Br. J. clin. Pharmac. (1990), **29**, 651–663

Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man

DOROTHEA SESARDIC, A. R. BOOBIS, B. P. MURRAY, S. MURRAY, J. SEGURA[1], R. DE LA TORRE[1] & D. S. DAVIES

Department of Clinical Pharmacology, Royal Postgraduate Medical School, London W12 0NN and [1]Institut Municipal D'Investigació Mèdica, Department of Pharmacology, Passeig Maritim 25–29, 08003 Barcelona, Spain

1 Furafylline (1,8-dimethyl-3-(2'-furfuryl)methylxanthine) is a methylxanthine derivative that was introduced as a long-acting replacement for theophylline in the treatment of asthma. Administration of furafylline was associated with an elevation in plasma levels of caffeine, due to inhibition of caffeine oxidation, a reaction catalysed by one or more hydrocarbon-inducible isoenzymes of P450. We have now investigated the selectivity of inhibition of human monooxygenase activities by furafylline.

2 Furafylline was a potent, non-competitive inhibitor of high affinity phenacetin *O*-deethylase activity of microsomal fractions of human liver, a reaction catalysed by P450IA2, with an IC_{50} value of 0.07 μM.

3 Furafylline had either very little or no effect on human monooxygenase activities catalysed by other isoenzymes of P450, including P450IID1, P450IIC, P450IIIA. Of particular interest, furafylline did not inhibit P450IA1, assessed from aryl hydrocarbon hydroxylase activity of placental samples from women who smoked cigarettes.

4 It is concluded that furafylline is a highly selective inhibitor of P450IA2 in man.

5 Furafylline was a potent inhibitor of the *N*3-demethylation of caffeine and of a component of the *N*1- and *N*7-demethylation. This confirms earlier suggestions that caffeine is a selective substrate of a hydrocarbon-inducible isoenzyme of P450 in man, and identifies this as P450IA2. Thus, caffeine *N*3-demethylation should provide a good measure of the activity of P450IA2 *in vivo* in man.

6 Although furafylline selectively inhibited P450IA2, relative to P450IA1, in the rat, this was at 1000-times the concentration required to inhibit the human isoenzyme, suggesting a major difference in the active site geometry between the human and the rat orthologues of P50IA2.

Keywords human monooxygenase activity cytochrome P450IA2 furafylline enzyme inhibition

Introduction

Cytochrome P450 comprises a superfamily of structurally and functionally related isoenzymes (Nebert *et al.*, 1989) which are involved in the metabolism of a diverse range of both exogenous and endogenous compounds (Yang & Lu, 1987). The products of metabolism by these isoenzymes are frequently less active than the parent compound, but in some instances the metabolite has

Correspondence: Dr A. R. Boobis, Department of Clinical Pharmacology, Royal Postgraduate Medical School, London W12 0NN

increased biological activity, and may even be responsible for the toxic effects of the substrate. Where the generation of a toxic metabolite is isoenzyme specific, this may be particularly important in determining the cell, tissue or species specificity of toxicity (see Nelson & Harvison, 1987).

Amongst those isoenzymes most implicated in the activation of foreign compounds to toxic or carcinogenic intermediates are members of the cytochrome P450IA subfamily, P450IA1 and P450IA2 (Kadlubar & Hammons, 1987). These isoenzymes are inducible by polycyclic aromatic hydrocarbons in a wide variety of species including the mouse, rabbit and rat (Gonzalez, 1989). P450IA1 is particularly active in the conversion of polycyclic aromatic hydrocarbons to genotoxic intermediates (Wilson *et al.*, 1984) whilst P450IA2 has a high specificity for the *N*-hydroxylation of a number of aromatic amines (Kadlubar & Hammons, 1987). There is now good evidence that both members of this subfamily of cytochrome P450 are also inducible by polycyclic aromatic hydrocarbons in man (Pasanen *et al.*, 1989; Sesardic *et al.*, 1988; Song *et al.*, 1985; Wong *et al.*, 1986; Wrighton *et al.*, 1986). In addition to increasing the specific content of the members of the P450IA subfamily in human tissues, cigarette smoking is associated with an elevation in the metabolism of a number of drugs and other chemicals. Amongst such substrates are phenacetin, via *O*-deethylation (Kahn *et al.*, 1985; Pantuck *et al.*, 1974) and caffeine, particularly via *N*3-demethylation (Campbell *et al.*, 1987; Kotake *et al.*, 1982).

In previous studies we have demonstrated that the hepatic *O*-deethylation of phenacetin is catalysed almost exclusively by P450IA2 and that cigarette smoking causes a parallel increase in the specific content of this isoenzyme and phenacetin *O*-deethylase activity (Sesardic *et al.*, 1988). Recently, Butler *et al.* (1989) have confirmed the extensive involvement of P450IA2 in high affinity phenacetin *O*-deethylase activity of human liver. There is increasing evidence that the demethylation of caffeine is also catalysed by a hydrocarbon inducible form of cytochrome P450 in man, although the exact identity of the isoenzyme has yet to be determined (Campbell *et al.*, 1987; Grant *et al.*, 1987). In view of the importance of polycyclic aromatic hydrocarbon inducible forms of P450 in toxicity and carcinogenicity, it would be of obvious benefit to identify specific substrates of P450IA1 and P450IA2 that could be administered to man so that their activities could be determined *in vivo*.

The specificity of cytochrome P450 can be studied in a variety of ways, perhaps the most rigorous of which is the use of highly specific inhibitory antibodies (Sesardic *et al.*, 1990). However, this suffers from the disadvantage that they cannot be administered *in vivo* to determine the contribution of a particular isoenzyme in the overall metabolism of a compound, in volunteers or patients. Thus, alternative methods of studying specificity have been sought, and amongst these is the use of highly specific chemical inhibitors. This approach has proved extremely successful for studying the P450IID isoenzyme responsible for the 4-hydroxylation of debrisoquine in man. It is this isoenzyme which is subject to monogenic variation, such that there is a genetic polymorphism in the 4-hydroxylation of debrisoquine (Gonzalez *et al.*, 1988). Quinidine is a highly selective inhibitor of this isoenzyme (Otton *et al.*, 1984; Speirs *et al.*, 1986) and can be utilised *in vivo* in healthy volunteers to determine the contribution of the polymorphic pathway in the elimination of a given drug (Speirs *et al.*, 1986).

A recent report has suggested that the metabolism of caffeine in man may be inhibited by the methylxanthine drug candidate furafylline (1,8-dimethyl-3-(2′-furfuryl)methylxanthine) (Tarrús *et al.*, 1987a). In healthy volunteers who received furafylline there was a considerable accumulation of caffeine, due to inhibition of its metabolism by demethylation (Tarrús *et al.*, 1987a). Since the demethylation of caffeine in man is inducible by cigarette smoking, and there is evidence *in vitro* to suggest the involvement of polycyclic aromatic hydrocarbon inducible forms of P450 in this reaction (Campbell *et al.*, 1987; Grant *et al.*, 1987), we have now investigated the specificity of furafylline as an inhibitor of human monooxygenase activity, utilising a variety of assays *in vitro*, selected for their specificity for different isoenzymes.

Methods

Abbreviations

AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; BzDM, benzphetamine *N*-demethylase; CN1, CN3 and CN7, caffeine *N*1-, *N*3- and *N*7-demethylase, respectively; CO, carbon monoxide; COH, coumarin 7-hydroxylase; D4OH, debrisoquine 4-hydroxylase; EDM, ethylmorphine *N*-demethylase; GC/NICI MS, gas chromatography/negative ion chemical ionisation mass spectrometry; HCHO, formaldehyde; IC_{50}, concentration of inhibitor producing 50% inhibition; MC, 3-methylcholanthrene; pNOH, 4-nitrophenol 3-hydroxylase; POD,

phenacetin *O*-deethylase; U.v.-h.p.l.c. high pressure liquid chromatography with u.v. detection.

Chemicals

Furafylline was a generous gift from Laboratorias Almirall (Barcelona, Spain). Debrisoquine hemisulphate and 4-hydroxydebrisoquine hemisulphate were generously provided by Roche Products Limited (Welwyn Garden City, UK). Tetradeutero-4-hydroxydebrisoquine, the internal standard for the assay of debrisoquine 4-hydroxylase activity, was kindly provided by C. O. Meese (Stuttgart, FRG). Paracetamol, formaldehyde and phenacetin, the last being recrystallised prior to use (Murray & Boobis, 1986), were purchased from BDH Chemicals Limited (Poole, UK). The internal standard for the assay of paracetamol, trideuteroparacetamol, was prepared as previously described (Murray & Boobis, 1986). Hexafluoroacetylacetone and 3,5-bistrifluoromethylbenzoyl chloride were obtained from Fluorochem Limited (Glossop, UK). Ethylmorphine was obtained from Macarthys Medical Limited (Romford, UK) and 7-hydroxycoumarin, 4-nitrophenol and 4-nitrocatechol were purchased from Aldrich Chemical Company Limited (Gillingham, UK). Sigma Chemical Company Limited (Poole, UK) supplied benzphetamine, benzo[*a*]pyrene, caffeine, coumarin, 3-methylcholanthrene, *n*-undecane, NADPH and quinine sulphate. All other chemicals and reagents were purchased from either Sigma Chemical Company or BDH Limited and were of the best grade available.

Human tissue samples were obtained from renal transplant donors (Boobis *et al.*, 1983), details of whom are provided in Table 1. All samples were shown to have normal histology prior to use. The use of such samples in these studies had local Research Ethics Committee permission and coroner's approval. The placental samples were generously provided by O. Pelkonen and M. Pasanen (Oulu, Finland) and were from women of known smoking status, based on plasma cotinine levels (Pasanen *et al.*, 1988). Details of the handling of these samples will be found in the literature (Pelkonen & Pasanen, 1981; Pasanen *et al.*, 1985).

Male Wistar rats (180–200 g) were obtained from Olac (Bicester, UK) and were maintained under constant conditions of heating and lighting. Animals were permitted free access to food (PRD diet, Labsure Animal Products, Poole, UK) and tap water *ad libitum*. The animals were treated with a single intraperitoneal injection of 3-methylcholanthene (MC) at a dose of 80 mg kg^{-1} body weight, 48 h before sacrifice. The animals were killed by stunning and exsanguination.

Microsomal fractions from the liver and placental samples were isolated by differential ultracentrifugation as previously described (Boobis *et al.*, 1980b), and resuspended in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol. Samples were stored in small aliquots at −80° C until required for assay. Under these conditions of preparation and storage, monooxygenase activities of microsomal fractions were stable for at least 6 months. All assays were performed within this time.

Determination of monooxygenase activities

Ten different oxidation pathways, catalysed by a variety of different isoenzymes of cytochrome P450, were assayed for human liver and placental samples *in vitro*. These activities were the high affinity component of phenacetin *O*-deethylase (P450IA2: Sesardic *et al.*, 1988), benzphetamine *N*-demethylase (P450IIIA in man: Guengerich *et al.*, 1986; P450IIB1 in rat: Reik *et al.*, 1985; possibly also in man), debrisoquine 4-hydroxylase (P450IID1: Distlerath *et al.*, 1985), 4-nitrophenol 3-hydroxylase (P450IIE1 in rat: Koop, 1986; not determined in man), ethylmorphine *N*-demethylase (P450IIIA in rat: Wrighton *et al.*, 1985; not determined in man), coumarin 7-hydroxylase ($P450_{Coh}$: Raunio *et al.*, 1988), aryl hydrocarbon hydroxylase (P450IIC and P450IIIA in liver: Kawano *et al.*, 1987; P450IA1

Table 1 Details of human liver samples

Sample code	*Sex*	*Age (years)*	*Cause of death*	*Drugs administered*
03005	F	37	Sub-arachnoid haemorrhage	Unknown
03006	M	64	Road traffic accident	Dexamethasone
03007	M	17	Road traffic accident	None
03008	F	38	Sub-arachnoid haemorrhage	None
03009	F	16	Cardiac arrest, coma	Dexamethasone

Human liver samples were obtained from renal transplant donors as described in **Methods**.

Table 2 Details of assays of monooxygenase activities

Activity	*Substrate concentration (μM)*	*Protein amount (mg)*	*Incubation time (min)*	*Method of analysis*	*Reference*
Phenacetin *O*-deethylase (POD)	4	0.2	10	GC/ NICI MS	Murray & Boobis (1986) Sesardic *et al.* (1988)
Coumarin 7-hydroxylase (COH)	5000	0.4	10	Spectro-fluorometric	Aitio (1978) Greenlee & Poland (1978)
Debrisoquine 4-hydroxylase (D4OH)	200	0.6	12	GC/ NICI MS	Boobis *et al.* (1983) Kobayashi *et al.* (1989)
4-Nitrophenol 3-hydroxylase (pNOH)	200	1.0	60	Spectro-photometry (Nitrocatechol)	Reinke & Moyer (1985)
Benzphetamine *N*-demethylase (BzDM)	1000	1.0	30	Spectro-photometry (HCHO)	Nash (1953) Boobis & Davies (1984)
Ethylmorphine *N*-demethylase (EDM)	5000	1.0	20	Spectro-photometry (HCHO)	Nash (1953) Boobis & Davies (1984)
Aryl hydrocarbon hydroxylase (AHH)	5	0.2	5	Spectro-fluorometric	Atlas *et al.* (1976) Nebert (1978)
Caffeine *N*-demethylase, (CN1, CN3, CN7)	1000	2.0	30	U.v.-h.p.l.c.	Grant *et al.* (1987) de la Torre *et al.* (in preparation)

All assays were performed using the incubation mixture described in **Methods**, under conditions that were linear with respect to protein concentration and time of incubation. Concentrations of substrate were selected to be 1–2 times the respective K_m, where known. Full details of the methods can be found in the cited references.

in placenta: Wong *et al.*, 1986) and caffeine *N*1-, *N*3- and *N*7-demethylase.

The procedure for all of the assays was similar, differences between the methods being indicated in Table 2. In general, the reaction mixture contained 75 mM Tris-hydrochloride buffer, pH 7.4, 3 mM magnesium chloride, 1.2 mM NADPH and between 0.2 to 1.0 mg of protein in 50 μl 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol in a final volume of 1 ml. Samples were preincubated at 37° C for 2 min and prewarmed substrate added at 10 or 50 times the final concentration, depending upon the solvent used to prepare the stock solution of substrate. The final concentrations of the substrates in the reaction mixtures for the various monooxygenase activities are shown in Table 2.

In inhibition studies, furafylline was added at either 50 or 100 times the desired final concentration, in 10 or 20 μl methanol as appropriate, to give a final concentration of 1–1000 μM, unless otherwise indicated. Samples were preincubated in the presence of inhibitor for 2 min at 37° C, prior to addition of substrate. All incubations were performed at 37° C in a shaking water bath in an atmosphere of air. The duration of incubation and the concentration of protein were adjusted for each assay, the values used being shown in Table 2, to be within the respective linear range. Blanks were incubated either without substrate, which was added after stopping the reaction, or without NADPH. The respective products were analysed, as indicated in Table 2, by previously published methods, with the exception of the demethylation products of caffeine, which were assayed using a modification of the method of Grant *et al.* (1987), which will be published in detail elsewhere. This involved h.p.l.c. separation of the metabolites using a Waters Model 510 pump (Waters Chromatography Division, Watford, UK) fitted with an Ultrasphere ODS reverse-phase column (25 cm × 4.6 mm i.d.) (Beckman Instruments (UK) Ltd, High Wycombe, UK) eluted isocratically at ambient temperature with a mobile phase of 0.05% acetic acid : methanol : acetonitrile (86 : 12 : 2) at 1.3 ml min^{-1}. Quantification was based on u.v. absorbance at 280 nm. The presence of furafylline in the incubations did not interfere with the quantification of the respective product(s) in any of the assays.

The effects of the solvent for furafylline, methanol, itself were determined on the different metabolic activities. Whilst very little effect was observed on POD activity, D4OH activity was inhibited by up to 40%, as reported previously (Boobis *et al.*, 1987). The presence of methanol increased the background production rate of formaldehyde in the assay of EDM and BzDM activities, but did not appear to have any effect on the rate of substrate metabolism. None of the other assays was affected by the addition of methanol at the volumes used in these studies.

Other assays

Protein concentration of microsomal fractions was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin (fraction V) as standard. Cytochrome P450 content was measured by reduced *vs* CO-reduced difference spectroscopy (Omura & Sato, 1964) on a model 555 split beam scanning spectrophotometer with microprocessor controlled background correction (Perkin Elmer Ltd, Beaconsfield, UK). An extinction coefficient of 91 mM^{-1} cm^{-1} was assumed for reduced CO-bound P450 between 450 and 480 nm.

Analysis of data

Results have been expressed as means ± s.d. where appropriate.

The concentration of inhibitor causing 50% reduction of activity, relative to the appropriate control value (IC_{50} value) was calculated, where possible, by interpolation of a regression line of the log concentration *vs* percent inhibition plot over at least three concentrations of inhibitor. The IC_{50} values for the inhibitable component of caffeine *N*1- and *N*-7 demethylation were determined by subtracting the contribution of the non-inhibitable component from total activity and then analysing the residual inhibitable activity as described above. Michaelis-Menten parameters were estimated by iterative non-linear regression analysis using a commercial software package (GraphPad, Institute for Scientific Information, Philadelphia, PA, USA) assuming a double rectangular hyperbola (Boobis *et al.*, 1981), which would fit the data by either one or two components, as appropriate. Inhibitory constants (K_i values) were obtained graphically from Dixon plots (Dixon, 1953) using regression analysis, and displayed using Eadie-Hofstee plots (Hofstee, 1952).

Results

The monooxygenase activities of individual human liver samples are shown in Table 3. The averages and ranges of these activities were similar to those obtained previously both by our own laboratory and those of others (Boobis *et al.*, 1980a,b, 1985; Farrell *et al.*, 1979; Kahn

Table 3 Monooxygenase activities of human liver samples

Sample	*POD*	*COH*	*D4OH*	*pNOH*	*BzDM*	*EDM*	*AHH*	*CN1*	*CN3*	*CN7*	*P450*
03005	67.1	87.9	30.0	226	1.00	1.81	38.5	ND	ND	ND	225
03006	99.8	96.1	35.0	363	1.23	1.54	32.3	ND	ND	ND	236
03007	135	135	60.0	701	1.26	1.36	24.9	0.43	1.00	0.31	340
03008	3.65	61.0	ND	449	0.75	0.44	19.7	0.37	0.33	0.28	139
03009	89.9	41.5	ND	723	0.88	1.21	20.4	0.52	1.14	0.28	194
Mean	79.2	84.2	41.7	492	1.02	1.27	27.2	0.44	0.82	0.29	227
s.d.	48.9	35.6	16.0	216	0.22	0.52	8.11	0.08	0.43	0.02	73.8

The abbreviations used for the monooxygenase activities are as described in Table 2.
Assay conditions were as described in **Methods**, and summarised in Table 2.
Activities have been expressed in pmol product formed mg^{-1} microsomal protein min^{-1}, except for BzDM and EDM activities, which have been expressed in nmol mg^{-1} min^{-1}. Cytochrome P450 content has been expressed in pmol mg^{-1} protein.
ND: Not determined.

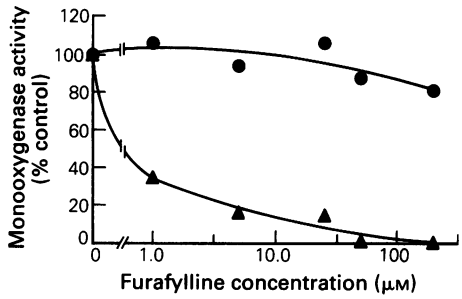

Figure 1 Effects of furafylline on human hepatic phenacetin *O*-deethylase and debrisoquine 4-hydroxylase activities.

The effects of furafylline on phenacetin *O*-deethylase (▼) and debrisoquine 4-hydroxylase (●) activities were determined on a pooled microsomal fraction from three different human liver samples. Furafylline was added to the samples at a concentration of 0–200 μM. The samples were then preincubated for 2 min at 37° C prior to addition of substrate. Activity in the presence of furafylline has been expressed as a percentage of the corresponding control value, in the presence of methanol alone. Control activities were: phenacetin *O*-deethylase, 90.4 pmol mg^{-1} min^{-1}; debrisoquine 4-hydroxylase, 19.5 pmol mg^{-1} min^{-1}.

et al., 1985; Kremers *et al.*, 1981; Pelkonen *et al.*, 1985).

Furafylline was a potent inhibitor of the high affinity component of POD activity (Figure 1). At concentrations of 1 μM more than 60% of activity was inhibited. At 4 μM phenacetin, the high affinity component of POD activity would contribute 82% of total activity. Thus, the observation that greater than 95% of POD was inhibited at concentrations of furafylline of 50–100 μM suggested that at such concentrations the compound inhibits by a less specific mechanism. Indeed, all of high affinity POD activity was inhibited at 5 μM furafylline. In marked contrast to the effect on POD activity, no inhibition of debrisoquine 4-hydroxylase activity was observed at concentrations of furafylline below 50 μM. At higher concentrations, a small degree of inhibition (up to 20%) was observed.

The effects of furafylline on several other monooxygenase activities, selected on the basis that they are believed to be catalysed largely by a number of different isoenzymes of cytochrome P450, were determined. The IC_{50} values (Table 4) for coumarin 7-hydroxylase, 4-nitrophenol 3-hydroxylase, benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase were all greater than 400 μM. The effects of furafylline were also determined on aryl hydrocarbon

Table 4 IC_{50} values for furafylline inhibition of human monooxygenase activities

Activity	IC_{50} (μM)
Phenacetin *O*-deethylase*	0.07 (0.05–0.09)
Debrisoquine 4-hydroxylase	> 200
Coumarin 7-hydroxylase	> 400
4-Nitrophenol hydroxylase	> 400
Benzphetamine *N*-demethylase	> 500
Ethylmorphine *N*-demethylase	> 500
Hepatic AHH	> 500
Placental AHH	> 500
Caffeine *N*1-demethylase (inhibitable 60%)**	< 0.10
Caffeine *N*3-demethylase	< 0.05
Caffeine *N*7-demethylase (inhibitable 70%)**	< 0.10

Monooxygenase activities were determined as described in **Methods**. For each activity, at least three different tissue samples were incubated with furafylline in methanol for 2 min at 37° C prior to initiating the reaction by addition of substrate. IC_{50} values (the concentration of furafylline causing 50% inhibition of activity relative to that in the presence of methanol alone) were determined by interpolation of semilog plots of inhibitor concentration against percentage of activity remaining. When no inhibition was observed up to the highest concentration of furafylline tested, the IC_{50} value was assumed to be greater than that concentration of the compound.
*Results are the mean (range) of determinations on three different liver samples.
Only a proportion of caffeine *N*1- and *N*7-demethylase activity was inhibitable by caffeine (see Figure 2 and text). The IC_{50} values for these activities have been calculated for that portion of activity that was inhibitable by furafylline as described in **Methods.

hydroxylase activity of both liver and placenta. Whilst AHH activity of liver is thought to be catalysed largely by P450IIC and P450IIIA (Kawano *et al.*, 1987), that of placenta from cigarette smoking women is now known to be catalysed largely by P450IA1 (Pasanen *et al.*, 1989; Wong *et al.*, 1986). Furafylline had virtually no effect on either activity, in both cases the IC_{50} value being greater than 500 μM (Table 4).

All three pathways of caffeine *N*-demethylation were inhibited by furafylline, but *N*3-demethylase activity was inhibited to a much greater extent than the other two demethylase pathways. Whereas 95% of caffeine *N*3-demethylase activity was inhibited by furafylline, only 70% of *N*7-demethylase and 60% of *N*1-demethylase activity, respectively, was inhibitable (Figure 2). However, in all cases the IC_{50} value for the inhibitable component of these activities (calculated as described in **Methods**) was < 0.1 μM (Table 4).

Whilst furafylline had little or no effect on many of the monooxygenase activities studied, there were some differences, depending on the

Table 5 Maximum effect of furafylline on human monooxygenase activities

Activity	*Maximum % change*
Phenacetin *O*-deethylase	−98
Debrisoquine 4-hydroxylase	−20
Coumarin 7-hydroxylase	+116 ± 44.2
4-Nitrophenol hydroxylase	−20.3 ± 5.7
Benzphetamine *N*-demethylase	−33.0 ± 18.3
Ethylmorphine *N*-demethylase	−6.4 ± 7.1
Hepatic AHH	+203
Placental AHH	−4.7
Caffeine *N*1-demethylase	−60
Caffeine *N*3-demethylase	−93.4 ± 1.5
Caffeine *N*7-demethylase	−70

Monooxygenase activities, assayed as described in **Methods**, were determined in the presence of a range of furafylline concentrations. Each activity was measured using at least three different tissue samples. Results are mean and, where shown, s.d. of the maximum change in activity relative to that in the presence of methanol alone. For each activity the direction of change was the same for all of the tissue samples studied.

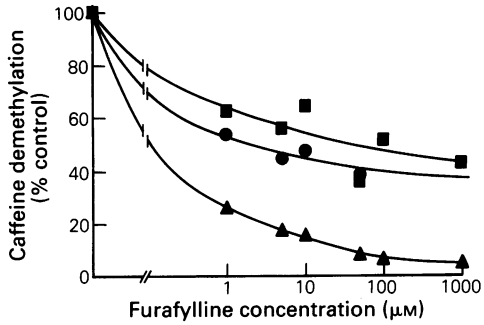

Figure 2 Effects of furafylline on the *N*1-, *N*3- and *N*7-demethylation of caffeine catalysed by hepatic microsomal fractions.

The effects of furafylline on caffeine *N*1- (●), *N*3- (▲) and *N*7- (■) demethylase activities were determined on a pooled microsomal fraction from three different human liver samples. Furafylline was added to the samples at a concentration of 0–1000 μM. The samples were than preincubated for 2 min at 37° C prior to addition of substrate. Activity in the presence of furafylline has been expressed as a percentage of the corresponding control value, in the presence of methanol alone. The **lines** were computed by non-linear iterative least squares regression analysis.

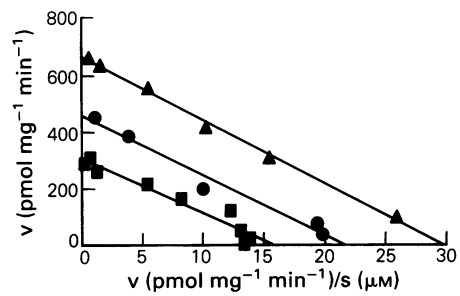

Figure 3 Kinetics of inhibition of the high affinity component of human hepatic phenacetin *O*-deethylase activity by furafylline.

Phenacetin *O*-deethylase activity of a pooled microsomal fraction from three different human liver samples was determined over a substrate concentration range of 0 to 1 mM, in the presence of 0 μM (▲), 0.3 μM (●) and 1.0 μM (■) furafylline. Samples were preincubated for 2 min at 37° C in the presence of furafylline prior to addition of the substrate. Data were analysed assuming biphasic kinetics (i.e. comprising high affinity and low affinity components of activity) as described in **Methods** and elsewhere (Boobis *et al.*, 1981). Data are shown only for the high affinity component of activity, in the form of Eadie-Hofstee plots for illustrative purposes. The **points** represent the observed values corrected for the contribution of the low affinity component of activity, estimated from the values of V_{max} and K_m obtained by computer analysis of the data. The **lines** are those predicted from the computer estimates of V_{max} and K_m of the high affinity component of activity.

monooxygenase activity. Thus, both coumarin 7-hydroxylase and hepatic AHH activities were stimulated by furafylline, even at high doses, whereas both benzphetamine *N*-demethylase and 4-nitrophenol 3-hydroxylase activities were weakly inhibited (Table 5). Neither ethylmorphine *N*-demethylase nor placental AHH activity was affected at any concentration of furafylline.

The kinetics of furafylline inhibition of the high affinity component of the *O*-deethylation of phenacetin by microsomal fractions of human liver were determined. Furafylline caused a dose dependent decrease in the apparent V_{max} for POD activity, with no change in apparent K_m (Table 6). This is apparent from the parallel shift in the Eadie-Hofstee plots shown in Figure 3. The K_i for inhibition of POD activity by furafylline was 0.7 μM (Table 6).

The effects of furafylline on POD and AHH activities of microsomal fractions from MC induced rats was also investigated. In previous studies (Sesardic *et al.*, 1990), we have shown that these activities are catalysed almost exclusively by P450IA2 and P450IA1, respectively, in the liver of MC induced rats. At concentrations of up to 50 μM, furafylline had no effect on either activity in the rat (Figure 4). However, at higher

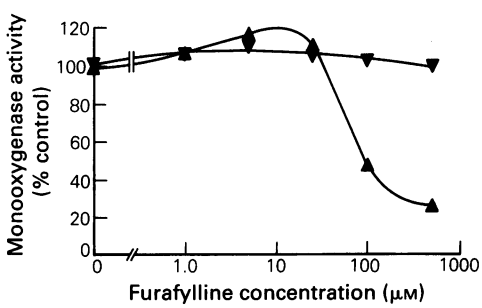

Figure 4 Effects of furafylline of hepatic phenacetin *O*-deethylase and aryl hydrocarbon hydroxylase activities of 3-methylcholanthrene treated rats.

The effects of furafylline were determined on phenacetin *O*-deethylase (▲) and aryl hydrocarbon hydroxylase (▼) activities of hepatic microsomal fractions from rats treated with a single i.p. dose of 80 mg kg^{-1} 3-methylcholanthrene 48 h prior to sacrifice. The pooled hepatic microsomal fraction from three different animals was preincubated with furafylline, from 0–500 μM, for 2 min at 37° C prior to addition of substrate, phenacetin or benzo[*a*]pyrene, to give a final concentration of 4 μM or 40 μM, respectively. The values shown are for activities in the presence of furafylline expressed as a percentage of control activity, in the presence of methanol alone, which was 487 pmol mg^{-1} min^{-1} for phenacetin *O*-deethylase activity and 1.56 nmol mg^{-1} min^{-1} for aryl hydrocarbon hydroxylase activity.

Table 6 Kinetics of inhibition of high affinity POD activity of human liver by furafylline

Furafylline concentration (μM)	*Apparent* V_{max} (*pmol mg*$^{-1}$ *min*$^{-1}$) (*Radex error*)	*Apparent* K_m (μM) (*Radex error*)	K_i (μM)
0	676 (40.8)	24.0 (2.6)	—
0.3	479 (335)	25.6 (20.2)	0.73
1.0	299 (113)	16.1 (7.7)	0.80

The kinetics of phenacetin *O*-deethylase activity were determined over a range of substrate concentrations, from 1 μM to 1 mM, in the presence and absence of furafylline, as described in **Methods**. The data were analysed assuming a 2-component, 4-parameter system, comprising low affinity and high affinity components of activity (Boobis *et al.*, 1981), using iterative non-linear least squares regression analysis to determine the respective values for V_{max} and K_m. Results are shown only for the high affinity component of activity. The figures in parentheses are the radex errors (Koeppe & Hamann, 1980), defined as the maximum amount by which the regression coefficient can deviate from its 'best' value without changing the sum of squares by more than 100% of its best (smallest) value.

concentrations, POD activity was selectively inhibited, and at 500 μM most of POD activity was inhibited, whilst furafylline was still virtually without effect on AHH activity.

Discussion

Furafylline was introduced 4 years ago as a long-acting substitute for theophylline in the treatment of asthma (Vega *et al.*, 1985). However, problems were encountered during phase 1 studies which were subsequently shown to be due to the inhibition of caffeine metabolism (Tarrús *et al.*, 1987a). This appeared to be due largely to inhibition of the major route of oxidation, *N*3-demethylation (Tarrús *et al.*, 1987a). Previous studies, both in animals (Bonati *et al.*, 1980) and in man (Campbell *et al.*, 1987), have provided evidence that this pathway is inducible by polycyclic aromatic hydrocarbons, and indeed is largely responsible for the increased clearance of caffeine observed in cigarette smokers (Kotake *et al.*, 1982). Further, it has been suggested that constitutive *N*3-demethylation of caffeine is also catalysed by a polycyclic hydrocarbon inducible isoenzyme (Campbell *et al.*, 1987). This is similar to the situation reported for phenacetin, which we have shown to be a highly specific substrate for the polycyclic hydrocarbon inducible isoenzyme P450IA2 in human liver (Sesardic *et al.*, 1988), as well as in rat liver (Sesardic *et al.*, 1990).

It was therefore of interest to determine how specific the inhibitory effects of furafylline are in man. To that end, a number of marker activities were selected, as representing predominantly different isoenzymes of cytochrome P450. Furafylline was an effective inhibitor of only the *O*-deethylation of phenacetin and the demethylation of caffeine. Virtually no inhibitory effect was observed on debrisoquine 4-hydroxylase activity, catalysed specifically by P450IID1 in human liver (Distlerath *et al.*, 1985), on the 7-hydroxylation of coumarin, catalysed by a unique isoenzyme designated $P450_{Coh}$ (Raunio *et al.*, 1988), benzphetamine *N*-demethylase, catalysed by P450IIB1 in PB-induced animals (Reik *et al.*, 1985) but probably catalysed by P450IIIA (Guengerich *et al.*, 1986), and possibly P450IIB1, in man, ethylmorphine *N*-demethylase, catalysed by P450IIIA (Guengerich *et al.*, 1986), and 4-nitrophenol 3-hydroxylase, which is catalysed by P450IIE1 in experimental animals (Koop, 1986) and may be catalysed by this isoenzyme in man. In addition, no effect was seen on aryl hydrocarbon hydroxylase activity of liver, catalysed by P450IIC and P450IIIA (Kawano *et al.*, 1987), nor on the AHH activity of placenta from women who smoked, catalysed by P450IA1 (Pasanen *et al.*, 1989). Thus, furafylline is a highly selective inhibitor of the hydrocarbon inducible isoenzyme P450IA2, but has no effect on the other major hydrocarbon inducible isoenzyme, P450IA1.

The data presented here thus show that fura-

fylline inhibits P450IA2, and not P450IA1. As a consequence, we can conclude that the *N*3-demethylation of caffeine is catalysed almost exclusively by P450IA2, and that P450IA1 does not contribute to this reaction in the liver. This is also true for hepatic phenacetin *O*-deethylation, which is catalysed by only P450IA2. Butler *et al.* (1989) have recently reached similar conclusions on the role of P450IA2 in these two reactions in the liver. However, the normal absence of P450IA1 from human liver, even in smokers (Sesardic *et al.*, 1988), makes it impossible to determine the contribution that this isoenzyme might make should it be induced (Wrighton *et al.*, 1986), for example such as might occur on exposure to chlorinated hydrocarbons. POD activity of extrahepatic tissues, such as the placenta of smokers, in which P450IA1 is expressed (Pasanen *et al.*, 1989), is catalysed by this isoenzyme. The inducibility of caffeine *N*3-demethylation by cigarette smoking (Campbell *et al.*, 1987), coupled with the results presented here, suggest that both constitutive and induced *N*3-demethylation of caffeine in human liver is catalysed by the polycyclic aromatic hydrocarbon inducible isoenzyme, P450IA2. A proportion of both the *N*1- and *N*7-demethylation of caffeine is potently inhibited by furafylline, and is presumably catalysed by P450IA2. It is likely that the residual activity is catalysed by one or more additional isoenzymes, not inducible by cigarette smoking.

Whilst furafylline was without any great effect on the monooxygenase activities studied, other than POD and caffeine demethylation, differences are observed in the effects on the other monooxygenase activities. Thus, both hepatic AHH and coumarin 7-hydroxylase activities are stimulated by furafylline. The phenomenon of enhancement of monooxygenase activity by the *in vitro* addition of a modifier to microsomal fractions of human liver has been observed previously (Kapitulnik *et al.*, 1977). The mechanism is still not clear, but is presumably related either to allosteric modification of the specific isoenzyme involved, or to inhibition of the further metabolism of the product. Under the conditions of the *in vitro* assay, it is highly unlikely that inhibition of competing pathways for the parent substrate play an important role in this effect. In view of the possibility that inhibition of product metabolism might be involved, it is not possible to draw any conclusions from these data regarding the similarity or otherwise of the isoenzymes catalysing coumarin 7-hydroxylase and AHH activities in human liver. However, there is evidence from previous studies that these two activities are catalysed primarily by different isoenzymes of P450 in human liver (Raunio *et al.*, 1988).

Both benzphetamine *N*-demethylase and 4-nitrophenol hydroxylase activities are inhibited to a minor extent by furafylline, as is the low affinity component of POD activity. This might be due either to a minor contribution of P450IA2 to the metabolism of these compounds or, more likely, to a weak inhibitory effect of furafylline on other isoenzymes of P450. However, there does appear to be specificity in any such weak inhibitory effect as neither the *N*-demethylation of ethylmorphine nor placental AHH activity was affected at all by furafylline.

Furafylline inhibits P450IA2 non-competitively which would readily explain the potency of its inhibitory effect *in vivo*. Indeed, the plasma concentrations achieved (Tarrús *et al.*, 1987a) are some 10 times its K_i value. Such a mechanism of inhibition is perhaps surprising given the high degree of specificity of furafylline for P450IA2. However, this might be explained by the similarity in structure between furafylline and some of the substrates for P450IA2, such as caffeine. It may be that furafylline initially interacts with the substrate binding site, but then forms a tight ligand through a basic nitrogen with the haem iron of P450. This mechanism of inhibition has been demonstrated previously for a number of important inhibitors, including ketoconazole (Sheets *et al.*, 1986) and possibly cimetidine (Murray, 1987). Whatever the mechanism, furafylline is undoubtedly a potent inhibitor of human P450IA2 catalysed activities.

Despite the similarity in the kinetics and specificity of P450IA2 for phenacetin *O*-deethylation in rat (Sesardic *et al.*, 1990) and man (Sesardic *et al.*, 1988), furafylline is a much weaker inhibitor of POD activity in the former species, the IC_{50} value differing by over 1,000-fold from that in man. However, the inhibition that is observed in the rat appears to be selective for P450IA2, at least between the hydrocarbon inducible isoenzymes of P450, as P450IA1 activity (AHH) is not affected by concentrations of furafylline of up to 500 μM. Thus, there appears to be a fundamental difference in the structure of the active site of these two orthologous isoenzymes, and this points further to the difficulties of extrapolating from results obtained in one species to another. Nevertheless, the rat has been shown to be a suitable species in which to identify inhibitors of caffeine metabolism *in vivo*, when high concentrations of potential inhibitors are used (Tarrús *et al.*, 1987b).

We have demonstrated that furafylline is an extremely potent inhibitor of an activity, POD, catalysed by P450IA2 in human liver. This in-

hibition is very selective, and no inhibition of the closely related P450IA1 is observed. Further, the fact that all of high affinity POD activity is inhibitable by furafylline confirms the specificity of P450IA2 for this reaction in human liver. Thus, inhibition by furafylline *in vivo* provides a means of determining the contribution of P450IA2 to any given oxidation pathway. The demethylation of caffeine, particularly at *N*3, is also catalysed almost entirely by P450IA2 in human liver and thus, the formation of the *N*3-demethylated product (paraxanthine) *in vivo* should provide a very useful means of assessing the activity of this isoenzyme in man.

We wish to thank Laboratorias Almirall (Barcelona, Spain) for providing furafylline, and Roche Products Ltd (Welwyn Garden City, UK) for debrisoquine and 4-hydroxydebrisoquine. Dr C. O. Meese (Stuttgart, FRG) very generously provided tetradeutero-4-hydroxydebrisoquine. We are extremely grateful to Professor O. Pelkonen and Dr M. Pasanen (Oulo, Finland) for making human placental samples available to us for these studies.

This study was supported by grants from the Medical Research Council, the Cancer Research Campaign and the Wellcome Trust.

This study was conducted within the framework of COST Action B1.

References

Aitio, A. (1978). A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal. Biochem.*, **85**, 488–491.

Atlas, S. A., Vesell, E. S. & Nebert, D. W. (1976). Genetic control of inter-individual variations in the inducibility of aryl hydrocarbon hydroxylase in cultured human lymphocytes. *Cancer Res.*, **36**, 4619–4630.

Bonati, M., Latani, R., Marzi, E., Cantoni, R. & Belvedere, G. (1980). [2-^{14}C]Caffeine metabolism in control and 3-methylcholanthrene induced rat liver microsomes by high pressure liquid chromatography. *Tox. Lett.*, **7**, 1–7.

Boobis, A. R. & Davies, D. S., (1984). Human cytochromes P-450. *Xenobiotica*, **14**, 151–185.

Boobis, A. R., Brodie, M. J., Kahn, G. C. & Davies, D. S. (1980a). Evidence for more than one form of cytochrome P-450 in hepatic microsomal fractions from human liver samples. In *Microsomes, drug oxidations, and chemical carcinogenesis*, eds Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R. & O'Brien, P. J., pp. 957–960. New York: Academic Press.

Boobis, A. R., Brodie, M. J., Kahn, G. C., Fletcher, D. R., Saunders, J. H. & Davies, D. S. (1980b). Monooxygenase activity of human liver in microsomal fractions of needle biopsy specimens. *Br. J. clin. Pharmac.*, **9**, 11–19.

Boobis, A. R., Hampden, C. E., Murray, S., Beaune, P. & Davies, D. S. (1985). Attempts to phenotype human liver samples *in vitro* for debrisoquine 4-hydroxylase activity. *Br. J. clin. Pharmac.*, **19**, 721–729.

Boobis, A. R., Kahn, G. C., Whyte, C., Brodie, M. J. & Davies, D. S. (1981). Biphasic *O*-deethylation of phenacetin and 7-ethoxycoumarin by human and rat liver microsomal fractions. *Biochem. Pharmac.*, **30**, 2451–2456.

Boobis, A. R., Murray, S., Kahn, G. C., Robertz, G.-M. & Davies, D. S. (1983). Substrate specificity of the form of cytochrome P-450 catalyzing the 4-hydroxylation of debrisoquine in man. *Mol. Pharmac.*, **23**, 474–481.

Boobis, A. R., Murray, S., Seddon, C. E. & Davies, D. S. (1987). *In vitro* studies of induction and inhibition of drug oxidation in man. *Pharmac. Ther.*, **33**, 101–198.

Butler, M. A., Iwasaki, M., Guengerich, F. P. & Kadlubar, F. K. (1989). Human cytochrome P-450$_{PA}$ (P-450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc. Nat. Acad. Sci.*, **86**, 7696–7700.

Campbell, M. E., Grant, D. M., Inaba, T. & Kalow, W. (1987). Biotransformation of caffeine, paraxanthine, theophylline, and theobromine by polycyclic aromatic hydrocarbon-inducible cytochrome(s) P-450 in human liver microsomes. *Drug Metab. Dispos.*, **15**, 237–249.

Distlerath, L. M., Reilly, P. E. B., Martin, M. V., Davis, G. G., Wilkinson, G. R. & Guengerich, F. P. (1985). Purification and characterization of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin *O*-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. *J. biol. Chem.*, **260**, 9057–9067.

Dixon, M. (1953). The determination of enzyme inhibitor constants. *Biochem. J.*, **55**, 170–171.

Farrell, G. C., Cooksley, W. G. E. & Powell, L. W. (1979). Drug metabolism in liver disease: Activity of hepatic microsomal metabolizing enzymes. *Clin. Pharmac. Ther.*, **26**, 483–492.

Gonzalez, F. P. (1989). The molecular biology of cytochrome P450s. *Pharmac. Rev.*, **40**, 243–288.

Gonzalez, F. J., Skoda, R., Kimura, S., Umeno, M., Zanger, U. M., Nebert, D. W., Gelboin, H. V., Hardwick, J. P. & Meyer, U. A. (1988). Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature*, **331**, 442–446.

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

Greenlee, W. F. & Poland, A. (1978). An improved

assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Pharmac. exp. Ther.*, **205**, 596–605.

Guengerich, F. P., Martin, M. V., Beaune, P. H., Kremers, P., Wolff, T. & Waxman, D. J. (1986). Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. biol. Chem.*, **261**, 5051–5060.

Hofstee, B. H. J. (1952). On the evaluation of the constants V_{max} and K_m in enzyme reactions. *Science*, **116**, 329–331.

Kadlubar, F. F. & Hammons, G. J. (1987). The role of cytochrome P-450 in the metabolism of chemical carcinogens. In *Mammalian cytochromes P-450*. Vol. II, ed. Guengerich, F. P., pp. 81–130. Boca Raton, FL: CRC Press.

Kahn, G. C., Boobis, A. R., Brodie, M. J., Toverud, E.-L., Murray, S. & Davies, D. S. (1985). Phenacetin *O*-deethylase: An activity of a cytochrome P-450 showing genetic linkage with that catalysing the 4-hydroxylation of debrisoquine? *Br. J. clin. Pharmac.*, **20**, 67–76.

Kapitulnik, J., Poppers, P. J., Buening, M. K., Fortner, J. G. & Conney, A. H. (1977). Activation of monooxygenases in human liver by 7,8-benzoflavone. *Clin. Pharmac. Ther.*, **22**, 475–484.

Kawano, S., Kamataki, T., Yasumori, T., Yamazoe, Y. & Kato, R. (1987). Purification of human liver cytochrome P-450 catalyzing testosterone 6β-hydroxylation. *J. Biochem. (Tokyo)*, **102**, 493–501.

Kobayashi, S., Murray, S., Watson, D., Sesardic, D., Davies, D. S. & Boobis, A. R. (1989). The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochem. Pharmac.*, **38**, 2795–2799.

Koeppe, P. & Hamann, C. (1980). A program for non-linear regression analysis to be used on desk-top computers. *Comput. Programs Biomed.*, **12**, 121–128.

Koop, D. R. (1986). Hydroxylation of *p*-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isoenzyme 3a. *Mol. Pharmac.*, **29**, 399–404.

Kotake, A. N., Schoeller, D. A., Lambert, G. H., Baker, A. L., Schaffer, D. C. & Josephs, H. (1982). The caffeine CO_2 breath test: Dose response and route of *N*-demethylation in smokers and non-smokers. *Clin. Pharmac. Ther.*, **32**, 261–269.

Kremers, P., Beaune, P., Cresteil, T., de Graeve, J., Columelli, S., Leroux, J.-P. & Gielen, J. E. (1981). Cytochrome P-450 monooxygenase activities in human and rat liver microsomes. *Eur. J. Biochem.*, **118**, 599–606.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

Murray, M. (1987). Mechanisms of the inhibition of cytochrome P-450-mediated drug oxidation by therapeutic agents. *Drug Metab. Rev.*, **18**, 55–81.

Murray, S. & Boobis, A. R. (1986). An assay for paracetamol, produced by the *O*-deethylation of phenacetin *in vitro*, using gas chromatography/electron capture negative ion chemical ionization mass spectrometry. *Biomed. environ. Mass Spec.*, **13**, 91–93.

Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.*, **55**, 416–421.

Nebert, D. W. (1978). Genetic differences in microsomal electron transport: The *Ah* locus. *Methods Enzym.*, **52**, 226–240.

Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, S. & Waterman, M. R. (1989). The P450 superfamily: Updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA*, **8**, 1–13.

Nelson, S. D. & Harvison, P. J. (1987). Roles of cytochromes P-450 in chemically induced cytotoxicity. In *Mammalian cytochromes P-450*. Vol. II, ed. Guengerich, F. P., pp. 19–79. Boca Raton, FL: CRC Press.

Omura, T. & Sato, R. (1964). The carbon monoxide-binding pigment in liver microsomes. I. Evidence for its hemoprotein nature. *J. biol. Chem.*, **239**, 2370–2378.

Otton, S. V., Inaba, T. & Kalow, W. (1984). Competitive inhibition of sparteine oxidation in human liver by β-adrenoceptor antagonists and other cardiovascular drugs. *Life Sci.*, **34**, 73–80.

Pantuck, E. J., Hsiao, K.-C. Maggio, A., Nakamura, K., Kuntzman, R. & Conney, A. H. (1974). Effect of cigarette smoking on phenacetin metabolism. *Clin. Pharmac. Ther.*, **15**, 9–17.

Pasanen, M., Raunio, H. & Pelkonen, O. (1985). Freezing affects the activity and subcellular distribution profile of human placental xenobiotic- and steroid-metabolizing enzymes. *Placenta*, **6**, 527–535.

Pasanen, M., Stenbäck, F., Taskinen, T., Raunio, H., Sivonen, P., Hines, R. & Pelkonen, O. (1989). Human placental 7-ethoxyresorufin *O*-deethylase activity: Inhibition studies and immunological and molecular biological detection of P-450IA1. In *Cytochrome P-450: Biochemistry and biophysics*, ed. Schuster, I. pp. 532–535. London: Taylor & Francis.

Pasanen, M., Vahakangas, K., Sotaniemi, E. A. & Pelkonen, O. (1988). The effect of cigarette smoking on drug metabolism in the liver and placenta: The use of cotinine in verifying smoking status. *Eur. J. Drug Metab. Pharmacokin.*, **13**, 41–45.

Pelkonen, O. & Pasanen, M. (1981). Effect of heparin on the subcellular distribution of human placental 7-ethoxycoumarin *O*-deethylase activity. *Biochem. Pharmac.*, **30**, 3254–3256.

Pelkonen, O., Sotaniemi, E. A. & Ahokas, J. T. (1985). Coumarin 7-hydroxylase activity in human liver microsomes. Properties of the enzyme and

interspecies comparison. *Br. J. clin. Pharmac.*, **19**, 59–66.

Raunio, H., Syngelmä, T., Pasanen, M., Juvonen, R., Honkakoski, P., Kairaluoma, M. A., Sotaniemi, E., Lang, M. A. & Pelkonen, O. (1988). Immunochemical and catalytical studies on hepatic coumarin 7-hydroxylase in man, rat, and mouse. *Biochem. Pharmac.*, **37**, 3889–3895.

Reik, L. M., Levin, W., Ryan, D., Maines, S. L. & Thomas, P. E. (1985). Monoclonal antibodies distinguish among isoenzymes of the cytochrome P-450b subfamily. *Arch. Biochem. Biophys.*, **242**, 365–382.

Reinke, L. A. & Moyer, M. J. (1985). *p*-Nitrophenol hydroxylation. A microsomal oxidation which is highly inducible by ethanol. *Drug Metab. Dispos.*, **13**, 548–552.

Sesardic, D., Boobis, A. R., Edwards, R. J. & Davies, D. S. (1988). A form of cytochrome P450 in man, orthologous to form d in the rat, catalyses the *O*-deethylation of phenacetin and is inducible by cigarette smoking. *Br. J. clin. Pharmac.*, **26**, 363–372.

Sesardic, D., Edwards, R. J., Davies, D. S., Thomas, P. E., Levin, W. & Boobis, A. R. (1990). High affinity phenacetin *O*-deethylase is catalysed specifically by cytochrome P450d (P450IA2) in the liver of the rat. *Biochem. Pharmac.* (in press).

Sheets, J. L., Mason, J. I., Wise, C. A. & Estabrook, R. W. (1986). Inhibition of rat liver microsomal cytochrome P-450 steroid hydroxylase reactions by imidazole antimycotic agents. *Biochem. Pharmac.*, **35**, 487–491.

Song, B. J., Friedman, F. K., Park, S. S., Tsokos, G. C. & Gelboin, H. V. (1985). Monoclonal antibody-directed radioimmunoassay detects cytochrome P-450 in human placenta and lymphocytes. *Science*, **228**, 490–492.

Speirs, C. J., Murray, S., Boobis, A. R., Seddon, C. E. & Davies, D. S. (1986). Quinidine and the identification of drugs whose elimination is impaired in subjects classified as poor metabolizers of debrisoquine. *Br. J. clin. Pharmac.*, **22**, 739–743.

Tarrús, E., Cami, J., Roberts, D. J., Spickett, R. G. W., Celdran, E. & Segura, J. (1987a). Accumulation of caffeine in healthy volunteers treated with furafylline. *Br. J. clin. Pharmac.*, **23**, 9–18.

Tarrús, E., García, I. & Segura, J. (1987b). An animal model to detect the inhibition of the metabolism of caffeine. *Methods Find. exp. clin. Pharmac.*, **9**, 311–316.

Vega, A., Prieto, J., Moragues, J., Spickett, R. G. W., Llupia, J., Llenas, J. & Roberts, D. J. (1985). Derivadas de xantina. Presented at *4th National Congress of the Sociedad Español de Quimica Terapéntica Sitges (Barcelona) Spain.*

Wilson, N. M., Christou, M., Turner, C. R., Wrighton, S. A. & Jefcoate, C. R. (1984). Binding and metabolism of benzo(a)pyrene and 7,12-dimethylbenz(a)-anthracene by seven purified forms of cytochrome P-450. *Carcinogenesis*, **5**, 1475–1483.

Wong, T. K., Domin, B. A., Bent, P. E., Blanton, T. E., Anderson, M. W. & Philpot, R. M. (1986). Correlation of placental microsomal activities with protein detected by antibodies to rabbit cytochrome P-450 isoenzyme 6 in preparations from humans exposed to polychlorinated biphenyls, quaterphenyls, and dibenzofurans. *Cancer Res.*, **46**, 999–1004.

Wrighton, S. A., Maurel, P., Schuetz, E. G., Watkins, P. B., Young, B. & Guzelian, P. S. (1985). Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450$_p$. *Biochemistry*, **24**, 2171–2178.

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. & Guzelian, P. S. (1986). Identification of a human liver cytochrome P-450 homologous to the major isosafrole-inducible cytochrome P-450 in the rat. *Mol. Pharmac.*, **29**, 405–410.

Yang, C. S. & Lu, A. Y. H. (1987). The diversity of substrates for cytochrome P-450. In *Mammalian cytochromes P-450*. Vol. II, ed. Guengerich, F. P., pp. 2–17. Boca Raton, FL: CRC Press.

(Received 20 October 1989, accepted 19 January 1990)