62	3.62	3.91 ^{3,4}
	<i>R</i>	5.17 d	3.62	3.62	3.62	3.91 ^{3,4}
3	<i>S</i>	5.59 d	3.51 dd	3.61 t	3.55 t	3.85 d
	<i>R</i>	5.56 d	3.45 t	3.55 t	3.52 t	3.85 d
4	<i>S</i>	5.59 d	3.50 dd	3.59 t	3.51 t	3.82 d
	<i>R</i>	5.57 d	3.50 dd	3.59 t	3.51 t	3.84 d
5	? ⁵	5.54 d	3.44 dd	3.57 t	3.49 t	3.83 d
	? ⁵	5.57 d	3.44 dd	3.59 t	3.53 t	4.00 d
6	<i>S</i>	5.60 d	3.51 dd	3.60 t	3.52 t	3.84 d
	<i>R</i>	5.57 d	3.46 t	3.57 t	3.53 t	3.86 d

¹ δ relative to TSP at $\delta 0$.²Multiplicities: m, multiplet; s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets.³Cluster of peaks obscures clear observation.⁴Chemical shift was confirmed by TOCSY and by integration.⁵Molecule has two chiral centres and the stereochemistry has not been defined at either.

report (Upton *et al.* 1980). This was confirmed by HPLC-MS analysis of urine immediately after collection.

Quantification of ketoprofen metabolites by ^1H -NMR spectroscopy

Whilst the detection of the metabolites of ketoprofen is readily achieved by both HPLC-UV and HPLC-MS quantification is not possible by either technique in the absence of authentic standards as the response factors are not known. However, this limitation does not apply to NMR-based methods, and quantification was therefore performed using this approach. The peaks of choice for quantification in urine are those from the anomeric protons ($\delta 5.6$) and from the methyl protons ($\delta 1.5$) as they appear in spectral regions of low peak overlap. Even so, a sample containing a mixture of the glucuronide metabolites could only give a measure of the total metabolite excretion in urine by integration of the sum of the chosen peaks, because it was impossible to estimate the relative metabolite proportions, due to the serious overlap of these peaks in the spectra. A further complication comes from the fact that all the metabolites are mixtures of diastereoisomeric pairs. Although enantiomers have identical NMR spectra, this is not true for diastereoisomers as they generally have observable differences in chemical shift (tables 1 and 2). To simplify analysis, the conjugates were hydrolysed back to their aglycones before any attempt at quantification, thus converting the diastereoisomers to their enantiomers by removing the glucuronide moiety. For this reason, the diastereoisomeric proportions of each metabolite could not be quantified. The bulk of the endogenous material was removed by SPEC (elution with 10% acetonitrile) before hydrolysis. Drug-related components were eluted with 100% acetonitrile, under the assumption that no ketoprofen-related compounds were left on the column. The absence of glucuronides in the 10%-eluted fraction and the huge amount of endogenous substances removed was verified by ^1H -NMR analysis.

Table 3. Relative proportions (%) of ketoprofen metabolites at 0–2, 2–4 and 4–6 h after oral administration of the drug. Proportions of compounds were estimated from the ratios of the methyl peaks of their aglycones after hydrolysis of the conjugates. The amount of unchanged parent drug excreted in urine and the ether-to-ester ratio for compounds 1–4 were estimated by high-performance liquid chromatography. The percentage of the dose recovered at each time interval was estimated from the integrals of the sum of the anomeric protons. Errors are of the order of 10%.

Metabolite	0–2 h	2–4 h	4–6 h
1	0	2.6	4.0
2	0	0.4	3.6
3	0.2	9.9	8.0
4	0	1.5	6.9
5	7.3	14.8	25.3
6	88.1	70	46.5
II	4.4	0.8	5.7
I, III, V	ND	ND	ND
Percentage of the eliminated dose	18.5	26.5	19.1

ND, not detected.

Methanol- d_4 was the solvent of choice for NMR analysis, as the aglycones were not extensively soluble in water. Table 3 shows the relative metabolite proportions and the total ketoprofen recovery in urine at each collection time. The recovery of ketoprofen in urine was calculated from the urine spectra by taking the integral of the sum of the glucuronide peaks at $\delta 5.6$. The relative concentrations were estimated using the peak heights of the methyl groups ($\delta 1.4$) of the aglycones. Although peak areas should be more accurate, this method was rejected because of the partial overlap of some of the signals, and because of the presence of small amounts of interfering substances that would add error to the integral values. Heights and areas are in good agreement provided the line widths are comparable. Errors were estimated from the ^1H -NMR spectra of standard samples containing ketoprofen and ibuprofen. The compounds were chosen because they both contain a propionic acid group, as do all of the metabolites. When comparing like protons (in this case the methyl protons), the error was not greater than 9% for peak height measurements and under 5% for peak areas. For estimation of the absolute compound concentration, comparison of the methyl signals to TSP gave errors of up to 10%. The amount of parent drug excreted unchanged in urine was estimated by HPLC of urine that was analysed immediately after collection. The methyl peaks of compounds I and III arose from the hydrolysis of both their ether and ester glucuronides. The ether-to-ester ratios were therefore estimated from their peak area ratios in the HPLC chromatograms of unhydrolysed samples (and are thus subject to further error as the reasonable assumption has to be made that molar absorptivities are the same). The proportions of the various glucuronide metabolites changed with the time of urine collection as seen in table 3. The major metabolite, ketoprofen acyl glucuronide, was rapidly excreted within the first couple of hours after drug intake and then the concentrations decreased with time. The opposite is observed with the other metabolites. Calculation of the recovery of ketoprofen in urine (table 3) gives a rough estimate of the renal excretion half-life as 4 h, not far from the reported elimination half-life (1–3 h) (Jamali *et al.* 1990). The fact that metabolite 5 is formed in a higher proportion than the other minor metabolites, is not a straightforward observation from the initial HPLC-UV

chromatogram. This is because reduction of the ketone group causes disruption in the double bond conjugation, hence changing the strong chromophore of ketoprofen.

Conclusions

In the present study, the identification and characterization of a number of major and minor human metabolites of ketoprofen excreted in urine was achieved using HPLC-MS and HPLC-NMR. The application of NMR enabled identification of the position of the hydroxyl group on the ring and the site of conjugation with D-glucuronic acid. The metabolic scheme is similar to that of the analogous substance fenoprofen, where a combination of ring hydroxylation and glucuronic acid conjugation also occurs (Volland *et al.* 1990). The ability of NMR to distinguish between ether and ester glucuronides allowed identification of two novel minor metabolites. These were not detected in previous analytical studies as these involved purification of the aglycone after hydrolysis under strongly basic conditions before NMR characterization. Moreover, as diastereoisomers of these compounds are spectroscopically distinct, they can be readily observed in NMR spectra of mixtures. The difference in diastereoisomeric ratios allowed stereochemistry assignment of metabolites. Finally, the dose of ketoprofen recovered and relative metabolite concentrations at different time intervals were estimated by ^1H -NMR spectroscopy, with the aid of HPLC where necessary.

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