ELSEVIER SCIENTIFIC PUBLISHERS IRELAND

Toxicology 82 (1993) 169–189

TOXICOLOGY

Biotransformation of methylxanthines in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. Allocation of metabolic pathways to isoforms and inhibitory effects of quinolones

Uwe Fuhr*[a], Johannes Doehmer[b], Narayana Battula[c], Catherine Wölfel[d], Ingrid Flick[d], Christine Kudla[a], Yango Keita[a], A. Horst Staib[a]

[a]*Department of Clinical Pharmacology, University Hospital, Theodor-Stern-Kai 7, D-60590, Frankfurt am Main, Germany*
[b]*Institut für Toxikologie und Umwelthygiene, Technische Universität München, München, Germany*
[c]*Division of Antiviral Drug Products, FDA, Kensington, MD 20895, USA*
[d]*Institute of Toxicology, University of Mainz, Mainz, Germany*

Abstract

V79 Chinese hamster cells genetically engineered for stable expression of single forms of rat cytochromes P450IA1, P450IA2, P450IIB1, human P450IA2, and rat liver epithelial cells expressing murine P450IA2 were used to allocate metabolic pathways of methylxanthines to specific isoforms and to test the suitability of such cell lines for investigations on drug interactions occurring at the cytochrome expressed. The cell lines were exposed to caffeine and/or theophylline and concentrations of metabolites formed in the medium were determined by HPLC. Caffeine was metabolized by human, rat and murine P450IA2, resulting in the formation of four primary demethylated and hydroxylated metabolites. However, there were

* Corresponding author.

Abbreviations: 1X, 1-methylxanthine; 1U, 1-methyluric acid; 3X, 3-methylxanthine; 3U, 3-methyluric acid; 7X, 7-methylxanthine; 7U, 7-methyluric acid; 13X, 1,3-dimethylxanthine (i.e. theophylline); 13U, 1,3-dimethyluric acid; 17X, 1,7-dimethylxanthine (i.e. paraxanthine); 17U, 1,7-dimethyluric acid; 37X, 3,7-dimethylxanthine (i.e. theobromine); 37U, 3,7-dimethyluric acid; 137X, 1,3,7-trimethylxanthine (i.e. caffeine); 137U, 1,3,7-trimethyluric acid; HPT, 7-(2-hydroxypropyl)-1,3-dimethylxanthine (i.e. hydroxypropyltheophylline); AFMU, 5-Acetylamino-6-formyl-amino-3-methyluracil; PPA, pipemidic acid; PEFL, pefloxacin.

0300-483X/93/$06.00
SSDI 0300-483X(93)02610-S

differences in the relative amounts of the metabolites. The human and the mouse P450IA2 isoforms predominantly mediated 3-demethylation of caffeine. The rat cytochrome P450IA2 mediated both 3-demethylation and 1-demethylation of caffeine to a similar extent. The results support the hypothesis that caffeine plasma clearance is a specific in vivo probe for determining human P450IA2 activity. Addition of the quinolone antibiotic agents pipemidic acid or pefloxacin, both known to inhibit caffeine metabolism in vivo and in human liver microsomes, reduced formation rates of all metabolites of caffeine in cells expressing rat and human P450IA2. Theophylline was mainly metabolized via 8-hydroxylation. All cell lines tested were able to carry out this reaction, with highest activities in cell lines expressing rat or human P450IA2, or rat P450IA1.

Key words: Caffeine; Cytochrome P450; Cytochrome P450IA2; Gene expression; Metabolism; Methylxanthines; Pefloxacin; Pipemidic acid; Quinolones; Rat liver epithelial cells; V79 Chinese hamster cells

1. Rationale for the use of cell lines expressing single cytochrome P450 isoforms in a drug metabolism study

The availability of genetically modified cells expressing high levels of isolated cytochrome P450 isoforms of several species is an important step towards a better understanding of both structure and function of these enzymes. To date the roles of the different isoforms in metabolism of endogenous substances or of xenobiotics in many cases still is only fragmentary elucidated due to the lack of an appropriate test system containing sufficient amounts of the pure enzyme. Here we present an example for the successful use of such cell lines in investigations on methylxanthine metabolism, with results consistent with those obtained by other methods, but providing important additional information.

Scientific interest in metabolism of both caffeine and theophylline experienced a recent revival. Caffeine is increasingly accepted as a model drug for testing hepatic capacity of drug metabolism in general and as an in vitro indicator of cytochrome P450IA2 activity, an isoform responsible for bioactivation of potent carcinogens (Butler et al. 1989; Gonzalez et al. 1990). Theophylline is involved in several clinically important drug interactions (Harder et al. 1988; Nielsen-Kudsk et al. 1990), a topic of growing importance in therapeutics as well as in drug regulatory affairs. A precise allocation of the various metabolic pathways to the enzymes involved is essential both to establish caffeine as a test drug for human P450IA2 activity in vivo and to predict methylxanthine interactions expected in vivo from in vitro data.

Primary steps in the metabolism of caffeine, i.e. 1,3,7-trimethylxanthine (137X) and theophylline, i.e. 1,3-dimethylxanthine (13X) in rodents and in man are cleavage of methyl groups and hydroxylation at position 8, mediated by cytochrome P450 isoforms (Berthou et al. 1988; Kalow and Campbell

1988; Robson et al. 1988; Berthou et al. 1991). The role of human P450IA2 in 3-demethylation of caffeine is well established (Butler et al. 1989; Sesardic et al. 1990), whereas it remains unclear to which extent this isoform contributes to the metabolism of caffeine to other metabolites or to that of theophylline.

To resolve this question, studies on drug metabolism by single P450 isoforms are essential. One of the major difficulties in the identification of cytochromes P450 responsible for methylxanthine degradation may be due to the relatively low in vitro activity of the enzymes in both liver microsomes and cultured hepatocytes (Robson et al. 1988; Berthou et al. 1989). To overcome problems arising in proper allocation of pathways to isoforms by conventional indirect techniques (specifity of antibodies, substrates, or inhibitors) and by isoform purification, we used recombinant cell lines genetically engineered for stable expression of rat, mouse, and human cytochromes to analyze metabolism of methylxanthines. The value of this approach has been addressed (Gelboin et al. 1985; Gonzlaez et al. 1990). V79 cell lines expressing functional rat P450IA1 (the cell line was called 'XEM2' (Dogra et al. 1990)), rat P450IA2 ('XEMdMz' (Wölfel et al. 1991)), rat P450IIB1 ('SD1' (Doehmer et al., 1988; Platt et al. 1989; Waxman et al. 1989)), human P450IA2 ('XEMHIA2.36' and 'XEMHIA2.43' (Wölfel et al. 1992)), rat liver epithelial cells expressing murine P450IA2 ('R52-16' (Battula, 1989)), and their corresponding controls were used to metabolize caffeine and/or theophylline. These mammalian cell lines have been genetically engineered to produce high levels of a single P450 isoform only. Thus, cytochrome P450 dependent pathways mediated by these cells can unequivocally be assigned to the appropriate isoform.

As an additional advantage, this cellular test system is expected to reflect in vivo conditions better than liver microsomes or reconstituted systems, since the enzymes are operating in a living cell with intact and functional structures, and the situation is less complex than, e.g. hepatocyte cultures, where the whole range of cytochrome P450 isoforms is present. Therefore, we tested inhibitory effects of two quinolone antibiotic agents, pipemidic acid and pefloxacin, on caffeine metabolism by the V79 cell lines expressing rat and/or human P450IA2. These compounds are inhibitors of caffeine degradation in vivo and in human liver microsomes, but the extent of inhibition differs between both systems, which makes it difficult to predict the effect of quinolone co-medication in therapy on methylxanthine kinetics by in vitro results.

2. Materials and methods

2.1. Chemicals

Methylxanthines were obtained from the following companies: Caffeine (Serva, Heidelberg, Germany), 17X, 13U (Aldrich, Milwaukee, USA), 13X

(Klinge, München, Germany), 137U, 37X, 1X, 3X, hydroxypropyltheophylline (Fluka, Buchs, Switzerland). Purity of substrates was checked by HPLC; caffeine contained <0.002% of 17X, of 37X and of 137U and 0.0088% of 13X. This impurity was taken into account for quantification of caffeine metabolites by subtracting this amount from 13X the concentrations measured. Theophylline chromatograms at 10 mM did not show peaks of possible metabolites (impurity <0.002%). Quinolone antibacterial agents were supplied by Madaus, Köln, Germany (pipemidic acid) and by Rhone-Poulenc, Antony, France (pefloxacin). Acetonitrile, 2-propanol, diisopropyl ether, and tetrahydrofuran were Merck chromatography grade products (Darmstadt, Germany). All other chemicals (analytical grade) were purchased from Merck.

2.2. Cell culture

V79 Chinese hamster cells (Glatt et al. 1987) and V79 derived cell lines expressing rat cytochrome P450IIB1, IA1, IA2, and human IA2 have been evaluated for enzymatic activity, for metabolic activation of promutagens and procarcinogens, and for drug metabolism (Doehmer et al. 1988; Platt et al. 1989; Waxman et al. 1989; Dogra et al. 1990; Wölfel et al. 1991; Wölfel et al. 1992). The cells were maintained in Dulbecco Vogt Eagle's Medium (DMEM, high glucose) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 1 mM L-glutamine and incubated at 37°C in 10% CO_2 in air at 85% humidity. V79 derived cell lines were maintained under the same conditions except that the medium was supplemented with 400 μg/ml of the antibiotic G418. Cells were free of Mycoplasma contamination (Russel et al. 1975).

For metabolism studies 2×10^6 cells were seeded in a 75 cm^2 flask in 10 ml of the same medium as described above without G418 and incubated for 2 more days. During this time cells grew to a confluency close to 100%, corresponding to an absolute number of about 4×10^7 cells. Thereafter, the medium was exchanged against medium without G418, containing 4 mM caffeine or 4 mM theophylline, or no test substrate for control. Three days later supernatant medium was harvested, centrifuged at $3000 \times g$ for separating cell debris and subjected to HPLC analysis. These conditions were optimized for maximum metabolism taking into account that caffeine and theophylline revealed cytostatic effects in all cell lines.

For inhibition studies in V79 derived cells expressing rat or human P450IA2, incubation conditions were as described for metabolism experiments, except that we used caffeine concentrations of 0.5 mM, which is near the K_M value for methylxanthine demethylations in human liver microsomes. Incubations with caffeine without inhibitor, with caffeine plus pipemidic acid, and with caffeine plus pefloxacin were carried out in duplicate. The concentration of the quinolones was 0.5 mM; under these conditions, cell growth was not impaired by addition of the inhibitors.

The rat liver epithelial cell lines (Battula 1989) also have already been used successfully in investigations on carcinogenesis and drug metabolism. These cells were cultured in DMEM containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), G418 (200 μg/ml) and incubated at 37°C in 5% CO_2 in air at 100% humidity. Only caffeine metabolism was investigated using this cell line, under conditions similar to those described for V79 derived cell lines, but incubation times were shorter (up to 24 h) and substrate concentration was 1 mM.

2.3. HPLC assay of metabolites

The HPLC method we used for determination of metabolites has been described (Fuhr et al. 1992b). We used a mixture of diisopropyl ether and 2-propanol to extract the methylxanthines and uric acids from the acidified sample. Following evaporation of the organic layer, an aliquot of the dissolved residue was injected onto a 250 × 4 mm column packed with Nucleosil 5 μm (Merck, Darmstadt, Germany). Metabolites were resolved by gradient elution with increasing percentage of organic solvents with time. The UV absorption of the methylxanthines was monitored at a wavelength of 278 nm (see Fig. 1). Peak height ratios with the internal standard (hydroxypropyltheophylline) were used to determine concentrations. These ratios were proportional to spiked concentrations of all substances measured from detection limit up to 30 μM ($r^2 > 0.998$). The lower limits of detection of the assay were defined as the concentrations giving a peak height greater than three times the noise fluctuation of the chromatographic baseline. Values were used for quantification when the coefficient of variation did not exceed 15% for the concentration determined (Fuhr et al., 1992b). Thus, limits were (μM): 1X, 0.3; 3X, 0.3; 13X, 0.1; 13U, 0.8; 17X, 0.1; 37X, 0.2; 137X, 0.1; 137U, 0.1.

Interactions with other methylxanthines can be ruled out because retention times of other possible (secondary) metabolites were different from retention times for the primary ones. The recovery of metabolites following extraction was determined by comparison of aqueous standard solution with spiked medium samples. Values for the various compounds ranged from 66 to 95% (Fuhr et al. 1992b). Activities of cytochromes expressed in the cell lines were calculated by subtracting metabolite concentrations formed by cells without cytochromes from those obtained by cell lines engineered for expression of single P450 isoforms.

3. Results

3.1. Identification of methylxanthine metabolites

The HPLC method we used for the determination of caffeine and theophylline metabolites is specific and sensitive to measure even low concentrations of unlabeled metabolites. Metabolic activity of the cells in methyl-

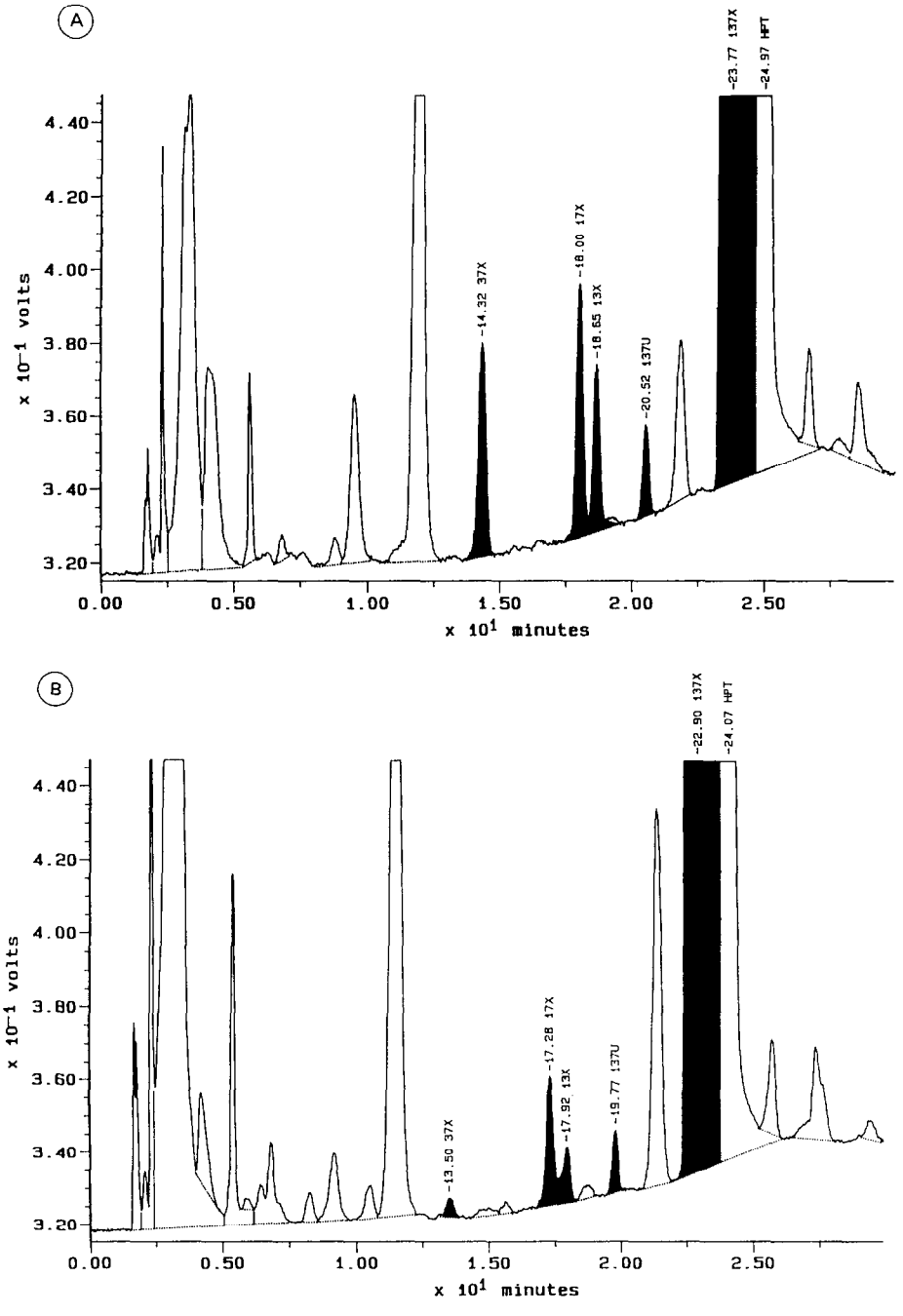

Fig. 1. HPLC-chromatograms of incubation medium following incubations of cell lines with 4 mM caffeine for 3 days (from Fuhr et al., 1992b, modified). Black, definite peaks of substrates and their metabolites; peak identification is written at the top of each peak (see list of abbreviations): (A) rat P450IA2 cell line and (B) human P450IA2 cell line.

Table 1
Caffeine metabolism by mammal cell lines expressing single cytochrome P450 isoforms and by controls (for incubation procedure see method section) (from Fuhr et al. 1992b, modified)

Cell line	Cyt.P450 isoform	Substrate conc.(mM)	Incubation time (h)	Metabolites formed (μM)			
				13X	17X	37X	137U
V79[b]	no P450	4	72	— [a]	— [a]	— [a]	0.3 ± 0.1
SD1[b]	rat P450IIB1	4	72	— [a]	— [a]	— [a]	0.2 ± 0.2
XEM2[b]	rat P450IA1	4	72	— [a]	0.3 ± 0.3	0.2 ± 0.2	0.4 ± 0.1
XEMdMz[b]	rat P450IA2	4	72	1.7 ± 0.4	4.1 ± 0.8	3.5 ± 0.8	0.8 ± 0.1
XEMHIA2.36[b]	human P450IA2	4	72	0.1 ± 0.0	2.2 ± 0.6	0.4 ± 0.1	0.3 ± 0.2
XEMHIA2.43[b]	human P450IA2	4	72	0.2 ± 0.0	2.7 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
R52–16[c]	mouse P450IA2	1	24	0.4	3.0	1.6	0.4

[a]Below detection limit, i.e (μM): 13X, 0.1; 17X, 0.1; 37X, 0.2; 137U, 0.1.
[b]Means and S.D. given for three incubations carried out on different days.
[c]Due to a different matrix and to small sample volumes, detection limits here were (μM): 13X, 0.2; 17X, 0.2; 37X, 0.2; 137U, 0.3.

xanthine metabolism was (approximate rates): V79 derived lines, <1 pmol/min/10^6 cells; rat liver epithelial cells, 4 pmol/min/10^6 cells. Thus, metabolite concentrations following incubations were low in general, a problem known from any in vitro test for metabolism of caffeine or theophylline. Additional problems in determination of metabolites were caused by peaks appearing when cells were incubated even without methylxanthines.

To optimize detection of metabolites, we used high substrate concentrations (4 mM) and prolonged periods of incubation (3 days) to obtain sufficient amounts of metabolites by V79 derived cell lines. These substrate concentrations had a cytostatic effect, which is a limitation to increase them further. The rat liver epithelial derived cell line showed higher catalytic activity, 1 mM of caffeine and shorter incubation times in this case were sufficient to produce easily measurable amounts of metabolites.

3.2. Concentrations of caffeine metabolites

Our results show (Table 1) that only cell lines expressing a P450IA2 isoform were able to demethylate caffeine to a significant extent. In all IA2 cell lines, demethylations are the predominant pathways accounting for 80% or more of total metabolites (see Fig. 1). Metabolite concentrations increased grossly linear with incubation time (Fig. 2). Minor hydroxylation activities exceeding that of the original cell lines were also found by IA1.

Interestingly, there were differences in the metabolic profiles among human, mouse and rat P450IA2 (Fig. 3). In the case of rat cytochrome P450IA2, both 17X and 37X contributed in equal parts to caffeine degradation, while the mouse P450IA2 and especially the human one predominatly produced 17X.

3.2.1. Effects of quinolones of caffeine metabolism. In inhibition experiments, the addition of the quinolone antibiotics pipemidic acid or pefloxacin at concentrations equal to that of caffeine (0.5 mM) decreased formation rates of metabolites (Fig. 4). In both rat and human IA2 cell lines, all metabolites were concerned. All demethylation rates mediated in a cell line declined by the same percentage when a quinolone was added to the incubation medium. The inhibition of the 8-hydroxylation pathway, however, was less marked than that of demethylations. Effects were more pronounced in the human 1A2 cell line, where additional differences between the two inhibitors were observed, a phenomenon not present in the rat IA2 cells (Fig. 4).

3.3. Concentrations of theophylline metabolites

In contrast to caffeine, theophylline was mainly metabolized via 8-hydroxylation, both by rat and human IA2 (Fig. 5), and with a relatively high activity by rat IA1 (Table 2). Demethylation activity was observed only by rat P450IA2. V79 cells without cytochromes also were able to 8-hydroxy-

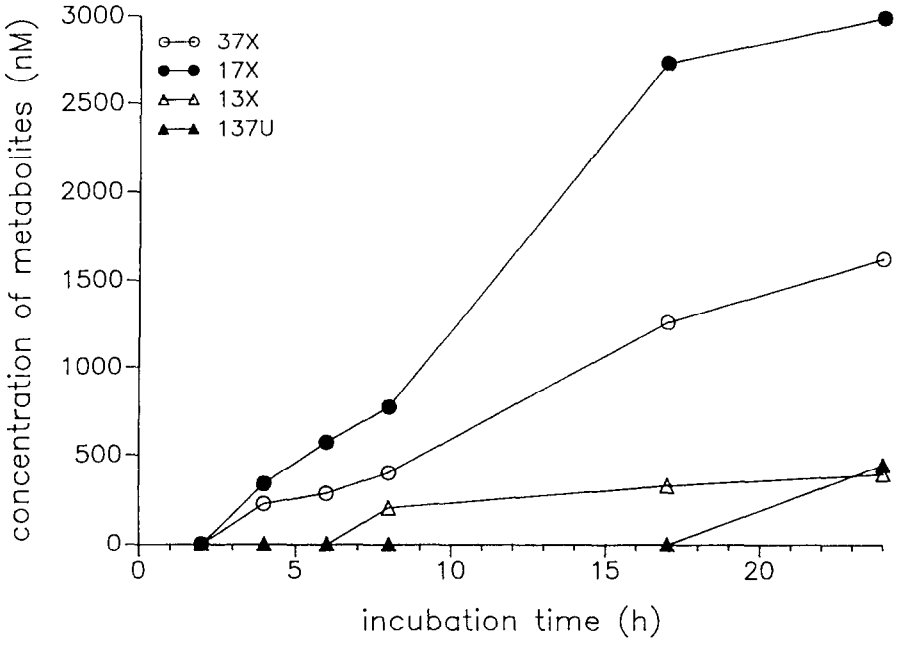

Fig. 2. Caffeine metabolism by rat liver epithelial cells expressing murine P450IA2 (caffeine concentration: 1 mM)

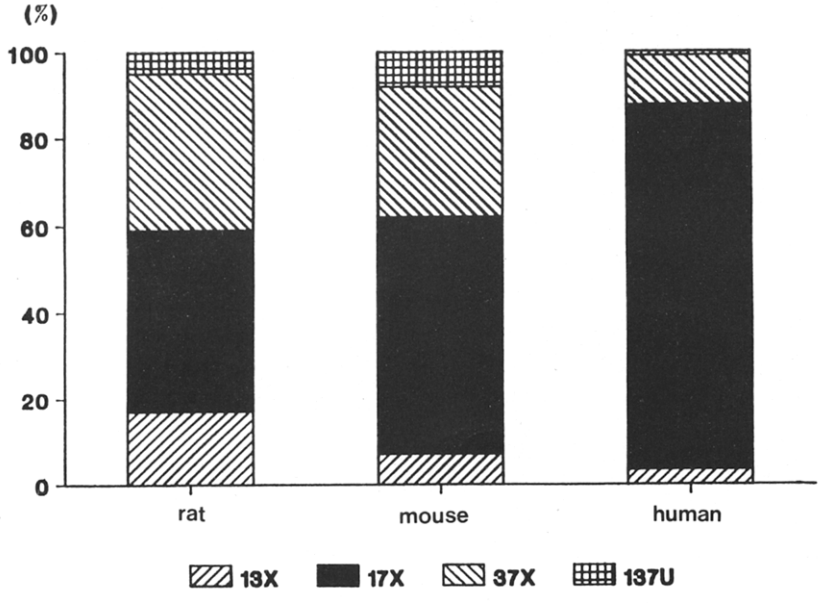

Fig. 3. Species differences in caffeine metabolism by P450IA2 cell lines (from Fuhr et al., 1992b). Relative contribution of each pathway is given as a percentage of all primary metabolites observed.

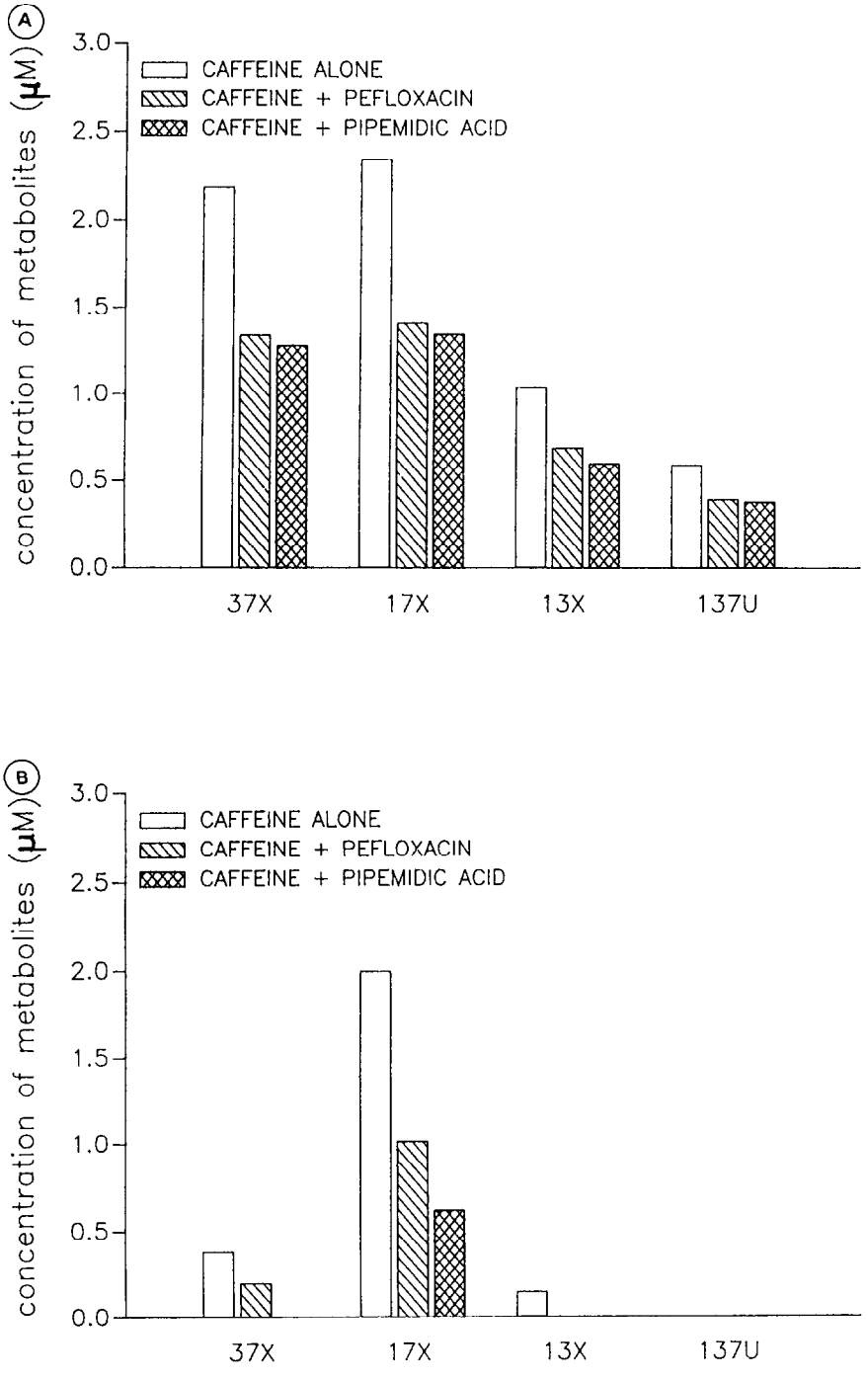

Fig. 4. Inhibition of caffeine metabolism in cytochrome P450IA2 cell lines by quinolone antibacterial agents (caffeine and inhibitor concentrations: 0.5 mM, incubation time: 3 days, mean of 2 incubations), (A) rat P450IA2 cell line; and (B) human P450IA2 cell line.

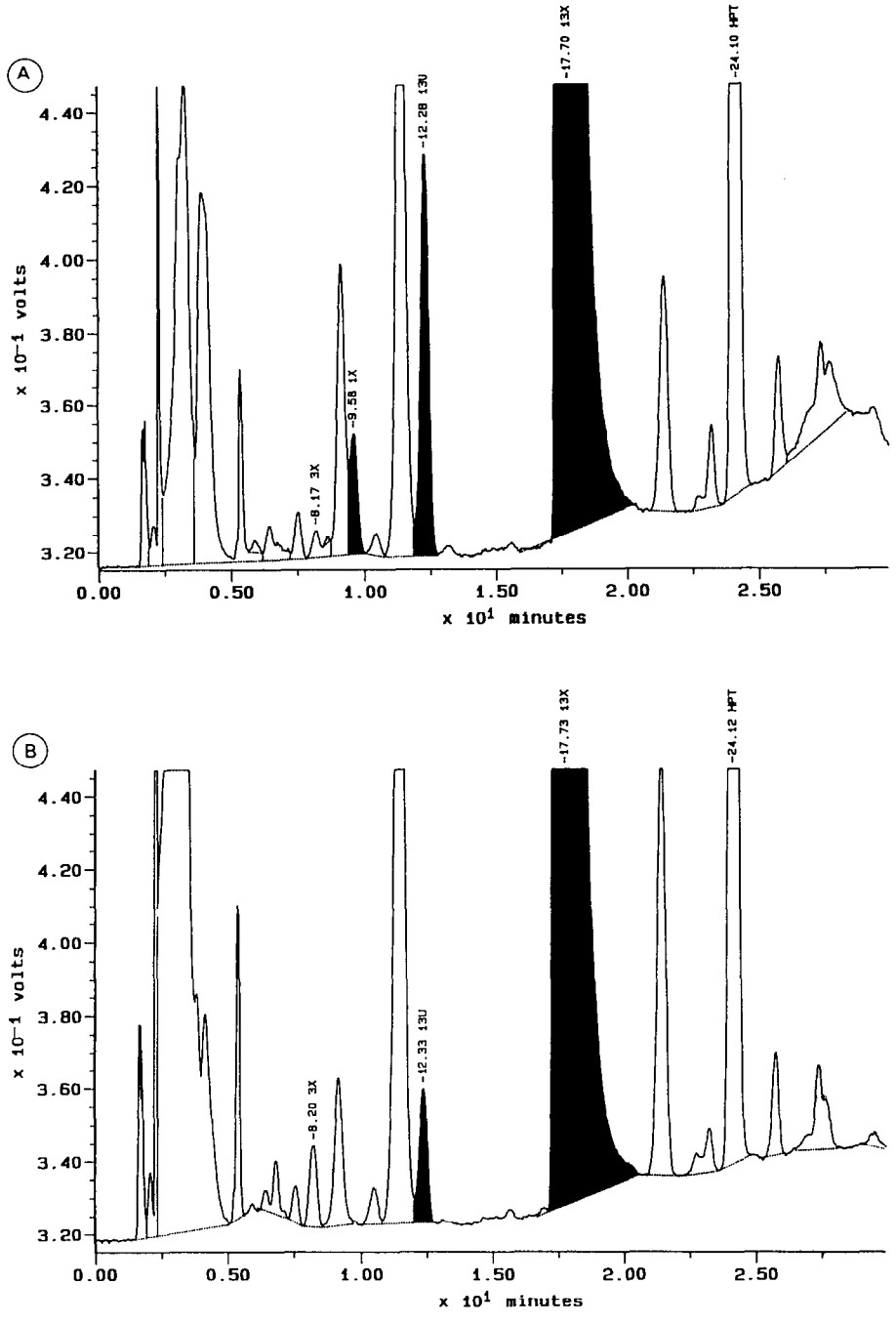

Fig. 5. HPLC-chromatograms of incubation medium following incubations of cell lines with 4 mM theophylline for 3 days (from Fuhr et al., 1992b, modified). Black, definite peaks of substrates and their metabolites; peak identification is written at the top of each peak (see list of abbreviations): (A) rat P450IA2 cell line and (B) human P450IA2 cell line.

Table 2
Theophylline metabolism by V79 chinese hamster cell lines expressing single cytochrome P450 isoforms (mean and S.D. given for three incubations carried out at different days; for incubation procedure see Materials and methods) (from Fuhr et al. 1992b)

Cell line	Cytochrome P450 isoform	Metabolite formed (μM)		
		1X	3X	13U
V79	no P450	[a]	[a]	1.9 ± 0.1
SD1	rat P450IIB1	[a]	[a]	3.7 ± 0.6
XEM2	rat P450IA1	[a]	[a]	8.9 ± 1.3
XEMdMz	rat P450IA2	2.3 ± 0.2	[a]	25.4 ±4.3
XEMHIA2.36	human P450IA2	[a]	[a]	5.2 ± 1.3
XEMHIA2.43	human P450IA2	[a]	[a]	8.6 ± 1.4

[a]Below detection limit (differing from that of the method due to interfering peaks), i.e. (μM): 1X, 0.6; 3X, 1.0; 13U, 0.8.

late theophylline. Expression of P450IIB1 also gave small additional amounts of the hydroxylated metabolite.

4. Discussion

The intention of this study was to define the role of cytochrome P450IA2 in the metabolism of caffeine and theophylline by means of genetically modified mammalian cells expressing single cytochrome P450 isoforms and to test the suitability of such cell lines for investigations on drug interactions occurring at the cytochrome expressed. Our results show that both rodent and human P450IA2 mediate primary caffeine degradation, which is mainly demethylation. Rat P450IA1 was unable to demethylate caffeine to a significant extent (Table 1). Theophylline was metabolized mainly via 8-hydroxylation; the reaction was most active in cells expressing rat P450IA1, or rat or human P450IA2 (Table 2). Thus, demethylation of caffeine seems to be a specific feature of P450IA2, whereas hydroxylation of the two methylxanthines may be mediated by a set of isoforms including both P450IA1 and IA2, of which some may be active only at high substrate concentrations in vitro. Addition of the quinolone antibiotic agents pipemidic acid and pefloxacin, both known to inhibit caffeine metabolism in vivo and in human liver microsomes, reduced formation rates of all metabolites of caffeine in cells expressing rat and human P450IA2.

4.1. Cell activity

The genetically engineered cell lines employed in this study have been validated in several studies (Doehmer et al. 1988; Battula 1989; Platt et al. 1989; Waxman et al. 1989; Doehmer et al. 1990; Dogra et al. 1990; Wölfel

et al. 1991; Wölfel et al. 1992). Metabolic activity of the cells in methylxanthine degradation was approximately one order of magnitude lower than that of cultured human hepatocytes reported by Berthou et al. (1989) who found an metabolic rate of (median) 12 pmol/min/10^6 cells when using similar concentrations of substrates.

4.2. Caffeine metabolism is mediated by P450IA2

We observed that the pattern of primary caffeine metabolites by isolated human P450IA2 is similar to that reported in vivo as well as to those obtained by human hepatocytes, by liver slices or by human liver microsomes (Table 3). By correlation studies with metabolic activity for specific pathways of human P450IA2 and with P450IA2 content, and by use of a specific antibody to inhibit enzyme activity, Butler et al. (1989) provided good evidence for this isoform to mediate caffeine 3-demethylation, which accounts for as much as 70–80% of caffeine metabolism in vivo (Arnaud et al. 1982; Berthou et al. 1989). A low and a high affinity form of caffeine metabolizing cytochromes were proposed (Campbell et al. 1987; Berthou et al. 1989) and an incomplete inhibition of 13X, 37X and 137U formation by P450IA2 inhibitors has been described (Robson et al. 1988; Fuhr et al. 1990; Sesardic et al. 1990). Furthermore the lack of correlation between 13X, 37X, and 137U formation and other reactions typical for P450IA2 (Berthou et al. 1991). These results obtained using human liver microsomes indicate that enzymes other than cytochrome P450IA2 are able to metabolize caffeine. The caffeine concentrations used for incubations in these studies were 1 mM and more. This is a relatively high concentration compared to 30–50 μM concentrations normally occurring in vivo. Substrate concentrations in the present study were also 1 mM and higher, but in this case, the absence of cytochromes P450 other than that introduced into the cells guarantees that all metabolites formed additionally when compared to controls are due to the isoform expressed.

Our results strongly suggest that the main part of caffeine 1-demethylation and a part of 7-demethylation in vivo is also mediated by P450IA2. Minor 8-hydroxylation of caffeine by human P450IA2 was observed only occasionally; thus the role of this isoform in vivo in the hydroxylation pathway, if any, is expected to be negligible. The assumption that the 1-demethylation of caffeine is catalyzed by human P450IA2 is further supported by significant correlations reported between 1- and 3-demethylation (Grant et al. 1987; Berthou et al. 1991). Lack of correlation between 7-demethylation and/or 8-hydroxylation and 3-demethylation of caffeine (Campbell et al. 1987; Grant et al. 1987; Berthou et al. 1991) again indicates that human P450IA2, although qualitatively shown here to be competent for these two reactions, may mediate only a minor part of these less important pathways in vivo.

Therefore, we suggest that the high affinity form primarily responsible for

Table 3
Contribution of demethylation and hydroxylation pathways to caffeine metabolism in different human systems (from Fuhr et al. 1992b)

Human system	Caffeine conc.	Primary metabolites formed (%)				Reference
		13X	17X	37X	137U	
In vivo	unknown	8	73	20	2	Arnaud 1982
Cultured hepatocytes	1 mM	12	68	18	2	Berthou 1989
Liver slices	12 μM	15[a]	70[a]	15[a]	[a]	Berthou 1989
Liver microsomes	1 mM	10	65	12	12	Berthou 1989
Human P450IA2 expressed in mammalian cells[b]	4 mM	4 ± 2	84 ± 1	12 ± 2	1 ± 2	Present study

[a] Only percentage of demethylation pathways were given.
[b] mean ± S.D. ($n = 6$).

caffeine demethylations, especially 1- and 3-demethylation, is P450IA2 and low affinity forms are other isoforms not able to carry out 3-demethylation of caffeine to a considerable extent. This hypothesis is supported by the observation that furafylline, a specific and very potent inhibitor of human P450IA2 (Sesardic et al. 1990), prolongs caffeine elimination in vivo exorbitantly (Tarrus et al. 1987). The more than 7-fold increase in caffeine half-live, despite a small fraction of caffeine directly excreted renally, corresponds to an inhibition of enzyme activities by 90% or more. This can be explained only by involvement of P450IA2 in the in vivo formation of primary caffeine metabolites other than 17X, since this pathway mediates less than 80% of primary caffeine metabolism (see above).

4.2.1. Caffeine as a test drug for P450IA2 activity in man. More than 90% of primary caffeine metabolism in man may be due to P450IA2, including all demethylations and perhaps even a small fraction of the hydroxylation pathway. Enzymes other than (hepatic) P450IA2 do not contribute significantly to the primary overall degradation of this drug in vivo. The data presented here suggest that caffeine plasma clearance following the application of a test dose can be used as a specific marker of P450IA2 activity in man. Caffeine test doses may be given as coffee or caffeinated soft drinks, as proposed earlier in investigations on N-acetylation (Grant et al. 1984), without loss of specifity for P450IA2. The presence of minor amounts of other methylxanthines in these beverages is not expected to interfere significantly with caffeine metabolism. Furthermore, it does not cause problems in distinguishing between methylxanthines other than caffeine already present in the drink and those resulting from caffeine breakdown, as would be the case in the determination of 3-demethylation of caffeine in vivo (Sesardic et al. 1990) (which is difficult by itself), rather than of caffeine elimination as a whole.

4.3. A new in vitro model to predict drug interactions occurring in vivo

We tested the effect of the quinolone antibiotic agents pefloxacin and pipemidic acid on caffeine metabolism in the cell lines. These substances both have been shown to reduce metabolic rates of caffeine in vivo (Harder et al. 1988; Carbo et al. 1989) and in human liver microsomes (Fuhr et al. 1992a). These congeners were chosen because the extent of their inhibitory effects differed between these systems. Whereas pipemidic acid showed an effect in microsomes three times that exerted by pefloxacin, the former was only 1.4-fold more effective than the latter in volunteers (Table 4). We presumed that, beside other factors, penetration of both substrate and inhibitor into the liver cell may be responsible for these discrepancies, which cause substantial limitations in prediction of drug interactions occurring in vivo by this in vitro test system. In search for a cellular system which is expected to mimic in vivo

Table 4
Inhibitory effects of pefloxacin (PEFL) and pipemidic acid (PPA) on caffeine metabolism in different human systems

Human system	Quinolone conc.	Caffeine conc.	Quinolone effect PEFL	PPA	Reference
In vivo	unknown[a]	unknown[a]	half life prolonged by 96%	by 136%	Harder 1988; Carbo 1989; Mahr 1990.
Liver microsomes	500 μM	500 μM	17X format. reduced by 22%	by 59%	Fuhr 1992a
Human P450IA2 expressed in mammalian cells	500 μM	500 μM	17X format. reduced by 49%	by 69%	Present study

[a]Although caffeine concentrations have been determined in this study, concentrations at the binding site in the liver cell are unknown; for doses, see references.

conditions better than liver microsomes do, we tested the V79 cell lines expressing P450IA2. Indeed, inhibitory effects of the two quinolones were much alike to those in the in vivo situation (see Table 4). These first promising results are currently being substantiated by testing a wide range of compounds acting as inhibitors of cytochrome P450IA2 in vivo.

4.4. Theophylline metabolism depends on several enzymes including P450IA2

The biotransformation of this methylxanthine differs in many respects from that of caffeine. Its major metabolite in all systems tested is 13U, the 8-hydroxylated product, reaching 61% of metabolites in vivo (see Table 5). There are conflicting results on a possible role of P450IA2 in theophylline metabolism. Inhibition of theophylline metabolism in human liver microsomes by caffeine (Robson et al. 1988) and by quinolone antibiotic agents (Sarkar et al. 1990) which have been shown to inhibit P450IA2 activity (Fuhr et al. 1990) indicates an involvement of P450IA2 in theophylline biodegradation, but more than one isoform seems to mediate primary metabolism of this drug (Robson et al. 1988). A good correlation between caffeine 3-demethylation and theophylline 1-demethylation, but not 8-hydroxylation (Campbell et al. 1987) provides evidence for P450IA2 to mediate only theophylline demethylations. This presumption is supported by results of Sarkar et al. (1990) using 10 mM theophylline concentrations in human liver microsomes who found an inhibition of demethylation rather than hydroxylation by quinolone antibiotics which are potent inhibitors of P450IA2 (Fuhr et al. 1990), but of unknown specifity. The K_I values are different for demethylation and hydroxylation using low theophylline concentrations in human liver microsomes again shows the involvement of more than one cytochrome P450 isoform (Robson et al. 1988). On the other hand, Mulder et al. (1988) reported inhibition of theophylline (1 mM) hydroxylation by a quinolone in

Table 5
Contribution of demethylation and hydroxylation pathways to theophylline metabolism in different human systems (from Fuhr et al. 1992b)

Human system	Theophylline conc.	Primary metabolites formed (%)			Reference
		1X	3X	13U	
In vivo	unknown	24	15	61	Birkett 1982
Liver microsomes	150 μM	8	5	87	Robson 1987
Liver microsomes	1.25 mM	16	15	69	Sarkar 1990
Human P450IA2 expressed in mammalian cells	4 mM	—[a]	—[a]	>81[a]	Present study

[a]other metabolites in incubations lower than detection limit.

rat hepatocytes; demethylations were unchanged. Human theophylline metabolism in vivo is prolonged by concomitant application of quinolones (Wijnands et al. 1986) to an extent requiring inhibition of the main metabolic pathway, i.e. 8-hydroxylation.

We could show that human cytochrome P450IA2 is able to mediate theophylline 8-hydroxylation as a major metabolic pathway. The contribution of further isoforms to primary theophylline metabolism both in vivo and in vitro remains to be evaluated. The formation of 13U (and of 137U out of caffeine) even by cells without cytochromes may be mediated by xanthine oxidase, an enzyme which is known to carry out 8-hydroxylation of 1X to 1U (Birkett et al. 1982).

4.5. Species differences in cytochrome P450IA2 function

One of the most interesting results of the present study is the difference between the species observed for caffeine and theophylline metabolism by cytochrome P450IA2 cell lines. Due to the presence of a single cytochrome P450, the pattern of metabolites is neither expected to depend on substrate concentration nor on incubation time and could be reproduced easily. A further degradation of metabolites is not to be expected, because the competition between the substrates added and the metabolites is in favour of the original substrates due to its more than 300-fold higher concentrations. Thus, it is possible to compare between cell lines with single P450 isoforms even when incubation conditions were not identical. Whereas the human P450IA2 gave a metabolic pattern of caffeine similar to that obtained in other human systems (see Table 3) with preference to the 3-demethylation pathway, rat P450 mediated 1-demethylation to a similar extent. Mouse P450IA2 holds an intermediate position.

The lack of detectable amounts of demethylated products by V79 derived cell lines other than XEMdMz allows no conclusion on species differences in the metabolic pattern of theophylline, but the high activity of P450IA1 in theophylline degradation found here is expected to cause a species diversity in theophylline degradation in vivo, because this isoform usually is not present in the human liver (Wrighton et al. 1986). Species diversities also became obvious in inhibition experiments with quinolones (Fig. 4), indicating that the affinity of inhibitors to the rat P450IA2 is not the same as to human P450IA2. These species differences observed for P450IA activity provide evidence that it is necessary to use caution in extrapolating animal data on drug metabolism and on interactions depending on this isoform to predict metabolism in man.

5. Acknowledgements

We thank Karin Hehlert for her technical assistance. This work is funded in part (J.D., I.F., C.W.) by the Deutsche Forschungsgemeinschaft, SFB 302,

the Bundesgesundheitsamt (ZEBET), Fo 2.1-1328-107 and the Naturwissenschaftlich-Medizinisches Forschungszentrum (NMFZ) of the Johannes Gutenberg-Universität Mainz.

6. References

Arnaud, M.J. and Welsch, C. (1982). Theophylline and caffeine metabolism in man. In: N. Rietbrock, B.G. Woodcock and A.H. Staib (Eds), Theophylline and other Methylxanthines, Vieweg Verlag, Braunschweig and Wiesbaden, pp. 135–148.

Battula, N. (1989). Transduction of cytochrome P_3-450 by retroviruses: Constitutive expression of enzymatically active microsomal hemoprotein in animal cells. J. Biol. Chem. 264, 2991–2996.

Berthou, F., Ratanasavanh, D., Alix, D., Carlhant, D., Riche, C. and Guillouzo, A. (1988). Caffeine and theophylline metabolism in newborn and adult human hepatocytes; comparison with adult rat hepatocytes. Biochem. Pharmacol. 37, 3691–3700.

Berthou, F., Ratanasavanh, D., Riche, C., Picart, D., Voirin, T. and Guillouzo, A. (1989). Comparison of caffeine metabolism by slices, microsomes and hepatocyte cultures from adult human liver. Xenobiotica 19, 401–417.

Berthou, F., Flinois, J.P., Ratanasavanh, D., Beaune, P., Riche, C. and Guillouzo, A. (1991). Evidence for the involvement of several cytochromes P450 in the first steps of caffeine metabolism by human liver microsomes. Drug Metab. Dispos. 19, 561–567.

Birkett, D.J., Grygiel, J.J. and Miners, J.O. (1982). Metabolic disposition of the methylxanthines in man. In: N. Rietbrock, B.G. Woodcock and A.H. Staib (Eds), Theophylline and other methylxanthines, Vieweg Verlag, Braunschweig and Wiesbaden, pp. 149–158.

Butler, M.A., Iwasaki, M., Guengerich, F.P. and Kadlubar, F.K. (1989). Human cytochrome $P450_{PA}$ (P450IA2), the phenacetin *O*-deethylase, is primary responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. Proc. Natl. Acad. Sci. USA 86, 7696–7700.

Campbell, M.E., Grant, M.D., Inaba, T. and Kalow, W. (1987). Biotransformation of caffeine, paraxanthine, theophylline, and theobromine by polycyclic aromatic hydrocarbon-inducible cytochrome(s) P450 in human liver microsomes. Drug Metabol. Dispos. 15, 237–249.

Carbo, M.L., Segura, J., de la Torre, R., Badenas, J.M. and Cami, J. (1989). Effect of quinolones on caffeine disposition. Clin. Pharmacol. Ther. 45, 234–240.

Doehmer, J., Dogra, S., Friedberg, T., Monier, S., Adesnik, M., Glatt, H. and Oesch, F. (1988). Stable expression of rat cytochrome P450IIB1 cDNA in chinese hamster cells (V79) and metabolic activation of aflatoxin B_1. Proc. Natl. Acad. Sci. USA 85, 5769–5773.

Doehmer, J., Seidel, A., Oesch, F. and Glatt, H.R. (1990). Genetically engineered V79 chinese hamster cells metabolically activate the cytostatic drugs cyclophosphamide and ifosfamide. Environ. Health Perspect. 88, 63–65.

Dogra, S., Doehmer, J., Glatt, H., Mölders, H., Siegert, P., Friedberg, T., Seidel, A. and Oesch, F. (1990). Stable expression of rat cytochrome P450IAI cDNA in V79 chinese hamster cells and their use in mutagenicity testing. Mol. Pharmacol. 37, 608–613.

Fuhr, U., Wolff, T., Harder, S., Schymanski, P. and Staib, A. H. (1990). Quinolone inhibition of cytochrome P450-dependent caffeine metabolism in human liver microsomes. Drug Metabol. Dispos. 18, 1005–1010.

Fuhr, U., Anders, E.M., Mahr, G., Sörgel, F. and Staib, A.H. (1992a). Inhibitory potency of quinolone antibiotics agents on cytochrome P450IA2 activity in vivo and in vitro. Antimicrob. Agents Chemother. 36, 942–948.

Fuhr, U., Doehmer, J., Battula, N., Wölfel, C., Kudla, C., Keita, Y., Staib, A.H. (1992b). Bio-

transformation of caffeine and theophylline in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. Biochem. Pharmacol. 43, 225–235.

Gelboin, H.V. and Friedman, F.K. (1985). Monoclonal antibodies for studies on xenobiotic and endobiotic metabolism. Biochem. Pharmacol. 34, 2225–2234.

Glatt, H.R., Gemperlein, I., Turchi, G., Heinritz, H., Doehmer, J. and Oesch, F. (1987). Search for cell culture systems with diverse xenobiotic-metabolizing activity and their use in toxicological studies. Mol. Toxicol. 1, 313–334.

Gonzalez, F.J., Aoyama, T. and Gelboin, H.V. (1990). Activation of promutagens by human cDNA-expressed cytochrome P450s. Mutat. Environm. B. 77–86.

Grant, D.M., Tang, B.K. and Kalow, W. (1984). A simple test for acetylator phenotype using caffeine. Br. J. Clin. Pharmacol. 17, 459–464.

Grant, D.M., Campbell, M.E., Tang, B.K. and Kalow, W. (1987). Biotransformation of caffeine by microsomes from human liver. Biochem. Pharmacol. 36, 1251–1260.

Harder, S., Staib, A.H., Beer, C., Papenburg, A., Stille, W. and Shah, P.M. (1988). 4-quinolones inhibit biotransformation of caffeine. Eur. J. Clin. Pharmacol. 35, 651–656.

Kalow, W. and Campbell, M.E. (1988). Biotransformation of caffeine by microsomes. ISI Atlas of Science: Pharmacology, pp. 381–386.

Mahr, G., Seelmann, R., Dammeyer, J., Seibel, M., Muth, P., Gottschalk, B., Stephan, U. and Sörgel, F. (1990). The effect of pefloxacin and enoxacin on the elimination of caffeine in human volunteers. Program Abstr. 3rd Intern. Symp. on New Quinolones, Vancouver, pp. 529.

Mulder, G.J., Nagelkerke, J.F., Tijdens, R.B., Wijnands, W. J.A. and van der Mark, E.J. (1988). Inhibition of the oxidative metabolism of theophylline in isolated rat hepatocytes by the quinolone antibiotic enoxacin and its metabolite oxoenoxacin, but not by ofloxacin. Biochem. Pharmacol. 37, 2565–2568.

Nielsen-Kudsk, J.E., Buhl, J.S. and Johannessen, A.C. (1990). Verapamil-induced inhibition of theophylline in healthy humans. Pharmacol. Toxicol. 66, 101–103.

Platt, K.L., Molitor, E., Döhmer, J., Dogra, S. and Oesch, F. (1989). Genetically engineered V79 chinese hamster cell expression of purified cytochrome P450IIB1 monooxygenase activity. J. Biochem. Toxicol. 4, 1–6.

Robson, R.A., Matthews, A.P., Miners, J.O., McManus, M.E., Meyer, U.A., Hall, P. de la M. and Birkett, D.J. (1987). Characterisation of theophylline metabolism in human liver microsomes. Inhibition and immunochemical studies. Br. J. Clin. Pharmacol. 24, 293–300.

Robson, R.A., Miners, J.O., Matthews, A.P., Stupans, I., Meller, D., McManus, M. and Birkett, D.J. (1988). Characterisation of theophylline metabolism by human liver microsomes. Inhibition and immunochemical studies. Biochem. Pharmacol. 37, 1651–1659.

Russel, W.C., Newman, C. and Williamson, D.H. (1975). A simple cytochemical technique for demonstration of DNA in cells infected with Mycoplasma and viruses. Nature 253, 461–462.

Sarkar, M., Polk, R.E., Guzelian, P.S., Hunt, C. and Karnes, H.T. (1990). In vitro effect of fluoroquinolones on theophylline metabolism in human liver microsomes. Antimicrob. Agents Chemother. 34, 594–599.

Sesardic, D., Boobis, A.R., Murray, B.P., Murray, S., Segura, J., de la Torre, R. and Davies, D.S. (1990). Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. Br. J. Clin. Pharmacol. 29, 651–663.

Tarrus, E., Cami, J., Roberts, D.J., Spickett, R.G.W., Celdran, E. and Segura, J. (1987). Accumulation of caffeine in healthy volunteers treated with furafylline. Br. J. Clin. Pharmacol. 23, 9–18.

Waxman, D.J., Lapenson, D.P., Morrissey, J.J., Park, S.S., Gelboin, H.V., Doehmer, J. and

Oesch, F. (1989). Androgen hydroxylation catalysed by a cell line (SD1) that stably expresses rat hepatic cytochrome P450 PB-4 (IIB1). Biochem. J. 260, 81–85.

Wijnands, W.J.A., Vree, T.B. and van Herwaarden, C.L.A. (1986). The influence of quinolone derivatives on theophylline clearance. Br. J. Clin. Pharmacol. 22, 677–683.

Wölfel, C., Platt, K.L., Dogra, S., Glatt, H., Wächter, F., Oesch, F. and Doehmer, J. (1991). Stable expression of rat cytochrome P450IA2 cDNA in V79 chinese hamster cells and hydroxylation of 17β-estradiol and 2-aminofluorene. Mol. Carcinogen. 4, 489–498.

Wölfel, C., Gonzalez, F.J., Glatt, H., Platt, K.L. and Doehmer, J. (1992). Stable expression of human cytochrome P450IA2 cDNA in V79 chinese hamster cells. Eur. J. Pharmacol., Environm. Toxicol., Pharmacol. Section 228, 95–102.

Wrighton, S.A., Campanile, C., Thomas, P.E., Maines, S.L., Watkins, P.B., Parker, G., Mendez-Picon, G., Haniu, M., Shively, J.E., Levin, W. and Guzelian, P.S. (1986). Identification of a human liver cytochrome P450 homologous to the major isosafrole-inducible cytochrome P450 in the rat. Mol. Pharmacol. 29, 405–410.