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BIOTRANSFORMATION OF CAFFEINE AND THEOPHYLLINE IN MAMMALIAN CELL LINES GENETICALLY ENGINEERED FOR EXPRESSION OF SINGLE CYTOCHROME P450 ISOFORMS

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Abstract—Primary steps in the metabolism of caffeine and theophylline are cleavage of methyl groups and/or hydroxylation at position 8, mediated by cytochromes P450. V79 Chinese hamster cells genetically engineered for stable expression of single forms of rat cytochromes P450IA1, P450IA2 and P450IIBI and human P450IA2 and rat liver epithelial cells expressing murine P450IA2 were used to overcome problems arising in the proper allocation of metabolic pathways to specific isoforms by conventional techniques. These 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 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. Theophylline was metabolized mainly 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. These results support the hypothesis that caffeine plasma clearance is a specific *in vivo* probe for determining human P450IA2 activity.

Scientific interest in the metabolism of both caffeine (1,3,7-trimethylxanthine, 137X‖) and theophylline has experienced a revival recently. Caffeine is accepted increasingly as a model drug for testing of the hepatic capacity of drug metabolism in general and as an *in vitro* indicator of cytochrome P450IA2 activity, an isoform responsible for the bioactivation of potent carcinogens [1, 2]. Theophylline is involved in several clinically important drug interactions [3, 4], a topic of growing importance in therapeutics as well as in drug regulatory affairs.

Therefore, a precise allocation of the various metabolic pathways to the enzymes involved is essential. The only direct way to achieve this goal is to study the metabolism of drugs by single enzymes and to monitor metabolites formed. Primary steps in the metabolism of caffeine and theophylline (for structures see Fig. 1) in rodents and in man are cleavage of methyl groups and hydroxylation at position 8, mediated by cytochrome P450 isoforms [5–8]. Low and high affinity isoforms have been proposed for the demethylation steps [9]. Hydroxylation was reported not to be reduced by inhibitors of demethylation and, therefore, to be carried out by other enzymes [6]. Our previous investigations with human liver microsomes indicated that a single cytochrome P450 is likely to mediate 3-demethylation, but at least two isoforms were involved in 7-demethylation and the hydroxylation of caffeine [10]. From correlation studies with metabolic activity for specific pathways of human P450IA2 and with P450IA2 content, and by the use of a specific antibody to inhibit enzyme activity Butler *et al.* [1] obtained good evidence that this isoform mediates caffeine 3-demethylation. Prolongation of caffeine half-life *in vivo* by coadministration of the specific P450IA2 inhibitor furafylline suggests that the predominant metabolic pathway(s) of caffeine (3-demethylation accounts for about 80% of primary caffeine degradation in man [11, 12] may be mediated almost exclusively by cytochrome P450IA2 [13].

Inhibition of theophylline metabolism in human

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‖ 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 (theophylline); 13U, 1,3-dimethyluric acid; 17X, 1,7-dimethylxanthine (paraxanthine); 17U, 1,7-dimethyluric acid; 37X, 3,7-dimethylxanthine (theobromine); 37U, 3,7-dimethyluric acid; 137X, 1,3,7-trimethylxanthine (caffeine); 137U, 1,3,7-trimethyluric acid; HPT, 7-(2-hydroxypropyl)-1,3-dimethylxanthine, hydroxypropyltheophylline.

liver microsomes by caffeine [6] and by quinolone antibiotic agents [14], which have been shown to inhibit P450IA2 activity [10], indicates an involvement of P450IA2 in theophylline biodegradation. However, more than one isoform seems to mediate primary metabolism of this drug [6].

Thus, the role of P450IA2 in the 3-demethylation of caffeine is well established whereas it remains unclear to what extent this isoform contributes to the metabolism of caffeine to other metabolites or to that of theophylline.

One of the major difficulties in the identification of cytochromes P450 responsible for methylxanthine degradation may be the relatively low *in vitro* activity of the enzymes in both liver microsomes and cultured hepatocytes [6, 12]. Studies using purified P450IA2 have not been published to our knowledge, probably as a result of further reduction of activity in reconstituted systems. To overcome problems arising in the proper allocation of pathways to isoforms by conventional indirect techniques (specificity of antibodies, substrates or inhibitors) and by isoform purification, we used recombinant cell lines genetically engineered for the stable expression of rat, mouse and human cytochromes to analyse the metabolism of caffeine and theophylline. The value of this approach has been reviewed [2, 15]. V79 cell lines expressing functional rat P450IA1 (the cell line was called "XEM2" [16], rat P450IA2 ("XEMdMz" [17]), rat P450IIB1 ("SD1" [18–20]), human P450IA2 ("XEMHIA2.36" and "XEMHIA2.43" [21]), rat liver epithelial cells expressing murine P450IA2 ("R52–16" [22]) 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.

MATERIALS AND METHODS

Cell culture

V79 Chinese hamster cells [23] and V79-derived cell lines expressing rat cytochrome P450IIB1, IA1 and IA2, and human IA2 have been evaluated for enzymatic activity, metabolic activation of promutagens and procarcinogens and drug metabolism [16–21]. The cells were maintained in Dulbecco Vogt Eagle's Medium (Dulbecco's Modified Eagle's Medium, high glucose) supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin 100 μg/mL) and 1 mM L-glutamine, and incubated at 37° 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 [24]. 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 the cells grew to a confluency close to 100%, corresponding to an absolute number of about 4×10^7 cells. Then, the medium was exchanged with medium without G418, containing 4 mM caffeine, 4 mM theophylline, or no test substrate as a control. Three days later the supernatant medium was harvested, centrifuged at 3000 *g* to separate cell debris and subjected to HPLC analysis. These conditions were optimized for maximum metabolism taking into account the fact that caffeine and theophylline had cytostatic effects in all cell lines.

The rat liver epithelial cell lines [22] have already been used successfully in investigations of carcinogenesis and drug metabolism. These cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL) and G418 (200 μg/mL), and incubated at 37° in 5% CO_2 in air at 100% humidity. Caffeine metabolism only was investigated using this cell line under conditions similar to those described for V79-derived cell lines with the exception that incubation times were shorter (up to 24 hr) and substrate concentration was 1 mM.

HPLC Assay of Metabolites

Chemicals. Methylxanthines were obtained from the following companies: 137X (Serva, Heidelberg, F.R.G.), 17X, 13U (Aldrich, Milwaukee, U.S.A.), 13X (Klinge, München, F.R.G.), 137U, 37X, 1X, 3X, HPT (Fluka, Buchs, Switzerland). Acetonitrile, 2-propanol, diisopropyl ether, and tetrahydrofuran were Merck chromatography grade products (Darmstadt, F.R.G.). 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 concentrations measured. Theophylline chromatograms at 10 mM did not show peaks of possible metabolites (impurity <0.002%). All other chemicals (analytical grade) were purchased from Merck.

Devices. Concentrations of methylxanthines and derivatives were measured using a Waters HPLC device (pump 510, detector UV-VIS 490E, autosampler WISP 710A with a NEC AP IV personal computer running the Waters Maxima® software).

Sample preparation. An aliquot of 750 μL of the sample, 75 μL of formic acid (100%), 250 mg of $(NH_4)H_2PO_4$, and 30 μL of internal standard (100 mg/L HPT, aqueous solution) were mixed and dissolved in a 10-mL glass test tube. Thereafter 5 mL of organic extraction solution (diisopropyl ether 70%, 2-propanol 30%, v/v) were added, followed by vigorous shaking for 30 sec on a vortex and another 2 min, manually. After centrifugation, the organic layer was transferred into a second glass test tube. The extraction step was repeated, and the two fractions were pooled and evaporated to dryness (air, 45°). The residue was dissolved in 150 μL of 0.1 M acetate buffer pH 4 containing 10% of acetonitrile.

Loading sample. Of a prepared sample, 35 μL were injected onto the column. The column (250 × 4 mm, packed with Nucleosil 5 μM (Merck)) was equilibrated to 27°. Metabolites were resolved by gradient elution with two solvents. Solvent A consisted of 50 mL 0.1 M sodium acetate buffer pH 4.0, 6 mL tetrahydrofuran and 944 mL aqua bidest.

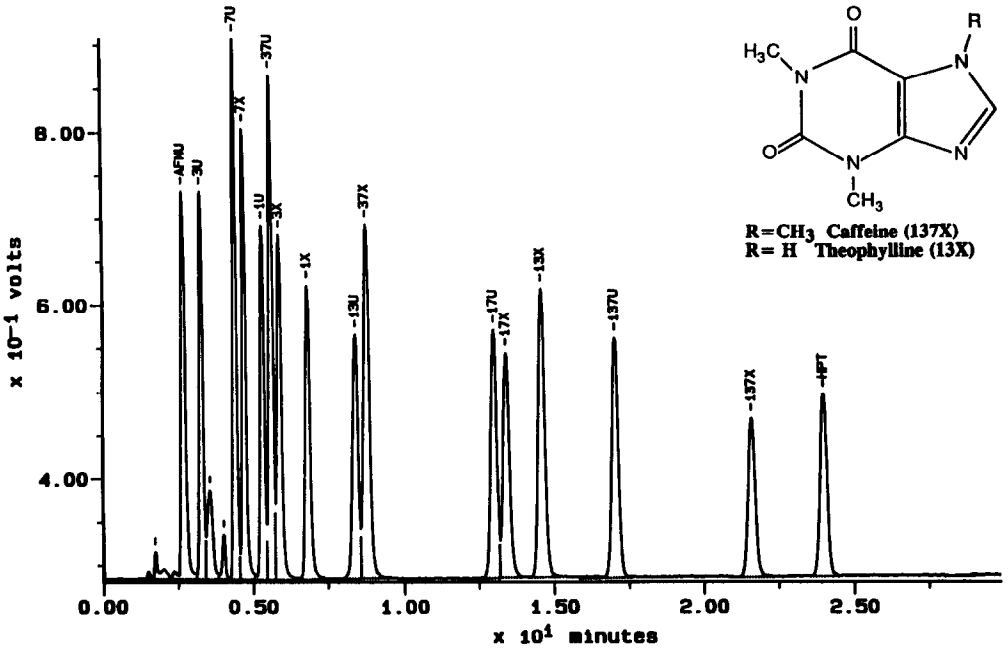


Fig. 1. HPLC-chromatogram of aqueous solution containing methylxanthines, methyluric acids and 5-acetylamino-6-formyl-amino-3-methyluracil (AFMU) (20 μg/ml each). Peak identification is written at the top of each peak.

Solvent B consisted of 50 mL 0.1 M sodium acetate buffer pH 4.0, 15 mL tetrahydrofuran, 70 mL acetonitrile and 865 mL water, and was subsequently adjusted to pH 3.0 with acetic acid. A flow rate of 1.4 mL/min was maintained during the run. HPLC was started with 100% of solvent A. After 4 min, solvent B was added reaching 10% by 8 min and 100% by 22 min. Solvent B (100%) was run to elute the remaining peaks off the column. Minor modifications due to differences in column characteristics and column ageing were made when necessary to optimize peak separation. The UV absorption of the methylxanthines was monitored at a wavelength of 278 nm (see Fig. 1). Peak height ratios with the internal standard (HPT) were used to determine concentrations. These ratios were proportional to spiked concentrations of all substances measured from detection limit up to 30 μM ($r^2 > 0.998$). Variability of the method was determined by repeated measurements of spiked samples (see Table 1). 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. 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.

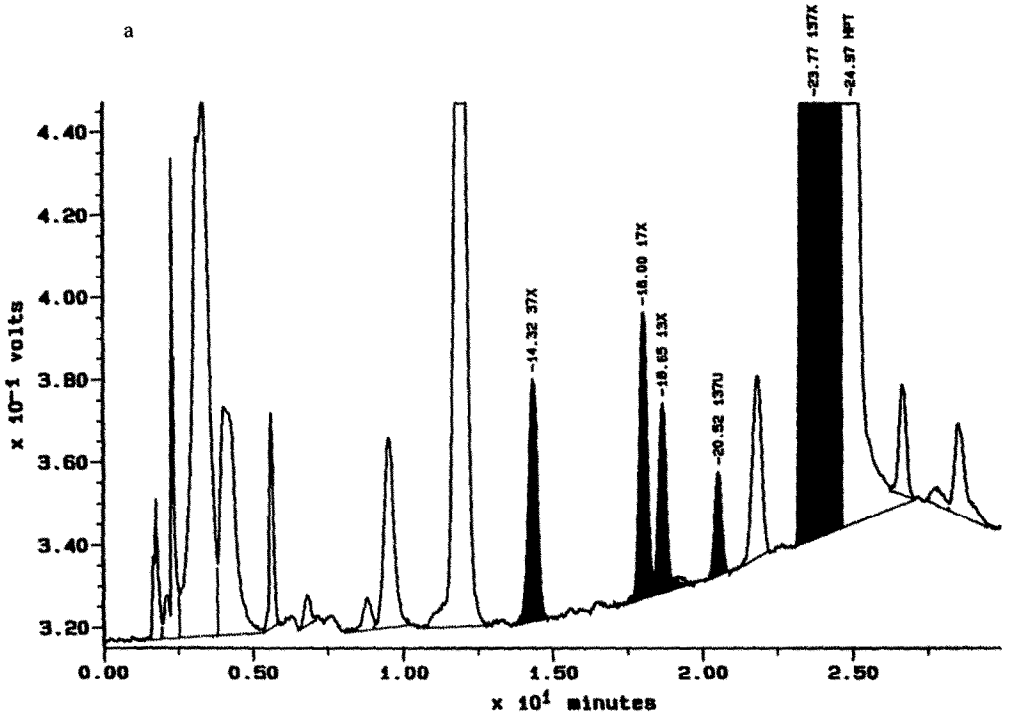
Interactions with further methylxanthines can be ruled out because the retention times of other possible (secondary) metabolites were different from those of the primary metabolites (see Fig. 1). The recovery of metabolites following extraction was determined by the comparison of aqueous standard solution with medium samples spiked with 300 and 300 nM of each metabolite (see Table 2) corresponding to the absolute amount of 52.5 and 525 pmol (in aqueous solution) of each metabolite injected onto the column, respectively. The quotient

Table 1. Determination of caffeine and theophylline metabolite concentrations by HPLC: coefficient of variation in repeated measurement of spiked samples

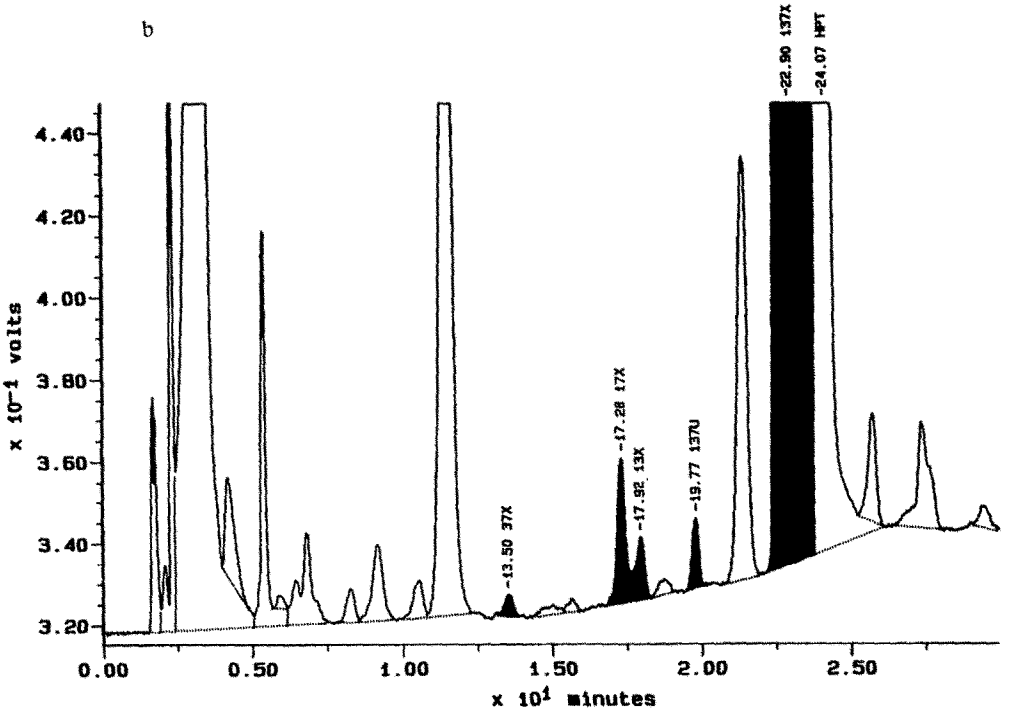
Spiked Concentration (nM)	N	Coefficient of variation of HPLC method (%)							
		1X	3X	13X	13U	17X	37X	137X	137U
100	6	ND	29.2	15.1	ND	9.3	19.7	9.1	13.7
300	6	4.5	7.9	6.8	ND	5.5	4.5	9.1	8.3
1000	6	3.3	2.4	2.7	6.8	2.3	1.5	3.3	1.7
3000	6	2.8	3.4	1.5	4.2	1.5	1.8	1.9	1.4
10000	6	3.5	3.3	3.8	1.6	3.4	5.2	2.9	4.0

N.D., peak not detected in most samples.

a


-23.77 137X
-24.97 HPT
-14.32 37X
-18.00 17X
-18.65 13X
-20.52 137U
4.40
4.20
4.00
3.80
3.60
3.40
3.20
x 10-1 volts
0.00
0.50
1.00
1.50
2.00
2.50
x 10¹ minutes

b


-22.90 137X
-24.07 HPT
-13.50 37X
-17.28 17X
-17.92 13X
-19.77 137U
4.40
4.20
4.00
3.80
3.60
3.40
3.20
x 10-1 volts
0.00
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x 10¹ minutes

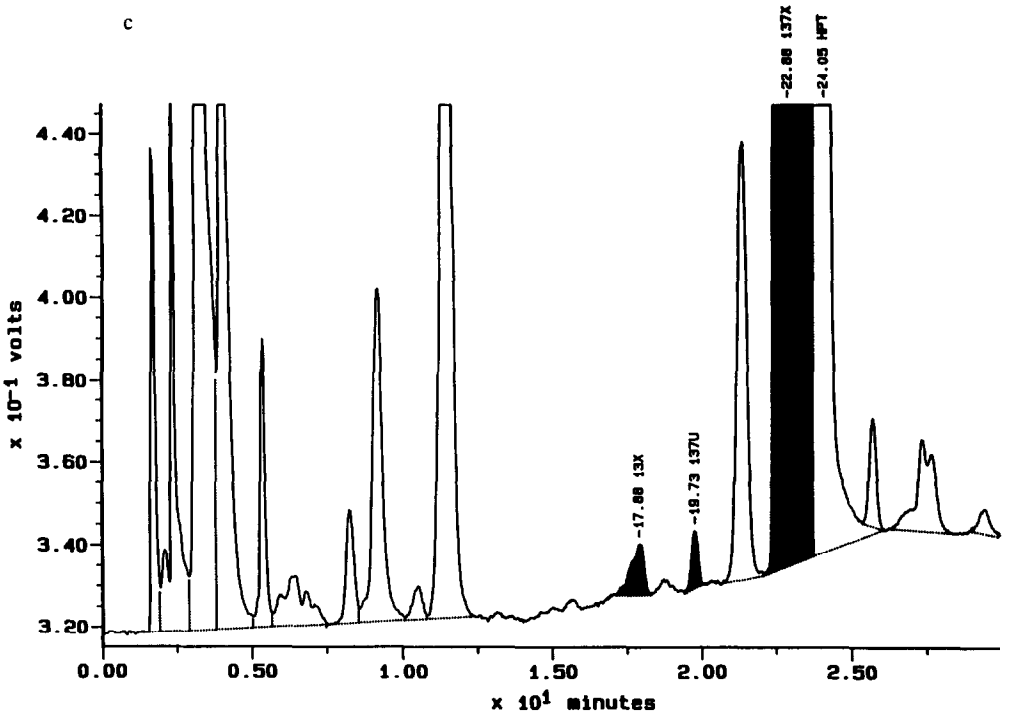


Fig. 2. HPLC-chromatograms of incubation medium following incubations of cell lines with 4 mM caffeine for 3 days. Black: definite peaks of substrates and their metabolites; peak identification is written at the top of each peak. (a) Rat P450IA2 cell line; (b) human P450IA2 cell line; (c) cell line without cytochromes P450.

of the means of three determinations (HPLC peak height) is displayed.

The 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 the expression of single P450 isoforms.

RESULTS

Improvements in the separation and identification of methylxanthine metabolites

Our attempts to reproduce current methods using chloroform/isopropanol extraction [12, 25] showed a very variable and poor recovery of uric acids, and the separation of metabolite peaks was unsatisfactory in some cases. The HPLC method we developed for the determination of caffeine and theophylline metabolites is specific and sensitive enough to measure even low concentrations of unlabeled metabolites. It provides a clear separation of all methylxanthines and methyluric acids (Fig. 1). The activity of methylxanthine metabolism (approximate rates) was for V79-derived lines, <1 pmol/min/10^6 cells and for 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 the metabolism of caffeine or theophylline. Additional problems were caused by peaks appearing even when cells were incubated without methylxanthines.

Caffeine metabolism

To optimize the detection of metabolites, we used high substrate concentrations (4 mM) and prolonged

Table 2. Recovery of caffeine and theophylline metabolites following extraction procedure (spiked medium samples versus aqueous solution)

Spiked amount (pmol)	Recovery following extraction procedure (%)								
	1X	3X	13X	13U	17X	37X	137X	137U	HPT
52.5	66.3	66.7	94.9	54.2*	76.8	63.0	85.9	76.4	81.1
525.0	73.7	66.4	85.3	51.7*	80.3	70.3	82.3	77.5	83.9

* Spiked amounts for 13U were 525 pmol and 1575 pmol.

Table 3. Caffeine metabolism by mammalian cell lines expressing single cytochrome P450 isoforms and by controls

Cell line	Cytochrome	Substrate concn (mM)	Incubation time (hr)	Metabolites formed (μM) 13X	17X	37X	137U
V79†	No P450	4	72	—*	—*	—*	0.3 ±0.1
SD1†	Rat P450IIB1	4	72	—*	—*	—*	0.2 ±0.2
XEM2†	Rat P450IA1	4	72	—*	0.3 ±0.3	0.2 ±0.2	0.4 ±0.1
XEMdMz†	Rat P450IA2	4	72	1.7 ±0.4	4.1 ±0.8	3.5 ±0.8	0.8 ±0.1
XEMHIA2.36†	Human P450IA2	4	72	0.1 ±0.0	2.2 ±0.6	0.4 ±0.1	0.3 ±0.2
XEMHIA2.43†	Human P450IA2	4	72	0.2 ±0.0	2.7 ±0.1	0.3 ±0.0	0.2 ±0.0
R52-16	Mouse P450IA2	1	2	—‡	—‡	—‡	—‡
R52-16	Mouse P450IA2	1	4	—‡	0.3	0.2	—‡
R52-16	Mouse P450IA2	1	6	—‡	0.5	0.3	—‡
R52-16	Mouse P450IA2	1	8	0.2	0.8	0.4	—‡
R52-16	Mouse P450IA2	1	17	0.3	2.7	1.3	—‡
R52-16	Mouse P450IA2	1	24	0.4	3.0	1.6	0.4

For incubation procedure, see Materials and Methods.
* Below detection limit, i.e. (μM): 13X, 0.1; 17X, 0.1; 37X, 0.2; 137U, 0.1.
† Means ± SD given for three incubations carried out on different days.
‡ 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.

Table 4. Theophylline metabolism by V79 Chinese hamster cell lines expressing single cytochrome P450 isoforms

Cell line	Cytochrome P450 isoform	Metabolite formed (μM) 1X	3X	13U
V79	No P450	—*	—*	1.9 ±0.1
SD1	Rat P450IIB1	—*	—*	3.7 ±0.6
XEM2	Rat P450IA1	—*	—*	8.9 ±1.3
XEMdMz	Rat P450IA2	2.3 ±0.2	—*	25.4 ±4.3
XEMHIA2.36	Human P450IA2	—*	—*	5.2 ±1.3
XEMHIA2.43	Human P450IA2	—*	—*	8.6 ±1.4

Means ± SD given for three incubations carried out on different days. See Materials and Methods for incubation procedure.
—, 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.

Table 5. Contribution of demethylation and hydroxylation pathways to caffeine metabolism in different human systems

Human system	Caffeine concn	Primary metabolites formed (%) 13X	17X	37X	137U	Ref.
In vivo	Unknown	8	73	20	2	11
Cultured hepatocytes	1 mM	12	68	18	2	12
Liver slices	12 μM	15*	70*	15*	*	12
Liver microsomes	1 mM	10	65	12	12	12
Human P450IA2 expressed in mammalian cells†	4 mM	4 ±2	84 ±1	12 ±2	1 ±2	Present study

* Only percentages of demethylation pathways were given; †means ± SD (N = 6).

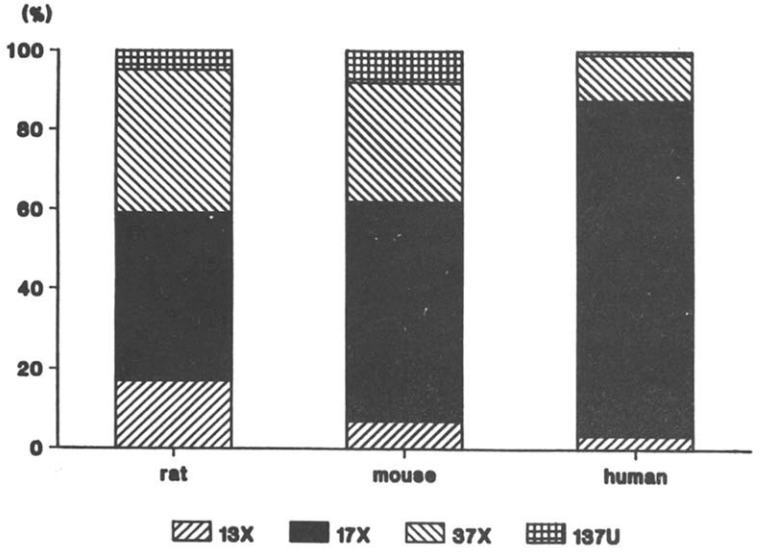


Fig. 3. Species differences in caffeine metabolism by P450IA2 cell lines. Relative contribution of each pathway is given as a percentage of all primary metabolites observed.

periods of incubation (3 days) to obtain sufficient amounts of metabolite by V79-derived cell lines. These substrate concentrations had a cytostatic effect which is a limitation to increasing 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 metabolite.

Our results show (Table 3) that only cell lines expressing a P450IA2 isoform were able to demethylate caffeine to a significant extent. Minor hydroxylation activities exceeding that of the original cell lines were also found by IA1. In all IA2 cell lines, demethylations are the predominant pathways accounting for 80% or more of the total metabolites (see Fig. 2).

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 and especially the human P450IA2 predominately produced 17X.

Theophylline metabolism

In contrast to caffeine, theophylline was metabolized mainly via 8-hydroxylation both by rat and human IA2 (Fig. 4), and with a relatively high activity by rat IA1 (Table 4). Demethylation activity was observed only by rat P450IA2. V79 cells without cytochromes were able also to hydroxylate theophylline. Expression of P450IIB1 gave also small additional amounts of the hydroxylated metabolite.

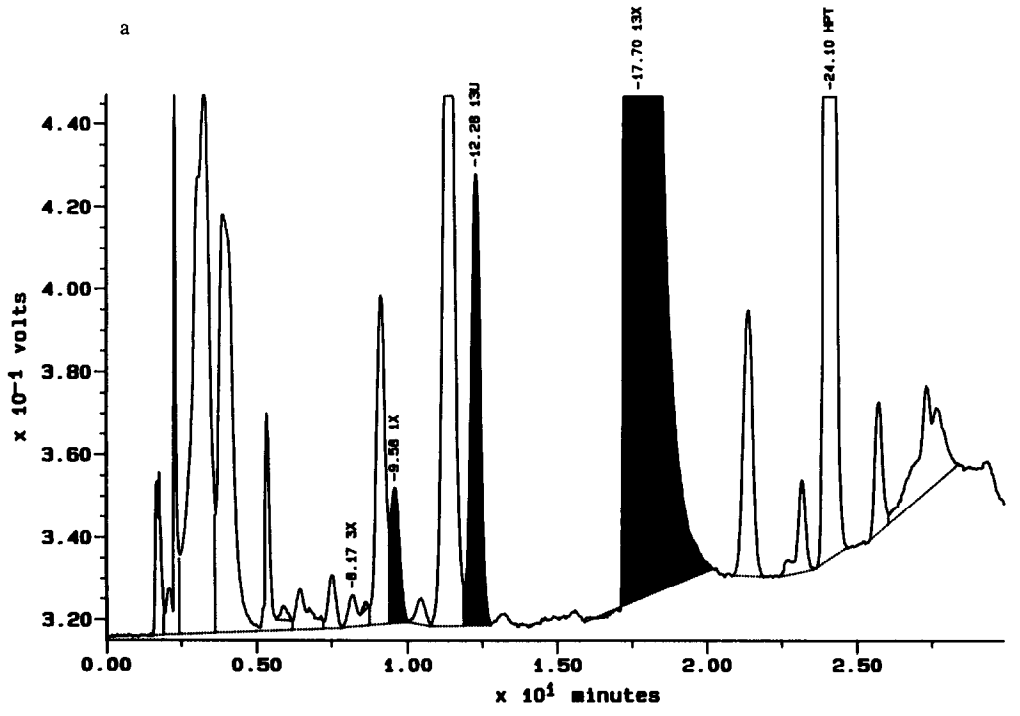
DISCUSSION

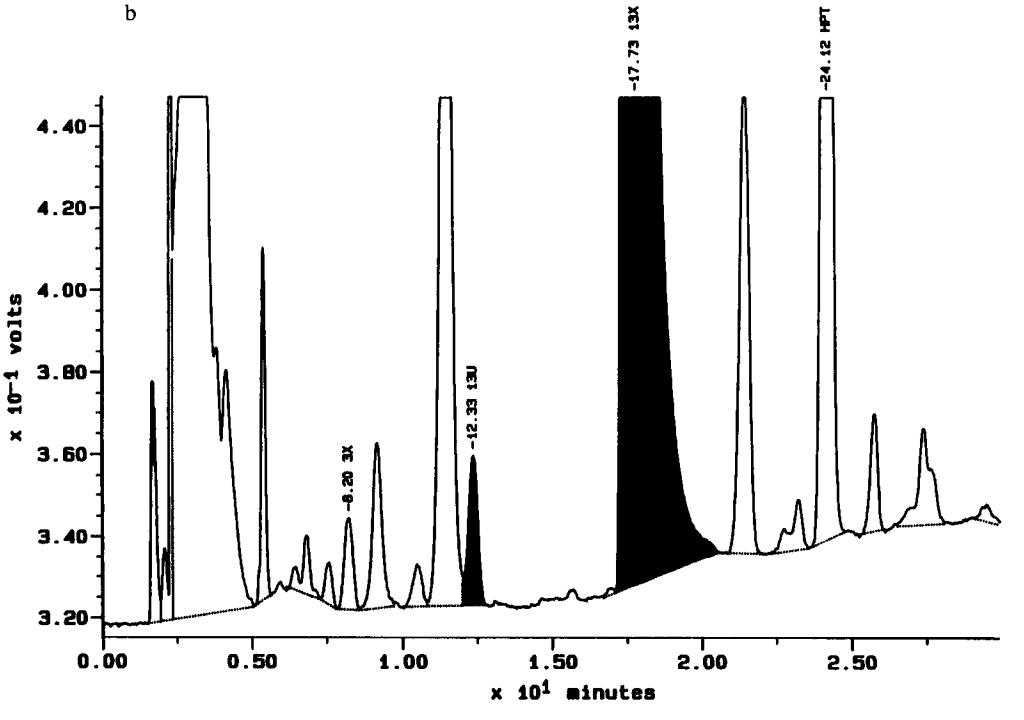
The intention of this study was to define the role of cytochrome P450IA2 in the metabolism of caffeine and theophylline. Our results with genetically modified mammalian cells expressing single cytochrome P450 isoforms show that both rodent and human P450IA2 mediate primary caffeine degradation, which is mainly demethylation. Rat P450IA1 was unable to demethylate caffeine to a significant extent (Table 3). Theophylline was metabolized mainly via 8-hydroxylation; the reaction

Table 6. Contribution of demethylation and hydroxylation pathways to theophylline metabolism in different human systems

Human system	Theophylline concn	Primary metabolites formed 1X	3X	13U	Ref.
In vivo	Unknown	24	15	61	30
Liver microsomes	150 μM	8	5	87	31
Liver microsomes	1.25 mM	16	15	69	14
Human P450IA2 expressed in mammalian cells	4 mM	—	—	>81*	Present study

* Other metabolites in incubations lower than detection limit.


a
-17.70 13X
-24.10 HPT
-12.28 13U
-8.56 1X
-8.17 3X
4.40
4.20
4.00
3.80
3.60
3.40
3.20
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
x 10¹ minutes


b
-17.73 13X
-24.12 HPT
-12.33 13U
-8.20 3X
4.40
4.20
4.00
3.80
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3.20
x 10⁻¹ volts
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x 10¹ minutes

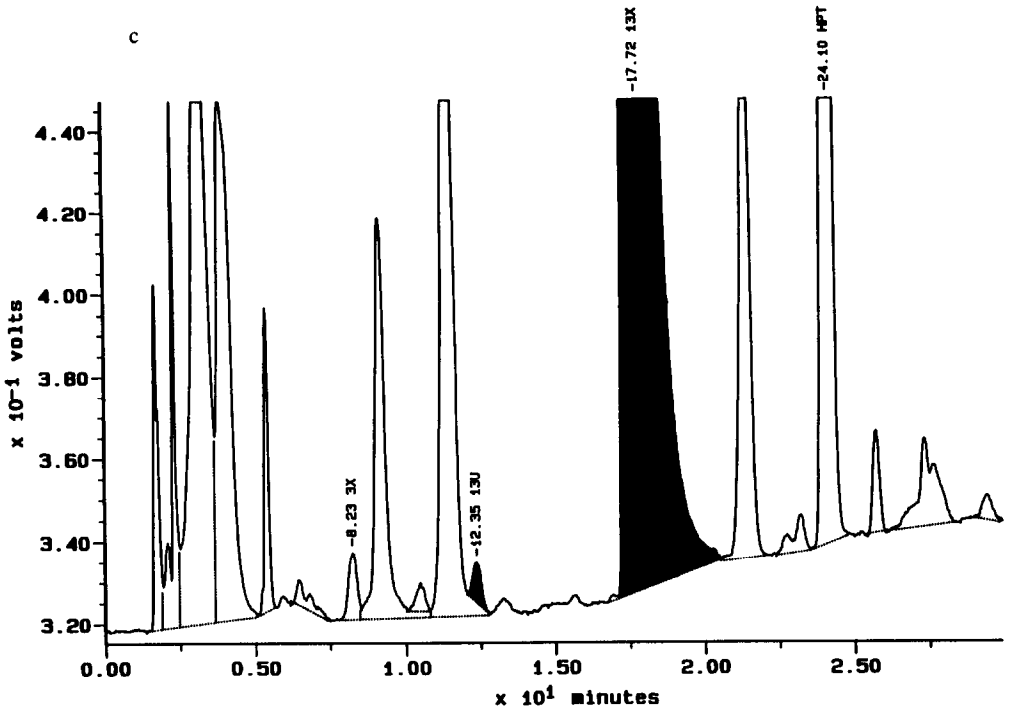

Fig. 4. HPLC-chromatograms of incubation medium following incubations of cell lines with 4 mM theophylline for 3 days. Black: definite peaks of substrates and their metabolites; peak identification is written at the top of each peak. (a) Rat P450IA2 cell line; (b) human P450IA2 cell line; (c) cell line without cytochromes P450.

was most active in cells expressing rat P450IA1, or rat or human P450IA2 (Table 4). 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*.

The genetically engineered cell lines employed in this study have been validated in several studies [16–22, 26]. Activity of methylxanthine metabolism in the cells was one order of magnitude lower than the activity of cultured human hepatocytes reported by Berthou *et al.* [12], who found a metabolic rate of (median) 12 pmol/min/10^6 cells when using similar substrate concentrations. We found that the pattern of primary caffeine metabolites obtained by isolated human P450IA2 is similar to that reported *in vivo* as well as to that obtained by human hepatocytes, liver slices or human liver microsomes (Table 5). It has been shown that the 3-demethylation of caffeine which accounts for as much as 70–80% of caffeine metabolism *in vivo* is mediated by human cytochrome P450IA2 [1]. Our results suggest strongly that caffeine 1-demethylation is mainly and 7-demethylation *in vivo* is partly mediated by P450IA2. Some 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 catalysed by human P450IA2 is supported by significant correlations reported between 1- and 3-demethethylation [8, 27]. Lack of correlation betwen 7-demethylation and/or 8-hydroxylation and 3-demethylation of caffeine [8, 9, 27] indicates again that human P450IA2, although shown here qualitatively to be competent for these two reactions, may mediate only a minor part of these less important pathways *in vivo*.

A low and a high affinity form of caffeine-metabolizing cytochrome was proposed [9, 12], and an incomplete inhibition of 13X, 37X and 137U formation by P450IA2 inhibitors has been described [10, 13]. Furthermore, a lack of correlation has been found between 13X, 37X and 137U formation, and other reactions typical for P450IA2 [8]. 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 occuring *in vivo*. Substrate concentrations in the present study were also 1 mM and higher but, in this case, the absence of cytochrome P450 other than that introduced into the cells guarantees that all metabolites formed additionally when compared to controls are due to the isoform expressed.

Thus, we suggest that the high affinity form primarily responsible for caffeine demethylation, especially 1- and 3-demethylation, is P450IA2, and low affinity forms are other isoforms unable to carry out the 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 [13], prolongs caffeine elimination *in vivo* exorbitantly [28]. The more than 7-fold increase in caffeine half-life despite the small fraction of caffeine excreted directly renally corresponds to an inhibition of enzyme activities by 90% or more. This can be explained only by the involvement of P450IA2 in the *in vivo* formation of primary caffeine metabolites other than 17X since the latter pathway mediates less than 80% of primary caffeine metabolism (see above).

Thus, 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 of N-acetylation [29], without loss of specificity for P450IA2. The presence of minor amounts of other methylxanthines in these beverages is not expected to interfere significantly with caffeine metabolism. Furthermore, this method does not cause problems in distinguishng between methylxanthines other than caffeine already present in the drink and those resulting from caffeine breakdown, as would be the case with the determination of the 3-demethylation of caffeine *in vivo* [13] (which is difficult by itself) rather than of caffeine elimination as a whole.

Theophylline metabolism 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 6).

There are conflicting results concerning a possible role of P450IA2 in theophylline metabolism. A good correlation between caffeine 3-demethylation and theophylline 1-demethylation but not 8-hydroxylation [9] provides evidence for P450IA2 mediating only theophylline demethylations. This presumption is supported by the results of Sarkar *et al.* [14] using a 10 mM theophylline concentration in human liver microsomes who found an inhibition of demethylation rather than hydroxylation by quinolone antibiotics which are potent inhibitors of P450IA2 [10], but of unknown specificity. The k_i values are different for demethylation and hydroxylation using low theophylline concentrations in human liver microsomes, showing again the involvement of more than one cytochrome P450 isoform [6]. On the other hand, Mulder *et al.* [32] reported inhibition of theophylline (1 mM) hydroxylation by a quinolone in rat hepatocytes; demethylations were unaffected. Human theophylline metabolism *in vivo* is prolonged by the concomitant application of quinolones [33] 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 is expected to be mediated by xanthine oxidase, an enzyme which is known to carry out the 8-hydroxylation of 1X to 1U [30].

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 not expected to depend on substrate concentration or 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 their 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 pattern of caffeine metabolism similar to that obtained in other human systems (see Table 5) with preference for the 3-demethylation pathway, rat P450 mediated 1-demethylation to a similar extent as 3-demethylation. 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 show a species diversity in theophylline degradation *in vivo*, since this isoform is not usually present in the human liver [34].

These species differences observed for P450IA activity provide evidence for the use of caution in using animal data on drug metabolism depending on this isoform to predict metabolism in man.

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REFERENCES

1. Butler MA, Iwasaki M, Guengerich FP and Kadlubar FK, Human cytochrome $P450_{PA}$ (P-450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc Natl Acad Sci USA* **86**: 7696–7700, 1989.
2. Gonzalez FJ, Aoyama T and Gelboin HV, Activation of promutagens by human cDNA-expressed cytochrome P450s. *Mutat Environ* **B**: 77–86, 1990.
3. Harder S, Staib AH, Beer C, Papenburg A, Stille W and Shah PM, 4-Quinolones inhibit biotransformation of caffeine. *Eur J Clin Pharmacol* **35**: 651–656, 1988.
4. Nielsen-Kudsk JE, Buhl JS and Johannessen AC, Verapamil-induced inhibition of theophylline in healthy humans. *Pharmacol Toxicol* **66**: 101–103, 1990.
5. Kalow W and Campbell ME, Biotransformation of caffeine by microsomes. *ISI Atlas of Science: Pharmacology:* 381–386, 1988.
6. Robson RA, Miners JO, Matthews AP, Stupans I,

Meller D, McManus M and Birkett DJ, Characterisation of theophylline metabolism by human liver microsomes. Inhibition and immunochemical studies. *Biochem Pharmacol* **37**: 1651–1659, 1988.
7. Berthou F, Ratanasavanh D, Alix D, Carlhant D, Riche C and Guillouzo A, Caffeine and theophylline metabolism in newborn and adult human hepatocytes; comparison with adult rat hepatocytes. *Biochem Pharmacol* **37**: 3691–3700, 1988.
8. Berthou F, Flinois JP, Ratanasavanh D, Beaune P, Riche C and Guillouzo A, Evidence for the involvement of several cytochromes P-450 in the first steps of caffeine metabolism by human liver microsomes. *Drug Metab Dispos*, **19**: 561–567, 1991.
9. Campbell ME, Grant MD, Inaba T and Kalow W, Biotransformation of caffeine, paraxanthine, theophylline, ande theobromine by polycyclic aromatic hydrocarbon-inducible cytochrome(s) P-450 in human liver microsomes. *Drug Metab Dispos* **15**: 237–249, 1987.
10. Fuhr U, Wolff T, Harder S, Schymanski P and Staib AH, Quinolone inhibition of cytochrome P-450-dependent caffeine metabolism in human liver microsomes. *Drug Metab Dispos* **18**: 1005–1010, 1990.
11. Arnaud MJ and Welsch C, Theophylline and caffeine metabolism in man. In: *Theophylline and Other Methylxanthines* (Eds. Rietbrock N, Woodcock BG and Staib AH), pp. 135–148. Vieweg, Braunschweig and Wiesbaden, 1982.
12. Berthou F, Ratanasavanh D, Riche C, Picart D, Voirin T and Guillouzo A, Comparison of caffeine metabolism by slices, microsomes and hepatocyte cultures from adult human liver. *Xenobiotica* **19**: 401–417, 1989.
13. Sesardic D, Boobis AR, Murray BP, Murray S, Segura J, de la Torre R and Davies DS, Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. *Br J Clin Pharmacol* **29**: 651–663, 1990.
14. Sarkar M, Polk RE, Guzelian PS, Hunt C and Karnes HT, *In vitro* effect of fluoroquinolones on theophylline metabolism in human liver microsomes. *Antimicrob Agents Chemother* **34**: 594–599, 1990.
15. Gelboin HV and Friedman FK, Monoclonal antibodies for studies on xenobiotic and endobiotic metabolism. *Biochem Pharmacol* **34**: 2225–2234, 1985.
16. Dogra S, Doehmer J, Glatt H, Mölders H, Siegert P, Friedberg T, Seidel A and Oesch F, Stable expression of rat cytochrome P-450IA1 cDNA in V79 chinese hamster cells and their use in mutagenicity testing. *Mol Pharmacol* **37**: 608–613, 1990.
17. Wölfel C, Platt KL, Dogra S, Glatt H, Wächter F, Oesch F and Doehmer J, Stable expression of rat cytochrome P-450IA2 cDNA in V79 chinese hamster cells and hydroxylation of 17β-estradiol and 2-aminofluorene. *Mol Carcinogen*, in press.
18. Doehmer J, Dogra S, Friedberg T, Monier S, Adesnik M, Glatt H and Oesch F, Stable expression of rat cytochrome P-450IIB1 cDNA in chinese hamster cells (V79) and metabolic activation of aflatoxin B_1. *Proc Natl Acad Sci USA* **85**: 5769–5773, 1988.
19. Platt KL, Molitor E, Döhmer J, Dogra S and Oesch F, Genetically engineered V79 chinese hamster cell expression of purified cytochrome P-450IIB1 monooxygenase activity. *J Biochem Toxicol* **4**: 1–6, 1989.
20. Waxman DJ, Lapenson DP, Morrissey JJ, Park SS, Gelboin HV, Doehmer J and Oesch F, Androgen hydroxylation catalysed by a cell line (SD1) that stably expresses rat hepatic cytochrome P-450 PB-4 (IIB1). *Biochem J* **260**: 81–85, 1989.
21. Wölfel C, Gonzalez FJ, Glatt H, Platt KL and Doehmer J, Stable expression of human cytochrome P-450IA2 cDNA in V79 chinese hamster cells, *Eur J Pharmacol*, in press.
22. Battula N, Transduction of cytochrome P_3-450 by retroviruses: constitutive expression of enzymatically active microsomal hemoprotein in animal cells. *J Biol Chem* **264**: 2991–2996, 1989.
23. Glatt HR, Gemperlein I, Turchi G, Heinritz H, Doehmer J and Oesch F, Search for cell culture systems with diverse xenobiotic-metabolizing activity and their use in toxicological studies. *Mol Toxicol* **1**: 313–334, 1987.
24. Russel WC, Newman C and Williamson DH, A simple cytochemical technique for demonstration of DNA in cells infected with *Mycoplasma* and viruses. *Nature* **253**: 461–462, 1975.
25. Grant DM, Tang BK and Kalow W, Variability in caffeine metabolism. *Clin Pharmacol Ther* **33**: 591–602, 1983.
26. Doehmer J, Seidel A, Oesch F and Glatt HR, Genetically engineered V79 chinese hamster cells metabolically activate the cytostatic drugs cyclophosphamide and ifosfamide. *Environ Health Perspect* **88**: 63–65, 1990.
27. Grant DM, Campbell ME, Tang BK and Kalow W, Biotransformation of caffeine by microsomes from human liver. *Biochem Pharmacol* **36**: 1251–1260, 1987.
28. Tarrus E, Cami J, Roberts DJ, Spickett RGW, Celdran E and Segura J, Accumulation of caffeine in healthy volunteers treated with furafylline. *Br J Clin Pharmacol* **23**: 9–18, 1987.
29. Grant DM, Tang BK and Kalow W, A simple test for acetylator phenotype using caffeine. *Br J Clin Pharmacol* **17**: 459–464, 1984.
30. Birkett DJ, Grygiel JJ and Miners JO, Metabolic disposition of the methylxanthines in man. In: *Theophylline and Other Methylxanthines* (Eds. Rietbrock N, Woodcock BG and Staib AH), p. 149–158. Vieweg, Braunschweig and Wiesbaden, 1982.
31. Robson RA, Matthews AP, Miners JO, McManus ME, Meyer UA, Hall P de la M and Birkett DJ, Characterisation of theophylline metabolism in human liver microsomes. Inhibition and immunochemical studies. *Br J Clin Pharmacol* **24**: 293–300, 1987.
32. Mulder GJ, Nagelkerke JF, Tijdens RB, Wijnands WJA and van der Mark EJ, 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, 1988.
33. Wijnands WJA, Vree TB and van Herwaarden CLA, The influence of quinolone derivatives on theophylline clearance. *Br J Clin Pharmacol* **22**: 677–683, 1986.
34. Wrighton SA, Campanile C, Thomas PE, Maines SL, Watkins PB, Parker G, Mendez-Picon G, Haniu M, Shively JE, Levin W and Guzelian PS, Identification of a human liver cytochrome P-450 homologous to the major isosafrole-inducible cytochrome P-450 in the rat. *Mol Pharmacol* **29**: 405–410, 1986.