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Original article

Caffeine as a probe for human cytochromes P450: validation using cDNA-expression, immunoinhibition and microsomal kinetic and inhibitor techniques

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The molecular basis for the use of caffeine (CA; 1,3,7-trimethylxanthine) as a probe for specific human cytochromes P450 has been investigated. The CA 1-, 3- and 7-demethylations (to form theobromine, paraxanthine and theophylline, respectively) all followed biphasic kinetics in human liver microsomes. Mean apparent K_m values for the high- and low-affinity components of the demethylations ranged from 0.13–0.31 mM and 19.2–30.0 mM, respectively. cDNA-expressed CYP1A2 catalysed all three CA demethylations, and the apparent K_m for CA 3-demethylation (the major metabolic pathway in humans) by the expressed enzyme was similar to the K_m for the high-affinity liver microsomal CA 3-demethylase. IC_{50} values for inhibition of the CA demethylations by α-naphthoflavone were similar for both expressed CYP1A2 and the high-affinity microsomal demethylases. Moreover, CA was a competitive inhibitor of expressed CYP1A2 catalysed phenacetin O-deethylation, with the apparent K_i (0.080 mM) closely matching the apparent K_m (0.082 mM) for CA 3-demethylation by the expressed enzyme. Expressed CYP1A1 was additionally shown to catalyse the 3-demethylation of CA, although activity was lower than that observed for CYP1A2. While these data indicate that CYP1A2 is responsible for the high-affinity component of human liver CA 3-demethylation, two limitations associated with the use of CA as an *in vitro* probe for CYP1A2 activity have been identified: (i) CA 3-demethylation reflects hepatic CYP1A2 activity only at appropriately low substrate concentrations; and (ii) CA is a non-specific CYP1A substrate and CYP1A1 may therefore contribute to CA 3-demethylase activity in tissues in which it is expressed. An anti-CYP3A antibody essentially abolished the 8-hydroxylation of CA to form trimethyluric acid, suggesting formation of this metabolite may potentially serve as a marker of CYP3A isozyme(s) activity.

Introduction

It is well established that cytochrome P450 comprises a gene superfamily, with individual gene products (isozymes) tending to differ in terms of substrate specificity, regulation and tissue distribution (Distlerath & Guengerich 1987; Gonzalez, 1989). Of the various cytochrome P450 isozymes identified to date, there has been particular interest in the function of the polycyclic aromatic hydrocarbon inducible 1A subfamily cytochromes P450 (i.e. CYP1A1 and CYP1A2). Owing to the apparent negligible expression of CYP1A1 in liver, most attention has focussed on CYP1A2. Although CYP1A2 metabolizes relatively few drugs, notably methylxanthines (Miners *et al.*, 1985a; Robson *et al.*, 1987a; Kalow & Campbell, 1988; Butler *et al.*, 1989) and phenacetin (Sesardic *et al.*, 1988, 1990a), it has been implicated in the *N*-hydroxylation of numerous aromatic amines, an obligatory step leading to mutagenesis or carcinogenesis. In particular, CYP1A2 has been demonstrated to catalyse the *N*-hydroxylation of 2-acetylamino-

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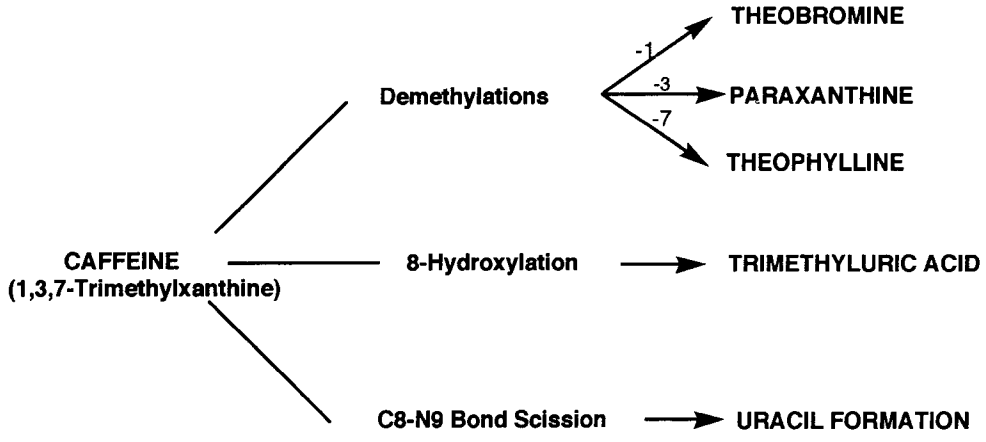

Fig. 1. Primary metabolic pathways of caffeine in humans.

fluorene, 2-aminoanthracene, 4-aminobiphenyl, 2-naphthylamine and a range of food-derived heterocyclic amines (Kadlubar & Hammons, 1987; Yamazoe *et al.*, 1988; Butler *et al.*, 1989; McManus *et al.*, 1989, 1990; Shimada *et al.*, 1989).

Given the potentially important role of CYP1A2 in chemical carcinogenesis, there has been considerable interest in identifying compounds which may be used as *in vivo* and *in vitro* probes for the population screening of the activity of this enzyme activity in humans. In this regard considerable attention has focussed on caffeine (CA; 1,3,7-trimethylxanthine). CA is one of the most widely consumed dietary chemicals in the world and therefore constitutes an ideal model substrate for assessing xenobiotic metabolizing enzyme activity in man. Although CA metabolism is complex, the various biotransformation pathways are now well characterized. The primary metabolic pathways of CA are illustrated schematically in Fig. 1. *N*3-Demethylation to form paraxanthine (PX; 1,7-dimethylxanthine) accounts for approximately 80% of CA metabolism in humans (Lelo *et al.*, 1986); *N*1-demethylation to theobromine (TB; 3,7-dimethylxanthine) and *N*7-demethylation to theophylline (TP; 1,3-dimethylxanthine) account for 11% and 4% of a CA dose, respectively. 8-Hydroxylation to form 1,3,7-trimethyluric acid (TMU), C8-N9 bond scission to give 6-amino-5-[N-formylmethylamino]-1,3-dimethyluric acid (DAU), and renal excretion of unchanged CA comprise the other primary elimination pathways (Callahan *et al.*, 1982; Lelo *et al.*, 1986). It should be noted that, once formed, PX, TB and TP undergo further extensive biotransformation resulting in the urinary excretion of at least seventeen CA-derived metabolites in humans ingesting this compound (Callahan *et al.*, 1982; Miners *et al.*, 1982; Birkett *et al.*, 1983; Lelo *et al.*, 1989).

Initial evidence suggesting the involvement of CYP1A2 in CA metabolism was provided by the observations that 3-methylcholanthrene treatment and cigarette smoking increased CA plasma clearance in the rat and in humans, respectively (Aldridge *et al.*, 1977; Parsons & Neims, 1978). More recently, associations between CYP1A2 and individual CA metabolic pathways have been investigated *in vitro* (Campbell *et al.*, 1987a; Grant *et al.*, 1987; Butler *et al.*, 1989; Sesardic *et al.*, 1990b; Berthou *et al.*, 1991). On the basis of results from these studies, measurement of CA 3-demethylase activity was proposed as a means of assessing levels of human liver CYP1A2 *in vitro*. Further to the use of CA as an *in vitro* probe, it has been reported that the ratio of certain PX-derived metabolites excreted in urine following CA administration to humans correlates with CA plasma clearance and hence reflects CA 3-demethylation (Campbell *et al.*, 1987b; Kalow & Campbell, 1988; Kalow & Tang, 1991). Accordingly, it has been argued that the so called CA urinary metabolite ratio provides a measure of *in vivo* hepatic CYP1A2 activity in humans.

Given the likely broad application of the CA 3-demethylation tests for the population screening of CYP1A2 activities *in vivo* and *in vitro*, the molecular basis for the use of CA as a probe for CYP1A2 and possibly other human cytochromes P450 has been investigated using cDNA-expression, immunoinhibition and human liver microsomal kinetic and inhibitor studies. The results have highlighted limitations associated with the use of CA as a probe for CYP1A2 activity. Importantly, it has been demonstrated for the first time that CYP1A1 also has the capacity to catalyse the 3-demethylation of CA and that the 8-hydroxylation pathway (to form TMU) may potentially serve as a marker of CYP3A isozyme(s) activity.

Materials and methods

Chemicals

CA, PX, 5-methoxypsoralen, 8-methoxypsoralen and phenacetin were purchased from the Sigma Chemical Co. (St Louis, MO), α-naphthoflavone from the Aldrich Chemical Co. (Milwaukee, WI), and TMU from Fluka AG (Buchs, Switzerland). [2-^{14}C] CA (specific activity 56 mCi mmol^{-1}) was synthesized by Moravek Biochemicals (Brea, CA). Other drugs were obtained from the following sources: erythromycin from FH Faulding & Co. (Adelaide, Australia), mephenytoin from Sandoz Ltd (Basle, Switzerland), nifedipine from Bayer Pharmaceutical (Sydney, Australia), phenylbutazone, sulphinpyrazone and sulphaphenazole from Ciba-Geigy Aust. (Sydney, Australia), propranolol HCl from ICI Aust. (Melbourne, Australia), quinidine sulphate from Burroughs Wellcome Aust. (Sydney, Australia) and TB and TP from Hamilton Laboratories (Adelaide, Australia). All other chemicals and solvents were of analytical reagent grade.

Human livers, antibodies and cDNAs

Human liver samples were obtained from renal transplant donors; relevant details of the donors of livers used in the present study (*viz.* F7, F8, F10, F15) have been published elsewhere (Robson *et al.*, 1987b; McManus *et al.*, 1990). Human hepatic microsomes were prepared as previously described (Robson *et al.*, 1987b). The use of human liver tissue for xenobiotic metabolism studies was approved by the Flinders Medical Centre Ethical Review Committee and, where appropriate, the Adelaide Coroner. The preparation of the two polyclonal antibodies used in this work, anti-human NADPH-P450 oxidoreductase and anti-rat cytochrome P450PCN1 (CYP3A1), has been described previously (McManus *et al.*, 1987a; Aoyama *et al.*, 1990). The anti-rat P4503A1 has been shown specifically to inhibit human cytochromes P450 3A3, 3A4 and 3A5 (Aoyama *et al.*, 1990). A full length human CYP1A2 cDNA and a human *CYP1A1* gene were isolated from human liver cDNA and genomic libraries, respectively, as previously described (Quattrochi *et al.*, 1986; Quattrochi & Tukey 1989; McManus *et al.*, 1990). The CYP1A2 cDNA and *CYP1A1* gene were subcloned into the pCMV4 expression vector and transfected into COS-7 cells according to published methods (McManus *et al.*, 1990; Veronese *et al.*, 1991). Cells were harvested 48 h posttransfection, resuspended in 0.1 M phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, and stored at −70°C until used. An anti-rabbit P4501A2 antibody, which recognizes both CYP1A1 and CYP1A2 (McManus *et al.*, 1990), was used to detect the expressed human proteins. Cells transfected with the pCMV4 vector alone served as negative controls for incubations of CYP1A1 and CYP1A2 transfected COS-7 cells. CYP1A1 and CYP1A2 were expressed simultaneously using the same batch of cells. Nascent enzyme was labelled with ^{35}S-methionine (Veronese *et al.*, 1991) and immunoadsorbed using the anti-rabbit P4501A2 antibody. The relative expression of CYP1A1 and CYP1A2 was 1:1.3.

Assay for measurement of CA metabolite formation

The conversion of CA to PX, TB, TP and TMU by human liver microsomes and by expressed CYP1A1 and CYP1A2 was determined using a modification of methods used previously for the study of methylxanthine biotransformation *in vitro* (Robson *et al.*, 1987b; Lelo *et al.*, 1988). A standard 0.5 ml incubation contained human liver microsomal protein (1 mg) or COS cell protein (2 mg) in phosphate buffer (0.2 M, pH 7.4), NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate, 2 IU glucose 6-phosphate dehydrogenase and 5 mM $MgCl_2$), and CA (0.1–30 mM). Incubations were started by the addition of generating system and carried out in air at 37° C in a shaking water bath. Incubation times were 2 h when human liver microsomes were used and 3 h when COS cell protein was used; reaction rates were shown to be linear to these times for the two protein sources. Reactions were stopped by the addition of 0.4 M HCl (0.25 ml) and cooling on ice. 8-Chlorotheophylline (4 μg; the assay internal standard) was added to the incubation mixture, which was then saturated with ammonium sulphate (800–900 mg) and extracted twice with dichloromethane-isopropanol (85:15 v/v, 5 ml). The pooled extracts were dried under N_2 and analysed for PX, TB, TP and TMU by high performance liquid chromatography (hplc). The chromatograph used was fitted with an Ultrasphere ODS column (25 cm × 4.6 mm i.d. 5 micron particle size; Beckman Instruments, San Ramon, CA) which was eluted isocratically with acetate buffer (10 mM, pH 4.0)-methanol-tetrahydrofuran (92.75:6.5:0.75) at a flow rate of 2 ml min^{-1}. Metabolites were monitored by ultraviolet absorbance at 276 nm. Under these chromatographic conditions, retention times for TB, PX, TP, TMU, CA and 8-chlorotheophylline were 2.4, 4.0, 4.6, 5.6, 7.2 and 15.7 min, respectively. For those experiments utilizing unlabelled CA, standard curves for each metabolite were constructed in the range 0.5–200 μM and unknown concentrations determined by comparison of peak height ratios with those of the standard curve. When [2-^{14}C]CA was used as substrate, chromatography fractions corresponding to each metabolite peak

Table 1. Derived Michaelis-Menten parameters for the conversion of caffeine to paraxanthine, theobromine and theophylline by human liver microsomes

Metabolite	K_{m1} (*mM*)	V_{max1} (*pmol min^{-1} mg^{-1}*)	K_{m2} (*mM*)	V_{max2} (*pmol min^{-1} mg^{-1}*)
Paraxanthine	0.18 ± 0.02 (0.16–0.20)	18.1 ± 5.5 (12.1–24.6)	30.0 ± 9.4 (17.3–37.8)	183.5 ± 72.1 (102.3–254.9)
Theobromine	0.31 ± 0.32 (0.06–0.77)	2.54 ± 1.61 (0.75–4.52)	20.2 ± 5.70 (11.8–24.8)	65.9 ± 16.7 (46.5–83.0)
Theophylline	0.13 ± 0.03 (0.10–0.15)	0.90 ± 0.49 (0.29–1.45)	19.2 ± 6.1 (13.4–27.8)	83.9 ± 18.2 (66.8–106.1)

Data are mean ± SD from four livers. K_{m1} and V_{max1} refer to Michaelis-Menten parameters for high-affinity component of the CA demethylations while K_{m2} and V_{max2} refer to the parameters for the low-affinity component of the CA demethylations. Ranges of values calculated for each parameter are shown in parenthesis.

were collected and counted using a liquid scintillation counter (Beckman LS3801, Beckman Instruments). In all experiments using [2-^{14}C]CA, >99.9% of the radioactivity added to incubations could be accounted for in the combined column fractions.

CA kinetic and inhibitor studies

In experiments performed to determine the Michaelis-Menten parameters for CA metabolite formation, activities were measured for thirteen CA concentrations over the range 0.1–30 mM. The inhibitory effects of xenobiotics and antibodies on the high- and low-affinity components of CA metabolite formation (see Results) were assessed using CA concentrations of 0.1 mM and 20 mM, respectively. At the low substrate concentration incubations contained [2-^{14}C]CA (4.5×10^5 dpm, equivalent to 7.3 μM) plus sufficient unlabelled CA to give the desired final concentration. Seven CA concentrations in the range 0.025–0.4 mM were used for the cDNA-expressed CYP1A2 kinetic study; concentrations up to 0.1 mM comprised [2-^{14}C]CA alone while the two highest concentrations (*viz.* 0.2 and 0.4 mM) comprised both [2-^{14}C]CA (equivalent to 0.15 mM) and unlabelled compound. It was necessary to use radiolabelled CA for the low substrate concentration inhibition studies and the kinetic studies using expressed CYP1A1 and 1A2 due to limitations of assay sensitivity. Immunoinhibition studies with the anti-rat 3A1 and anti-human NADPH-P450 oxidoreductase were carried out by preincubating antiserum with microsomes for 15 min prior to measurement of CA metabolite formation. Control incubations contained an equivalent amount of preimmune serum protein. Xenobiotics screened for inhibitory effects on CA metabolism were erythromycin, mephenytoin, 5- and 8-methoxypsoralin, α-naphthoflavone, nifedipine, phenacetin, phenylbutazone, propranolol, quinidine, sulphaphenazole and sulphinpyrazone. With the exception of propranolol and quinidine, compounds were added to incubations as solutions in DMSO. The final concentration of DMSO in incubations was 1% v/v; an equivalent amount was added to control incubations.

In addition to the experiments investigating the inhibitory effects of xenobiotics on CA metabolism, the kinetics of inhibition of cDNA-expressed CYP1A2 catalysed phenacetin *O*-deethylation were also characterized. Inhibition of phenacetin *O*-deethylation by four concentrations of CA in the range 0.3–1.2 mM was assessed in incubations of transfected COS cell homogenate containing phenacetin (10, 20 or 30 μM). Phenacetin *O*-deethylase activity was determined by measurement of paracetamol (acetaminophen) formation using a specific hplc procedure; full details of this method will be described elsewhere.

Analysis of results

All results are presented as mean ± SD. Initial estimates of the Michaelis-Menten parameters, apparent K_m and V_{max}, were obtained from graphical analysis of Eadie-Hofstee and/or Lineweaver-Burke plots. These values were then used as first estimates for MKMODEL, an extended least squares modelling programme (Holford, 1985). The method of Dixon (1953) was used for the calculation of apparent K_i values.

Results

PX, TB and TP formation exhibited biphasic kinetics in all four human livers studied. Derived Michaelis-Menten parameters are summarized in Table 1 and a representative Eadie-Hofstee plot (for liver F15) is illustrated in Fig. 2. Mean apparent K_m values for the high- and low-affinity components of the CA

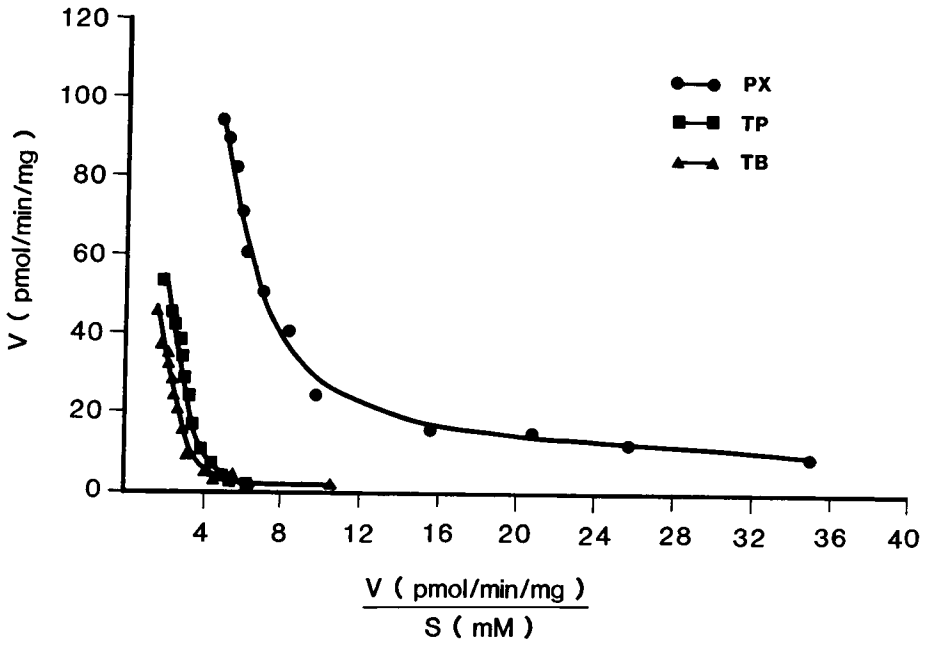

Fig. 2. Representative Eadie-Hofstee plots for the conversion of caffeine to paraxanthine (PX), theophylline (TP) and theobromine (TB) by human liver microsomes (liver F15). Points are experimentally determined values while solid lines are the computer-generated curves of best fit.

demethylations ranged from 0.13–0.31 mM and 19.21–30.02 mM, respectively. Whereas the mean V_{max} for the high-affinity component of PX formation was 7.1- and 20.1-fold higher than the respective V_{max} values for TB and TP formation, the mean V_{max} for the low-affinity component of PX formation was only 2.2- to 2.8-fold higher than the corresponding values for TP and TB. It should be recognized, however, that the derived Michaelis-Menten parameters (and activities shown below) for TB and TP should be regarded as estimates only. A co-chromatographing substance, believed to be 6-amino-5-[*N*-formylmethylamine]-1,3-dimethyluracil, occasionally interfered with the hplc quantification of TB while the low level of TP formation made measurement of this metabolite difficult at low substrate concentrations.

Substitution of the mean apparent K_m and V_{max} values in the Michaelis-Menten equation for a two enzyme system indicated that the high-affinity component of PX, TB and TP formation accounted for 91%, 66% and 48% of total activity along the respective pathways when the substrate concentration was 0.1 mM. Thus, at this substrate concentration the high-affinity component accounts for almost all PX formation, the majority of TB formation, but possibly (see previous paragraph) only approximately half of TP formation. In contrast, the low-affinity components of the CA demethylations are responsible for 80–90% of total activities at a substrate concentration of 20 mM and measurement of metabolite formation at this concentration can therefore be taken as representative of the low-affinity enzyme activity(s).

Omission of generating system from incubations abolished formation of PX, TB, TP and TMU at both the low and high substrate concentrations. Anti-human NADPH-P450 oxidoreductase (antiserum protein to microsomal protein ratio 10:1) reduced formation of the various CA metabolites by 83–98% at a substrate concentration of 0.1 mM and by 57–82% at a substrate concentration of 20 mM. The anti-reductase antibody employed has previously been shown to inhibit 2-acetylaminofluorene hydroxylations by 75–85% (McManus *et al.*, 1987b), theophylline *N*-demethylations and 8-hydroxylation by 80–85% (Robson *et al.*, 1987a), and tolbutamide hydroxylation by 55% (Miners *et al.*, 1988).

α-Naphthoflavone was a potent inhibitor of the high-affinity component of all three CA demethylations, with IC_{50} values of 0.4 μM, 0.7 μM and 0.7 μM for PX, TB and TP formation, respectively (Fig. 3A). 5- and 8-methoxypsoralen were approximately an order of magnitude less potent as inhibitors than was α-naphthoflavone, while nifedipine, phenacetin and propranolol were all able essentially to abolish the high-affinity component of PX formation at added concentrations of 0.25–1.0 mM. High concentrations (1 mM) of erythromycin, mephenytoin, and quinidine inhibited the CA demethylations to a minor extent,

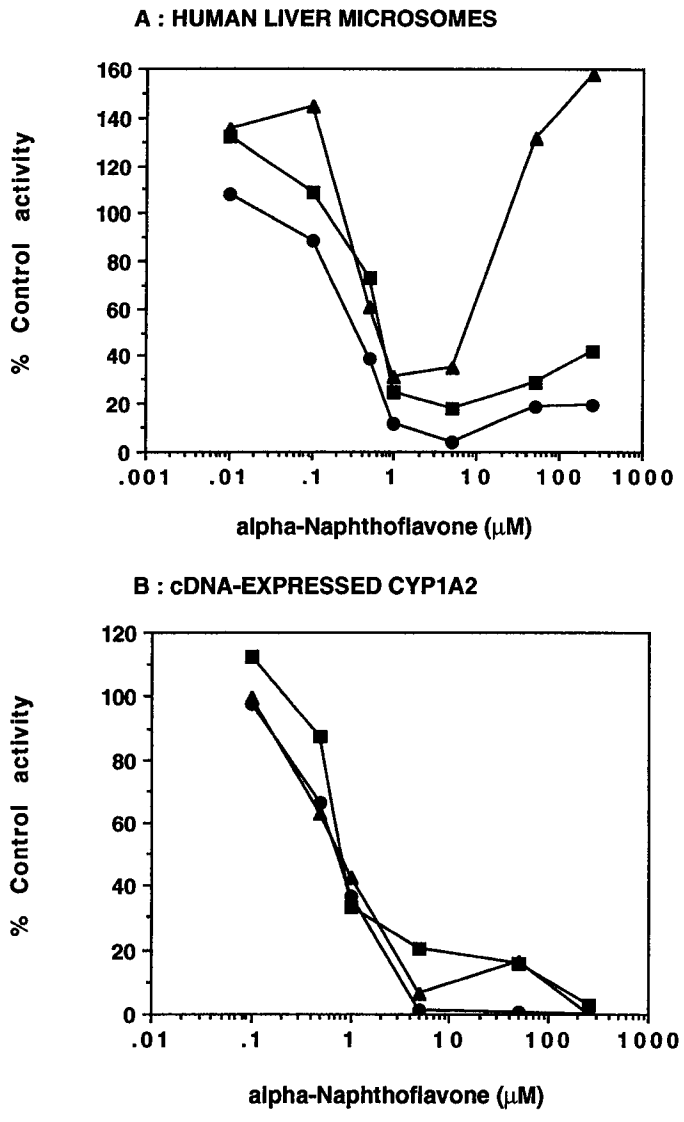

Fig. 3. Inhibition of caffeine demethylations by α-naphthoflavone. Panel A: human liver microsomes. Panel B: cDNA-expressed CYP1A2. ●—● Paraxanthine formation, ■—■ theophylline formation, ▲—▲ theobromine formation.

while phenylbutazone, sulphaphenazole and sulphinpyrazone were essentially without effect. Of the xenobiotics screened as inhibitors, only propranolol inhibited the low-affinity component of the CA demethylations to a substantial extent (*viz.* 56% of control activity at an added concentration of 1 mM). Notably, α-naphthoflavone was without effect on these reactions.

cDNA-expressed CYP1A2 catalysed the conversion of CA to PX, TB and TP (Table 2). Rates of formation for PX, TB and TP by expressed CYP1A2 exhibited the same rank order as the V_{max} values for the high-affinity components of the CA demethylations in human liver microsomes (Table 1). The apparent K_m for PX formation by expressed CYP1A2, determined over the substrate concentration range 0.025–0.4 mM, was 0.082 mM. This is of similar order to the apparent K_m (i.e. 0.18 mM) for the high-affinity PX-forming enzyme activity of human liver microsomes (Table 1). Limitations of assay sensitivity precluded calculation of accurate apparent K_m values for TB and TP formation by expressed CYP1A2. However, the IC_{50} values for α-naphthoflavone inhibition of all three CA demethylations (i.e. PX, TB and TP formation) could be determined and were all essentially identical, 0.7–0.75 μM (Fig. 3B). CA competitively inhibited expressed CYP1A2 catalysed phenacetin *O*-deethylation (Fig. 4). The calculated apparent K_i, 0.080 mM, was essentially identical to the apparent K_m (0.082 mM) for CA 3-demethylation by expressed CYP1A2.

Table 2. Comparative rates of DNA/cDNA-expressed CYP1A1 and CYP1A2 catalysed caffeine demethylations

	Rate of formation (pmol min^{-1} mg^{-1}) of:		
Enzyme	*Paraxanthine*	*Theobromine*	*Theophylline*
CYP1A1	0.08 ± 0.01	0.06 ± 0.01	ND
CYP1A2	0.55 ± 0.06	0.08 ± 0.02	0.02 ± 0.01

Substrate (caffeine) concentration in incubations 0.1 mM. Results expressed as mean ± SD from three incubations (using cells from same transfection).
ND: no activity detected.

Expressed CYP1A1 was additionally shown to catalyse the conversion of CA to PX and TB (Table 2).

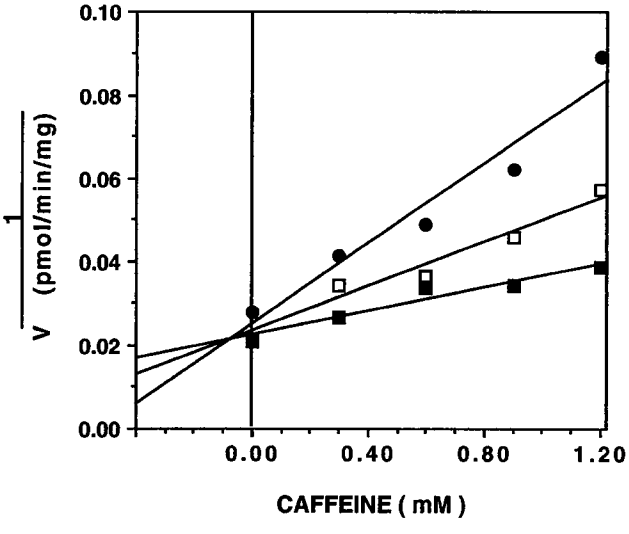

Fig. 4. Dixon plot for the inhibition of cDNA-expressed CYP1A2 catalysed phenacetin *O*-deethylation by caffeine. Concentrations of phenacetin were 10 μM (●), 20 μM (□) and 30 μM (■).

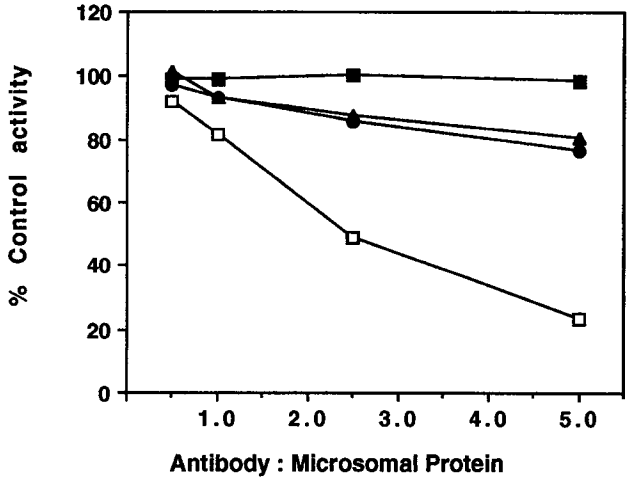

Fig. 5. Effect of anti-rat CYP3A1 antibody on caffeine demethylations and 8-hydroxylation in human liver microsomes (liver F15). □—□ Trimethyluric acid formation, ●—● paraxanthine formation, ■—■ theophylline formation, ▲—▲ theobromine formation.

At a substrate concentration of 0.1 mM, the rate of formation of PX by CYP1A1 was one seventh that observed for CYP1A2, although the rate of formation of TB was comparable for both expressed enzymes. Although TP was not detected as a CA metabolic produce using expressed CYP1A1, this may be due to the assay sensitivity limitation alluded to above and the possibility that CYP1A1 is able to convert CA to TP should not be excluded on the basis of this result.

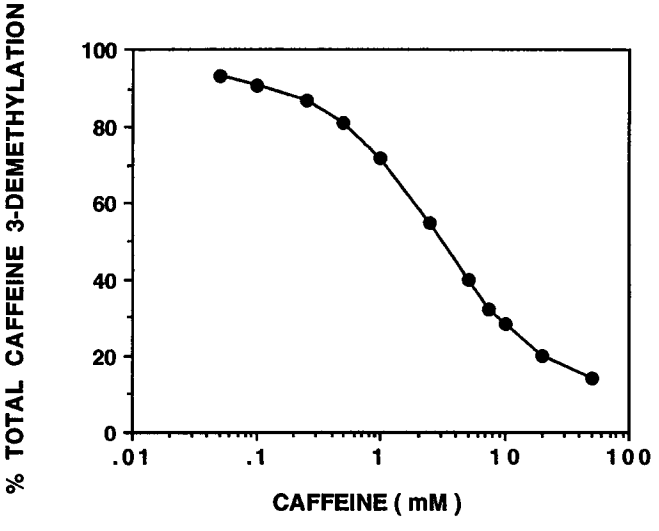

Fig. 6. Calculated contribution of the high-affinity component of caffeine 3-demethylation (CYP1A2) to total paraxanthine formation as a function of caffeine concentration. Calculated by substituting mean kinetic parameters for paraxanthine formation (Table 1) in the Michaelis-Menten equation for a two-enzyme system.

In addition to the CA demethylations, CA 8-hydroxylation to form TMU was also investigated. In all four livers investigated, Eadie-Hofstee plots for TMU formation were curvi-linear. The various xenobiotics screened for inhibitory effects towards the CA demethylations were also assessed as potential inhibitors of TMU formation at a substrate concentration of 20 mM and a number of compounds, including erythromycin, nifedipine, propranolol, quinidine, sulphaphenazole and sulphinpyrazone caused >40% inhibition at an added concentration of 1 mM. Of these compounds, erythromycin was the most potent inhibitor. Notably, α-naphthoflavone and 5- and 8-methoxypsoralen did not inhibit TMU formation. Indeed, at α-naphthoflavone concentrations above 0.01 mM activation of TMU formation occurred (data not shown). An anti-rat 3A1 antibody inhibited the conversion of CA to TMU in a concentration-dependent manner (Fig. 5). This antibody was without effect on the CA demethylations, either at a high (Fig. 5) or low substrate concentration (data not shown).

Discussion

CA 3-demethylase activity (i.e. the conversion of CA to PX) has been proposed as a marker of hepatic CYP1A2 activity, primarily on the basis of the results of human liver microsomal studies. The present comparative kinetic and inhibitor studies with cDNA-expressed CYP1A2 and human liver microsomes have enabled definition of the role of CYP1A2 in CA 3-demethylation and highlighted the limitations of using this pathway as a measure of CYP1A2 activity *in vitro*. While CYP1A2 is primarily responsible for the high-affinity component of PX formation, it is apparent that an additional P450 isozyme(s) may also 3-demethylate CA. The contribution of this additional isozyme(s) manifests as the low-affinity human liver microsomal CA 3-demethylase. Thus, PX formation only reflects CYP1A2 activity when an appropriately low substrate concentration is employed (Fig. 6); the low-affinity isozyme(s) will contribute >10% of CA 3-demethylase activity at substrate concentrations >0.1 mM. In addition, human CYP1A1 has been shown to be capable of catalysing the 3-demethylation of CA. Although of limited importance to hepatic CA metabolism, CYP1A1 will contribute to *in vitro* CA 3-demethylation in tissues in which it is expressed. From the results of the present work CYP1A2 also appears to be largely responsible for the high-affinity components of the human liver microsomal CA 1- and 7-demethylations (i.e. TB and TP formation). In contrast, an isozyme(s) of the 3A subfamily appears to mediate TMU formation (i.e. CA 8-hydroxylation).

Evidence to support CYP1A2 as the main enzyme responsible for the high-affinity component of human liver microsomal CA 3-demethylation is compelling: (i) apparent K_m values for the high-affinity liver microsomal CA 3-demethylase (0.18 ± 0.02 mM) and cDNA-expressed CYP1A2 catalysed CA 3-demethylation (0.082 mM) were of similar order; (ii) IC_{50} values for α-naphthoflavone inhibition of CA 3-demethylation by the two enzyme sources were similar (*viz.* 0.4 and 0.7 μM); (iii) phenacetin (a CYP1A2 substrate), propranolol, nifedipine and 5- and 8-methoxypsoralen (known inhibitors of CYP1A2 catalysed reactions *in vivo*, see below) all inhibited the high-affinity component of PX formation; and (iv) CA was a competitive inhibitor of expressed CYP1A2 catalysed phenacetin *O*-deethylation, with the apparent K_i closely matching the apparent K_m for CA 3-demethylation by the expressed enzyme. As would be expected, non-inhibitors of CYP1A2 (erythromycin, phenylbutazone, sulphaphenazole, sulphinpyrazone and anti-rat 3A1 antibody) were without effect on the high-affinity CA 3-demethylase.

Biphasic human liver microsomal CA demethylation kinetics have been described previously (Campbell *et al.*, 1987a; Grant *et al.*, 1987; Berthou *et al.*, 1989), although Michaelis-Menten parameters were reported for only a limited number of livers. In one of the studies kinetic parameters were calculated for the high-affinity component of CA 3-demethylation for microsomes from two separate livers (Campbell *et al.*, 1987a); apparent K_m values were both approximately 0.5 mM but the V_{max} values ranged from 140–570 pmol min^{-1} mg^{-1}. In another of the studies calculations apparently based on disappearance of substrate rather than product formation gave a composite (i.e. for the combined 1-, 3- and 7-demethylations) K_m of 1.32 mM and a V_{max} of 101 pmol min^{-1} mg^{-1} (Berthou *et al.*, 1989). Thus, both the apparent K_m and V_{max} values for CA 3-demethylation determined in the present study are lower than those reported previously. It is noteworthy, however, that the two livers with high V_{max} values reported by Campbell *et al.* (1987a) appeared to have unusually high CA 3-demethylase activity. The reason for the discrepancy in apparent K_m values between the various studies is not clear, but it should be emphasized that the lower apparent K_m for the high-affinity component of liver microsomal CA 3-demethylation calculated in the present study (0.18 mM) is in agreement with the low apparent K_m (0.082 mM) determined using cDNA-expressed CYP1A2 and with the apparent K_i calculated for CA inhibition of phenacetin *O*-deethylation (0.080 mM). A number of the previously published *in vitro* studies (Campbell *et al.*, 1987a; Grant *et al.*, 1987; Berthou *et al.*, 1989, 1991; Butler *et al.*, 1989; Sesardic *et al.*, 1990b) have additionally investigated the effects of known cytochrome P450 inhibitors on CA metabolism or compared rates of CA 3-demethylation to other CYP1A2 mediated reactions. A substrate concentration of 1 mM was typically used in these investigations and, according to data presented here (Fig. 6), a significant contribution (at least 30%) of the low-affinity component of CA 3-demethylation would be expected under these conditions. Nevertheless, the results of inhibition studies with acetanilide, α-naphthoflavone, 7-ethoxyresorufin, furafylline and phenacetin, and correlations with immunoreactive CYP1A2 and rates of a range of 1A2 catalysed reactions (Campbell *et al.*, 1987a; Grant *et al.*, 1987; Berthou *et al.*, 1989, 1991; Butler *et al.*, 1989; Sesardic *et al.*, 1990b) are all consistent with CYP1A2 being the high-affinity CA 3-demethylase.

The comparative kinetic and inhibitor studies with cDNA-expressed CYP1A2 and human liver microsomes reported here further indicate that CYP1A2 is most likely to be responsible for the high-affinity components of CA 1- and 7-demethylation (i.e., TB and TP formation). Relative rates of formation of TP, TB and PX were similar for both expressed CYP1A2 and human liver microsomes (high-affinity reaction components). Similar data have been reported recently by Fuhr *et al.* (1992) for human CYP1A2 stably expressed in a V79 Chinese hamster cell line. IC_{50} values for inhibition of TP and TB formation by α-naphthoflavone were identical (0.7–0.75 μM) for the two enzyme sources and other compounds which inhibited the high-affinity component of CA 3-demethylation (i.e. propranolol, nifedipine, phenacetin and 5- and 8-methoxypsoralen) also inhibited the high-affinity components of microsomal CA 1- and 7-demethylation. (Quantitative differences in the effects of a number of the inhibitors on the CA 1-, 3- and 7-demethylations were apparent, due probably to varying contributions of the low-affinity components to the respective demethylations at the substrate concentration employed; see Results section.) These latter data are generally consistent with the results of *in vitro* inhibitor studies reported previously by Grant *et al.* (1987) and Campbell *et al.* (1987a). A role for CYP2E1 in the CA 1- and 7-demethylations has recently been suggested on the basis of results from cDNA-expression studies (Gu *et al.*, 1992). Other reports of *in vitro* CA inhibition and activity correlation studies have also concluded that CYP1A2 is not responsible for TB and TP formation (Berthou *et al.*, 1989; Ratanasavanh *et al.*, 1990), but it is possible that a higher contribution of the low-affinity components of CA 1- and 7-demethylation (due to the use of 1 mM

substrate concentration) in these studies obscured the selectivity of inhibition and relationships with 1A2-mediated reactions. In this regard it is noteworthy that comparisons of the high-affinity components of the CA 1-, 3- and 7-demethylations in microsomes from 18 human livers have demonstrated excellent correlations ($r > 0.80$) between all three pathways at a substrate concentration of 0.1 mM (W. Tassaneeyakul, D. J. Birkett and J. O. Miners, unpublished results).

Apart from α-naphthoflavone, a proposed specific inhibitor of 1A sub-family P450s, other putative CYP1A2 inhibitors were shown to affect the high-affinity component of CA 3-demethylation. Phenacetin, an alternate CYP1A2 substrate (Sesardic *et al.*, 1988, 1990a), and propranolol and nifedipine, known inhibitors of human theophylline metabolism (Miners *et al.*, 1985b; Robson *et al.*, 1987a), inhibited the high-affinity component of PX formation. Acute administration of 8-methoxypsoralen markedly impairs caffeine (Mays *et al.*, 1987) and theophylline (Apseloff *et al.*, 1990) plasma clearances in humans, and in the present study added concentrations of 5 μM of both 5- and 8-methoxypsoralen essentially abolished CA 3-demethylation.

The very high apparent K_m values (19–30 mM) for the low-affinity components of the CA demethylations is suggestive of the non-specific involvement of a number of cytochromes P450 at high substrate concentrations. Despite screening a number of selective substrates and inhibitors of cytochrome P450 for effects on the low-affinity components of the CA demethylations, the identity of the isozyme(s) involved in these reactions remains obscure. The lack of substantial inhibition by mephenytoin, α-naphthoflavone, quinidine, sulphaphenazole (and the related inhibitors phenylbutazone and sulphinpyrazone) and the anti-rat CYP3A1 effectively excludes a significant contribution of S-mephenytoin hydroxylase, CYP1A1/1A2, CYP2D6, CYP2C9/2C10, and CYP3A subfamily isozymes to the low-affinity components of the CA demethylations.

Expressed CYP1A1 has been shown here to additionally convert CA to PX and TB. While the rate of PX formation by CYP1A2 was seven-fold higher than that by CYP1A1, rates of TB formation were similar for both expressed enzymes. TP was not observed as a demethylation product of CA by the CYP1A1 transfected cells, but the capacity of CYP1A1 to 7-demethylate CA should not be excluded due to the limitations of assay sensitivity referred to earlier. Recent analysis of human liver mRNA using a CYP1A1 specific riboprobe indicated that approximately 50% of human livers express this message (McKinnon *et al.*, 1991). The level of expression of CYP1A1 mRNA was, however, considerably lower (generally 20-fold) than that of CYP1A2 and hence CYP1A1 would generally not be expected to contribute significantly to human liver CA demethylation. The concurrence between the results of the human liver microsomal and CYP1A2 expression studies outlined above supports this conclusion. Nevertheless, it is apparent that CYP1A1 could contribute to the CA demethylations in tissues in which it is expressed. Moreover, there may be situations where CYP1A1 is induced leading to a significant contribution to *in vivo* caffeine demethylation.

In contrast to the CA demethylations, CYP1A isozymes appear not to contribute to CA 8-hydroxylation. TMU formation was unaffected by α-naphthoflavone and the methoxypsoralens at concentrations which inhibited CYP1A2. Moreover, TMU was not observed as a product of cDNA-expressed CYP1A2 mediated CA oxidation. TMU formation by human liver microsomes was, however, almost completely abolished by an anti-rat CYP3A1 antibody which specifically inhibits human CYP3A3, 3A4 and 3A5 (Aoyama *et al.*, 1990). Consistent with this observation, erythromycin was the most potent inhibitor of microsomal TMU formation. Thus, CYP3A4, the major 3A subfamily isozyme [expressed in almost all human livers examined to date (Waxman *et al.*, 1988; Aoyama *et al.*, 1989; Scheutz *et al.*, 1989)] and possibly CYP3A5, which is expressed in approximately 20% of human livers (Waxman *et al.*, 1988; Aoyama *et al.*, 1989), would appear to catalyse TMU formation. Consistent with this observation, a recent report (Gu *et al.*, 1992) has shown that cDNA-expressed CYP3A4 and CYP3A5 both catalysed the 8-hydroxylation of caffeine. Although CYP2E1 and CYP4B1 also carried out this reaction, the antibody inhibition data reported here suggest that a CYP3A isozyme(s) is of greatest quantitative importance in TMU formation. The atypical CA 8-hydroxylation kinetics, due possibly to substrate activation, apparent in the present study were noted without comment in a previous study of CA metabolism (Grant *et al.*, 1987).

In summary, it has been demonstrated that both CYP1A1 and 1A2 may catalyse the 3-demethylation of CA. Consideration of the non-specificity of CA as a CYP1A substrate will be necessary when this compound is used to measure *in vitro* enzyme activity in tissues which express CYP1A1. Since CYP1A1 is negligibly expressed in human liver, CYP1A2 is the principal enzyme responsible for the high-affinity component of human liver microsomal CA 3-demethylation and probably also the high-affinity components of CA 1- and 7-demethylation. Thus, CA 3-demethylation may serve as an *in vitro* measure of hepatic

CYP1A2 activity providing an appropriately low (*viz.* 0.1 mM) substrate concentration is employed. The use of substrate concentrations of 1 mM or more, which have been reported in numerous recent publications (Campbell *et al.*, 1987a; Grant *et al.*, 1987; Berthou *et al.*, 1989, 1991; Butler *et al.*, 1989; Ratanasavanh *et al.*, 1990), will result in a significant contribution of the low-affinity CA demethylase(s) to PX, TB and TP formation. In contrast to the CA demethylations, a CYP3A subfamily isozyme(s) appears to be responsible for CA 8-hydroxylation, and TMU formation may well serve as an *in vitro* probe for the relevant isozyme activity. This, however, requires further validation.

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