BIOTRANSFORMATION OF BENFLURON BY RAT HEPATIC CYTOCHROME P450. IDENTIFICATION OF INDIVIDUAL CYP-ENZYMES INVOLVED IN BIOTRANSFORMATION OF BENFLURON, PROSPECTIVE ANTINEOPLASTIC BASED ON BENZO[*c*]FLUORENE

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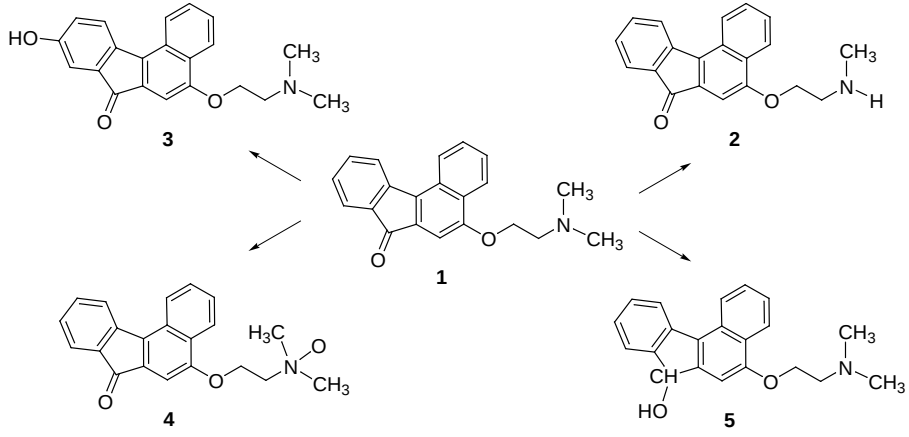
Benfluron, 5-[2-(dimethylamino)ethoxy]-7*H*-benzo[*c*]fluoren-7-one hydrochloride, a prospective antineoplastic agent, is metabolised by cytochromes P450 to *N*-demethyl and 9-hydroxy derivatives. To prove the participation of individual cytochrome P450 isoforms in formation of these metabolites, selective induction of cytochromes P450, inhibition of benfluron biotransformation using inhibitors specific for individual cytochromes P450, and inhibition by benfluron of "marker" enzyme activities characteristic of certain cytochromes P450 were used. *N*-Demethylbenfluron appears to be formed mainly by the cytochromes P450 of the 3A, 2B and 2C subfamilies with possible participation of the isoform 2E1; 9-hydroxybenfluron is formed with participation of cytochromes P450 belonging to 1A, and most probably to 3A and 2E1 enzymes. The fact that benfluron is in this respect a relatively promiscuous substrate may be an advantage because its metabolism should not be influenced by the absence or low activity of some cytochrome P450 isoforms and by possible drug interactions.

Key words: Cytochrome P450; Benzo[*c*]fluorenes; Biotransformations; Microsomes; Antitumor activity; Metabolism.

A potential benzo[*c*]fluorene antineoplastic agent, benfluron, 5-[2-(dimethylamino)ethoxy]-7*H*-benzo[*c*]fluoren-7-one hydrochloride, was prepared eighteen years ago in the Research Institute of Pharmacy and Biochemistry in Prague[1]. The pharmacodynamic properties of this compound were determined in experiments carried out *in vitro* (with animal and human cells) and *in vivo* (with Ehrlich ascites carcinoma and murine P388 leukemia)[1–5]. Pharmacokinetics of benfluron was studied on the basis

of TLC-densitometric analyses of serum extracts after oral and intravenous administration of the compound in four animal species[6]. The absorption, distribution and elimination of [^{3}H]benfluron following its oral and intravenous administration to rats was also studied[7]. Biotransformation of benfluron has been investigated *in vitro* and *in vivo*[8–11] and chemical structures of several metabolites were elucidated using infrared, FT-NMR and mass spectrometry[8,12–14].

Relatively little is known about enzymatic systems involved in oxidative biotransformation of benfluron. Three main oxidative metabolites of benfluron (**1**) – benfluron *N*-oxide (*N*-ox, **4**), 9-hydroxybenfluron (9-OH, **3**) and *N*-demethylated benfluron (*N*-dem, **2**) are formed by flavin-containing monooxygenase (FMO) and cytochrome P450 (CYP) systems; 9-OH and *N*-dem are CYP-mediated metabolites, *N*-ox is mainly the FMO-mediated one[8] (Scheme 1).



SCHEME 1

CYP is the main system of oxidative biotransformation of xenobiotics. Several *in vitro* approaches are available for identification of CYP isoforms involved in the biotransformation of compounds of interest. These approaches include: (i) correlation of the activity under consideration with known marker activities for individual CYP isoforms or with immunochemically determined levels of individual CYP isoforms, (ii) selective inhibition or induction of an individual CYP isoform, (iii) specific immunoinhibition, (iv) purification and reconstitution of a CYP system with the isoform, and (v) heterologous expression. The first two of these approaches, namely, the effect of selective induction and inhibition of CYP on the biotransformation of benfluron and the effect of benfluron on two

marker enzyme activities, that of ethoxycoumarin *O*-deethylase (7-ethoxy-1,2-benzopyrone *O*-deethylase, ECOD, specific for CYP1A1/2) and pentoxy-resorufin *O*-dependentylase (7-pentyloxy-3-phenoxazone *O*-dependentylase, PROD, specific for CYP2B1isoform), were investigated in this study. The purpose of this study was to identify CYP isoforms involved in formation of 9-OH, *N*-dem and partly of *N*-ox metabolites (together with FMO).

EXPERIMENTAL

Materials

Benfluron was from the Research Institute of Pharmacy and Biochemistry in Prague (Czech Republic). NADP, NADPH, isocitrate dehydrogenase, isocitric acid, tris(hydroxymethyl)-aminomethane (Tris), α-naphthoflavone (αNF), β-naphthoflavone (βNF), dexamethasone (DEX), triacetyloleandomycin (TAO), 7-ethoxycoumarin, 7-hydroxycoumarin, 7-pentoxy-resorufin and resorufin were purchased from Sigma (Prague, Czech Republic). Metyrapon (MET) and nonylamine were from Fluka (Buchs, Switzerland). Acetonitrile and dimethyl sulfoxide (DMSO) were from Merck (Darmstadt, Germany). Sodium diethyldithiocarbamate (DI) was from Aldrich (Sigma–Aldrich, Prague, Czech Republic). Sulfaphenazole (4-amino-benzensulfonamido-2′-fenylpyrazon, SUL) was a generous gift of Dr T. Vontor. All other chemicals were from local suppliers and they were of reagent grade purity.

Animals

Male Wistar rats from the Konárovice breeding station (BioTest, Konárovice) were used. They weighed 230–260 g and were allowed access to water and food pellets *ad libitum*. Rats were starved 24 h before the preparation of microsomal fraction. The experiments were approved by a local ethics commitee.

Induction of CYP, Preparation of Microsomes

Rats were treated by phenobarbital (5-ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine, PB, 1 g/l in aqueous solution p.o. *ad libitum* in drinking water one week prior to preparation of microsomes), ethanol (10% (v/v) solution p.o. *ad libitum* in drinking water two weeks prior to preparation of microsomes), βNF (40 mg/kg in corn oil i.p. one day prior to preparation of microsomes) and DEX (100 mg/kg in corn oil i.p. three days prior to preparation of microsomes). Rats were sacrificed by cervical dislocation 24 h after the final treatment and microsomes were prepared by differential centrifugation. Protein concentration was determined by the bicinchoninic acid (4,4′-dicarboxy-2,2′-biquinoline) method[15] using a commercial kit (Pierce, Rockford (IL), U.S.A.). Concentration of CYP was determined spectrophotometrically according to Omura and Sato[16]. Microsomes were resuspended in 0.25 M saccharose containing 1 mM EDTA and 50 mM Tris (pH 7.4) and were frozen in aliquots at –70 °C for subsequent experiments.

Measurement of Microsomal Activities

The 7-ethoxycoumarin *O*-deethylase (ECOD) and 7-pentoxyresorufin *O*-dependentylase (PROD) activities were determined by following the change in fluorescence using a Perkin–Elmer LS50B fluorescence spectrophotometer according to Lubet *et al.*[17]. The ECOD activity was determined in microsomes from β-naphthoflavone-induced rats, whereas PROD was measured in microsomes from phenobarbital-induced rats. The composition of reaction mixtures and conditions of incubations were:

ECOD: 250 μg microsomal protein, 0.5 mM 7-ethoxycoumarin, 1 mM NADPH, 10 mM isocitric acid, 2 U isocitric dehydrogenase and 1mM EDTA in 50 mM potassium phosphate buffer (pH 7.5). Final volume of incubation was 600 μl. The mixture was preincubated at 37 °C for 5 min, then NADPH was added and the 7-hydroxycoumarin production was monitored at excitation and emission wavelengths of 370 and 448 nm, respectively. Calibration was done by adding 10 μl of 0.1 mM 7-hydroxycoumarin. Control incubations were without NADPH. The effect of 50 and 500 μM benfluron on ECOD activity was investigated.

PROD: 50 μg microsomal protein, 5 μM 7-pentoxyresorufin and 0.25 mM NADPH in 100 mM sodium potassium phosphate buffer (pH 7.6). The final volume of incubation was 600 μl. The mixture was preincubated at 37 °C for 5 min, then NADPH was added and the resorufin production was measured at excitation and emission wavelengths of 530 and 585 nm, respectively. Calibration was done by adding 10 μl of 10 μM resorufin. Control incubations were again without NADPH. The effect of 0.5, 5 and 50 μM benfluron on the PROD activity was investigated in these experiments.

Incubations with Benfluron

Conditions of assays were: 0.5 mM benfluron, 2 mg microsomal protein, 0.5 mM NADPH, 3.75 mM isocitric acid, 1 U isocitric dehydrogenase and magnesium phosphate in 50 mM Tris buffer pH 7.8 in final volume of 2 ml. After preincubation (5 min at 37 °C in water bath while shaking), benfluron was added and mixture was incubated at 37 °C for 30 min. Incubation was stopped by adding 7 ml of 5% of ammonium hydroxide (1.5% NH_3) and by cooling. When the effect of inhibitors on benfluron biotransformation was investigated, experimental protocols according to established procedures[18] were used. Usually, 25 μl of an inhibitor solution (final concentrations 0.05, 0.5 and 5 mM) was added and the reaction mixture was preincubated for 5 min before adding benfluron (with TAO, 30 min of preincubation). MET, SUL and TAO were dissolved in methanol and used in PB-induced microsomes, TAO was used also in DEX-induced ones. αNF was dissolved in DMSO and used in βNF-induced microsomes. DI was dissolved in water and used in ethanol-induced microsomes. Control incubations were performed with pure solvents.

Analysis of Benfluron Metabolites

Alkalinized samples were extracted three times with ethyl acetate. The extracts were collected and evaporated *in vacuo* (maximum 40 °C) to dryness. The residues were dissolved in 1 ml of the mobile phase to be used in HPLC.

A Spectra Physics (now Thermo Separation Products) chromatograph was used for the HPLC. The configuration used was as follows: solvent degasser SCM400, quaternary pump P4000, autosampler AS3500 with a 100 μl sample loop, Spectra FOCUS high-speed scanning UV detector, system controller SN4000, computer Spectra 386E with PC1000 analytical soft-

ware working under OS-2. Analyses were performed according to Nobilis *et al.*[14] on a HPLC column LiChroCART 125 × 4 mm i.d. with precolumn LiChroCART 4 × 4 mm i.d., containing LiChrospher 100 RP-18 (5 mm, all from Merck). The mobile phase consisted of 0.2% (v/v) nonylamine buffer pH 7.4, acetonitrile and propan-2-ol (2 : 2 : 1 (v/v)). The flow-rate of the mobile phase was 0.9 ml/min. Detection was performed in a high-speed scanning mode (range 195–365 nm, with 5 nm steps).

Statistical Analysis

The effect of benfluron on enzyme activities and the effect of induction and inhibition on benfluron biotransformation was statistically evaluated. The data are expressed as a mean ±S.E.M., and statistical significance determined using Student's *t*-test. A $p < 0.05$ was considered significant.

RESULTS

Effect of Benfluron on Activities of CYP Isoforms

ECOD activity (characteristic of the CYP1A1/2 isoforms) was measured in βNF-induced microsomes. The results (Table I) indicate a marked effect of the benfluron on ECOD: 50 μM benfluron (one tenth of the concentration of 7-ethoxycoumarin) significantly decreased the ECOD activity to 27% with regard to control; 500 μM benfluron (equal to the 7-ethoxycoumarin concentration) caused a decrease in the ECOD activity of 6% of the control.

TABLE I
Effect of benfluron on 7-pentoxyresorufin *O*-dependentylase and 7-ethoxyresorufin *O*-deethylase activities

PROD activity, nmol/min/mg of protein			
Control	benfluron 0.5 μM	benfluron 5 μM	benfluron 50 μM
1.47 ± 0.52	1.00 ± 0.24	0.78 ± 0.20	0.13 ±0.07[a]
ECOD activity, nmol/min/mg of protein			
Control	benfluron 50 μM	benfluron 500 μM	
1.52 ± 0.43	0.41 ± 0.18[a]	0.09 ± 0.06[b]	

All values are means ±SD for three experiments. [a] $p < 0.05$. [b] 0.01. Substrate concentrations: 5 μM both.

The PROD activity (marker of the CYP2B enzymes) was measured in PB-induced microsomes (Table I). Both 0.5 and 5 μM benfluron (one tenth and equimolar concentration of the substrate) exhibited an effect on PROD activity, its significance was confirmed with higher (50 μM) benfluron concentration (ten-fold excess over the 7-pentoxyresorufin concentration) which decreased PROD activity to 9%.

Effect of Induction of CYP Isoforms on Benfluron Biotransformation

The effect of four CYP inducers (PB typical of CYP2B and, to a lesser extent, of 2C and 3A isoforms, βNF inducing the CYP1A isoforms, ethanol the 2E1 isoform and DEX the 3A and weakly the 2C family of CYP enzymes) on benfluron biotransformation was investigated. The effect was compared with control incubations with microsomes from untreated rats. The structures of four main benfluron metabolites after incubation with uninduced microsomes are shown in Scheme 1. It should be mentioned here again that the reduced benfluron (redB) is not a CYP-mediated metabolite, and that the formation of the *N*-ox is mainly FMO-dependent[19].

The effect of CYP induction on the formation of *N*-dem is presented in Table II. Induction by PB and DEX significantly increased the *N*-dem formation (225 and 257% of the control, respectively). For the other metabolite, the 9-OH, any inductor caused a significant increase in its formation, although the effect of DEX was again the most pronounced. Finally, in the

TABLE II
Effect of induction of rat hepatic cytochrome P450 on the formation of benfluron metabolites

Inductor	Metabolite concentration, nmol/ml		
	N-dem	9-OH	*N*-ox
Control	0.38 ± 0.19	0.10 ± 0.04	1.47 ± 1.18
Phenobarbital	0.96 ± 0.27[a]	0.06 ± 0.05	2.25 ± 1.23
β-Naphthoflavone	0.29 ± 0.14	0.07 ± 0.01	1.70 ± 1.35
Ethanol	0.23 ± 0.14	0.07 ± 0.03	0.88 ± 0.45
Dexamathasone	0.98 ± 0.23[b]	0.18 ± 0.09	1.38 ± 2.04

All values are means ±SD for five experiments. [a] $p < 0.05$. [b] 0.02.

case of the formation of *N*-ox (Table II), the results show no effect of induction and, moreover, marked variation in the *N*-ox formation. A spontaneous formation of *N*-ox in aqueous solution in the absence of enzyme, which has been confirmed by a parallel experiment (unpublished results), could be an explanation. This is why the discussion is oriented to the formation of the two main CYP-dependent metabolites, namely 9-OH and *N*-dem.

Effect of Specific Inhibitors of CYP Isoforms on Benfluron Biotransformation

The effect of five specific CYP inhibitors (SUL for rat CYP2C, MET for CYP2B and also 3A, TAO highly specific for CYP3A, αNF for CYP1A2 and DI for CYP2E1 and 2A isoforms) on benfluron biotransformation was investigated. In order to maximize the effect of specific inhibitors of CYP isoforms, microsomes from animals pretreated with inducer of the same isoform were used. PB-Induced microsomes were used for experiments with SUL, MET and TAO. βNF-Induced microsomes were used for experiments with αNF. Ethanol-induced microsomes were used for experiments with DI, and DEX-induced microsomes were used for experiments with TAO. Three concentrations of inhibitors were used: 0.05, 0.5 and 5 mM (one tenth, equal and ten-fold, respectively, of the benfluron concentration). The results are summarized in Table III.

5 mM MET caused a significant decrease (24% of the control) in the formation of *N*-dem; biotransformation of benfluron to 9-OH was not affected. 5 mM SUL significantly decreased (24% of the control) the formation of *N*-dem, whereas the levels of 9-OH were not changed. The use of 0.05, 0.5 and 5 mM TAO caused significant changes in the biotransformation of benfluron to *N*-dem (46, 37 and 23% of the control, respectively) in PB-induced microsomes. The marked decrease in the *N*-dem formation caused by TAO in DEX-induced microsomes (26, 27 and 21% of the control using the 0.05, 0.5 and 5 mM concentration, respectively) was not considered significant most probably due to rather high variation in the *N*-dem formation in control experiments. The 9-OH formation was not influenced by TAO in both PB- and DEX-induced microsomes. 5 mM αNF caused significant decrease (18% of the control) in the formation of 9-OH, whereas it did not influenced biotransformation of benfluron to *N*-dem. DI significantly decreased the formation of both 9-OH and *N*-dem: 0.05, 0.5 and 5 mM DI caused a gradual decrease in the formation of *N*-dem (55, 41 and 11% of the control). The biotransformation to 9-OH was also decreased as it dropped to 19 and 33% of the control using 0.5 and 5 mM DI, respectively.

TABLE III
Effect of isoform – selective inhibition of rat hepatic cytochrome P450 on the formation of benfluron metabolites

Inhibitor	Metabolite concentration, nmol/ml	
	9-hydroxybenfluron	N-demethylated benfluron
Control[d]	4.00 ± 1.89 (100)	25.12 ± 6.63 (100)
MET 0.05 mM	2.77 ± 1.23 (69)	13.23 ± 7.39 (53)
MET 0.5 mM	1.90 ± 1.94 (48)	9.78 ± 4.62 (39)
MET 5.0 mM	3.86 ± 1.22 (97)	5.97 ± 3.04 (24)[a]
SUL 0.05 mM	2.72 ± 0.99 (68)	24.51 ± 16.49 (98)
SUL 0.5 mM	1.85 ± 1.94 (46)	10.43 ± 4.48 (42)
SUL 5.0 mM	2.56 ± 2.36 (64)	6.13 ± 3.24 (24)[a]
TAO 0.05 mM	5.00 ± 2.42 (125)	11.60 ± 1.77 (46)[a]
TAO 0.5 mM	6.54 ± 2.94 (164)	9.21 ± 2.72 (37)[b]
TAO 5.0 mM	5.39 ± 2.46 (135)	5.81 ± 2.85 (23)[c]
Control[e]	6.63 ± 2.47 (100)	34.61 ± 21.78 (100)
TAO 0.05 mM	4.82 ± 3.65 (73)	9.11 ± 0.97 (26)
TAO 0.5 mM	4.78 ± 3.47 (72)	9.47 ± 0.59 (27)
TAO 5.0 mM	3.22 ± 2.46 (49)	7.41 ± 1.21 (21)
Control[f]	4.76 ± 1.50 (100)	8.03 ± 8.06 (100)
αNF 0.05 mM	3.97 ± 0.68 (83)	8.63 ± 7.72 (107)
αNF 0.5 mM	2.10 ± 0.83 (44)	9.16 ± 7.99 (114)
αNF 5.0 mM	0.85 ± 0.25 (18)[a]	8.94 ± 6.32 (111)
Control[g]	8.60 ± 1.53 (100)	9.73 ± 1.77 (100)
DI 0.05 mM	9.92 ± 3.86 (115)	5.31 ± 0.97 (55)[a]
DI 0.5 mM	1.61 ± 1.39 (19)[c]	3.97 ± 0.87 (41)[b]
DI 5.0 mM	2.88 ± 1.36 (33)[b]	1.03 ± 0.37 (11)[c]

All values are means ±S.E.M. for three experiments, per cent of the control in parentheses. [a] $p < 0.05$. [b] 0.02. [c] 0.01. [d] Phenobarbital-induced. [e] Dexamethasone-induced. [f] β-Naphthoflavone-induced. [g] Ethanol-induced microsomes used.

DISCUSSION

Three basic experimental approaches were used in the study of benfluron biotransformation by rat hepatic CYP:

1) Enzyme induction. Four typical inducers were used: PB, inducing mainly CYP 2B1/2 (and, further, the forms 2C6/7 and 3A1/2); βNF, inducing CYP1A1/2; ethanol, inducing typically CYP2E1; DEX, inducing CYP3A1 and 2B2 (ref.[20]).

2) Selective enzyme inhibition. Five specific inhibitors were used: αNF, inhibiting CYP1A1/2; MET, a typical inhibitor of CYP2B and 3A isoforms[21]; SUL, inhibiting isoforms of the CYP2C subfamily; DI, an inhibitor of CYP2E1and CYP2A; TAO, inhibiting isoforms of the CYP3A subfamily[22].

3) Measurement of specific enzyme activities. Two enzyme activities were measured – ECOD and PROD; the effect of benfluron on these activities was investigated. The ECOD activity was measured in βNF-induced microsomes (*i.e.* with elevated levels of the isoforms of the CYP1A subfamily), where it is an indicator of the CYP1A1/2 activity[23]. PROD activity, a measure of CYP2B1 activity[24], was studied in PB-induced microsomes with elevated levels of isoforms of the CYP2B subfamily.

A combination of these three approaches was used to shed some light on ways in which the metabolites, namely, the *N*-demethylated (*N*-dem) and the 9-hydroxylated ones, are formed by individual CYP isoforms.

First, the formation of *N*-dem is discussed. Both the increase in the *N*-dem formation by PB-induction and inhibition of the *N*-dem formation by MET are in favor of contribution of CYP2B1/2 and 3A1/2 to the formation of the metabolite (Tables II and III). Moreover, the PROD activity, a specific marker of the CYP2B1 activity, was decreased by benfluron (Table I), which supports involvement of CYP2B1 isoforms in the benfluron *N*-demethylation.

As to the role of CYP3A1/2 in the *N*-dem formation, it is suggested by a significant increase in levels of this metabolite by PB- and mainly by DEX-induction (Table II). It has been also confirmed by the use of a selective inhibitor of CYP3A1/2, TAO, in PB-induced microsomes (Table III, this induction is known to elevate the levels of mainly 2B1/2, 3A1/2 and 2C6/7 isoforms). Another support for involvement of CYP3A1 in *N*-demethylation of benfluron was expected to come from inhibition of this isoform by TAO in DEX-induced microsomes (here the induction of 2B2 and mainly the 3A1 isoforms was reported[21]). In fact, although a marked decrease in the *N*-dem formation was observed (26, 27 and 21% of the control, respectively: see Results and Table III), this change could not be taken as signifi-

cant, most probably due to rather high variation in the *N*-dem formation in control experiments. Taken together, these two types of experiments seem to confirm the role of CYP3A in the formation of 3-demethylated metabolite of benfluron.

The effect of SUL, an inhibitor of CYP2C isoenzymes, was tested in experiments with PB-induced microsomes. It has been shown to decrease the formation of *N*-dem (Table III). This fact may be interpreted in favour of possible involvement of CYP 2C6/7 (inducible by PB) in the formation of *N*-dem.

Discussion dealing with formation of 9-hydroxybenfluron (9-OH) and with involvement of other forms of CYP enzymes should be started again with experiments on induction and inhibition. Induction of CYP by βNF did not lead to a significant increase in formation of any CYP-mediated metabolite (Table II). Two isoforms, CYP 1A1 and 1A2, are inducible by βNF; CYP1A1 is the predominant one in untreated rats[25]. On the other hand, αNF, specific inhibitor of CYP1A1/2 in rats, significantly decreased the formation of 9-OH (Table III). In addition, the ECOD activity in βNF-induced microsomes (known to primarily reflect the activity of CYP1A isoenzymes[23]) was significantly decreased by benfluron (Table I). Hence, a contribution of CYP1A1/2 in biotransformation of benfluron to 9-OH is highly probable. It may be speculated that the CYP1A1/2 isoforms contribute to the overall 9-OH formation together with other isoforms (*e.g.*, with CYP3A1/2, see Table II, induction by DEX and Table III, inhibition by TAO in DEX-induced microsomes).

Although the induction of CYP 2E1 by ethanol did not lead to enhanced formation of any CYP-mediated metabolite (Table II), use of DI (a specific inhibitor of the 2E1 and 2A isoform) had a marked effect on the benfluron biotransformation. Both the *N*-dem and 9-OH formation were significantly decreased by this inhibitor (Table III).

On the basis of the presented results, we may conclude that benfluron itself is a promiscuous substrate, the biotransformation of which to the 9-hydroxy metabolite is mediated by CYP isoforms belonging to the 1A and 3A subfamilies and by the CYP2E1; the *N*-demethylated metabolite is formed by CYP isoforms of the 2B1/2, 3A1/2, 2C6/7 subfamilies and also by the CYP2E1. Final elucidation of contribution of particular CYP isoforms would need further experiments, in particular those with isolated isoforms and/or immunoinhibition experiments with specific antibodies.

Recently, a consensus has been reached about general strategies for determining catalytic activities of CYPs (ref.[26]). Of several approaches available, the selective CYP inhibition and induction and the effect of xenobiotics on

marker enzyme activities was used in this study. Each approach has inherent advantages and disadvantages. The use of inducers is often confounded with the problem of lack of selectivity, but the inducer may be useful to address pharmacological and toxicological consequences of an increased metabolism of a compound by various CYPs (ref.[27]).

An incomplete specificity may be the problem also in the use of immunoinhibition experiments. The composition of polyclonal antibodies produced by the same immunogen may vary with each immunization and, hence, they cannot be considered stable or pure reagents[28]. In the case of monoclonal antibodies (MAbs), MAb can definitely neither immunoprecipitate CYP proteins nor inhibit their catalytic activity[28]. Also, Mabs to one CYP isoform have been unexpectedly found to cross-react with different CYP variants[29,30]. Isolation of an individual CYP can be tedious and reconstitution is not always straightforward[31]. An interpretation of reaction rates in reconstituted systems may be difficult[32].

A clear advantage of the use of inhibitors for determining substrate specificity – as it has been applied in this study – is that these compounds are simple to use and readily available. In addition, chemical inhibitors can be used with intact cells or *in vivo*. Until recently, the major disadvantage with most available CYP inhibitors was a poorly defined or incomplete selectivity: however, the selectivity of recently used inhibitors is clearly defined and sufficient to preferentially target individual CYP isoforms[33–35].

Although definitive assessment of the role of an individual CYP in a given metabolic pathway can only be accomplished by using the entire battery of experimental approaches[26,33,36], experiments using CYP inducers and inhibitors are a useful and reliable step in defining CYP specificity in biotransformation of a given compound and provide insight into some mechanisms of possible pharmacological and toxicological interactions accompanied by biotransformation of the compound of interest. From this point of view, the low specificity of benfluron biotransformation catalysed by cytochromes P450 could be beneficial, and this fact could make benfluron biotransformation insensitive to the effects of various possible drug–drug interactions.

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