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CHARACTERISATION OF THEOPHYLLINE METABOLISM BY HUMAN LIVER MICROSOMES

INHIBITION AND IMMUNOCHEMICAL STUDIES

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Abstract—Anti-human NADPH-cytochrome P-450 reductase inhibited all theophylline metabolic pathways confirming the involvement of cytochrome P-450 isozymes in the metabolism of theophylline. Tolbutamide, debrisoquine, mephenytoin, theobromine, phenylbutazone, sulphaphenazole and sulphinpyrazone did not inhibit theophylline metabolism by human liver microsomes. Verapamil and dextropropoxyphene were non-selective competitive inhibitors of theophylline metabolism. Cimetidine and caffeine selectively inhibited the two demethylations as K_i values for these two pathways were lower than for the 8-hydroxylation pathway. The effects of nifedipine, propranolol and alpha-naphthoflavone were atypical. The degree of inhibition by propranolol reached a plateau, which was greater for the two demethylations than for the 8-hydroxylation. Alpha-naphthoflavone (ANF) at low concentrations inhibited the demethylations to a greater extent than the 8-hydroxylation. At higher concentrations ANF activated all pathways, with this effect being most marked for the 8-hydroxylation. Nifedipine inhibited the theophylline demethylations but not the 8-hydroxylation. In some livers the 8-hydroxylation was markedly activated.

The results confirm that there are at least two distinct cytochrome P-450 isozymes involved in theophylline metabolism, one isozyme being involved with the demethylations and a different isozyme involved in the 8-hydroxylation pathway. Preliminary correlation studies suggest that the human orthologue to the rabbit polycyclic hydrocarbon inducible P-450 Form 4 may be involved in the *N*-demethylations of theophylline.

Theophylline is metabolised in man by two *N*-demethylations and by 8-hydroxylation to form 1-methylxanthine (1MX), 3-methylxanthine (3MX) and 1,3-dimethyluric acid (DMU). 1MX is subsequently metabolised by xanthine oxidase to 1-methyluric acid (1MU) [1, 2]. Indirect evidence from *in vitro* animal studies [3] and *in vivo* induction/inhibition studies [4] suggests that the cytochrome P-450 system is responsible for the *N*-demethylation and 8-hydroxylation reactions.

In vivo inhibitor and inducer studies with cimetidine [5], propranolol [6] and cigarette smoking [7] suggest that theophylline is metabolised by at least two isozymes of cytochrome P-450; one isozyme predominantly performing the *N*-demethylations and another isozyme the 8-hydroxylation. The high degree of correlation observed in healthy subjects between metabolic clearances of theophylline and other methylxanthines (caffeine, theobromine and paraxanthine) suggests that these compounds are metabolised by a common "methylxanthine" group of cytochrome P-450 isozymes. In contrast, other *in vivo* results strongly suggest that tolbutamide and theophylline are hydroxylated by a different cytochrome P-450 isozyme. Compounds which markedly inhibit tolbutamide metabolism *in vivo* either induce methylxanthine metabolism (sulphinpyrazone [8]) or have no effect (sulphaphenazole [9]). *In vivo* data also suggest that theophylline metabolism is not linked to the debrisoquine polymorphism associated with cytochrome P-450 DB [10], but no *in vivo* data are available on the relationship between methylxanthine metabolism and other identified polymorphic forms of cytochrome P-450 (e.g. nifedipine and mephenytoin).

Cigarette smoking has been shown to increase the clearance of theophylline suggesting that polycyclic hydrocarbon responsive isozymes of cytochrome P-450 may be involved in its metabolism in man [7]. This effect is more marked with theophylline *N*-demethylation pathways.

The aim of the present study was to investigate, at the human liver microsomal level, the relationship between the isozymes of cytochrome P-450 metabolising theophylline with those involved in the metabolism of other methylxanthines and drugs oxidised polymorphically in man. We have also investigated the effect of other therapeutic agents and known inhibitors of the cytochrome P-450 system on theophylline metabolism *in vitro* to gain insight into mechanisms of drug–drug interactions *in vivo*. In addition, the human isozymes involved in theophylline metabolism were investigated by measurement of the contents in human livers of orthologues to rabbit polycyclic hydrocarbon inducible Forms 4 and 6 and correlation with rates of theophylline 1- and 3-demethylation and 8-hydroxylation.

MATERIALS AND METHODS

Human liver samples. Human liver was obtained from renal transplant donors with the approval of

Table 1. Liver donor details

Patient	Sex	Age (yr)	Cytochrome P-450 (nmol mg protein^{-1})	Cause of death	Drug history
F5	M	62	0.47	Gunshot	Phenytoin* Dexamethasone† Non-smoker
F6	M	18	0.34	Motor vehicle accident	Non-smoker
F7	F	44	0.41	Subarachnoid haemorrhage	Dexamethasone‡ Non-smoker
F8	M	24	0.32	Subarachnoid haemorrhage	Smoker
F9	M	18	0.34	Motor vehicle accident	Non-smoker

* 500 mg day^{-1} for 9 days.
† 48 mg day^{-1} for 9 days.
‡ 24 mg day^{-1} for 1 day.

the Clinical Investigation Committee of Flinders Medical Centre. Table 1 contains details of the renal donors. Microsomes were prepared by the differential ultracentrifugation method as previously described by McManus *et al.* [11]. Microsomal protein concentrations were determined by the method of Lowry *et al.* [12] using crystalline bovine albumin as a standard. Cytochrome P-450 concentrations were determined by the method of Omura and Sato [13].

In vitro *theophylline metabolism assay*. The *in vitro* metabolism of theophylline was measured according to the method of Robson *et al.* [14]. Briefly, 12.5 nmol 8-^{14}C theophylline plus unlabelled theophylline to give final concentrations of 200 μM or 500 μM, human liver microsomes 4 mg/ml and 0.1 M phosphate buffer pH 7.4 in a final volume of 0.5 ml were incubated for 2 hr with an NADPH-generating system. Reactions were stopped by cooling on ice and by the addition of 50 μl 5 M hydrochloric acid. Authentic standards of 3MX, 1MX and DMU were added to visualise absorbance peaks during chromatography.

The aqueous reaction mixture was saturated with ammonium sulphate and extracted twice with 10 ml of dichloromethane : isopropranolol (80 : 20 v/v). The pooled extracts were evaporated under nitrogen and the residue redissolved in 0.1 ml mobile phase for chromatographic analysis. Reconstituted sample (0.085 ml) was injected onto a reversed-phase C-18 column (4 mm × 15 cm). The initial mobile phase composition was 1% methanol: 1% acetonitrile: 98% 0.01 M acetic acid, pH 3.5. Absorbance was monitored at 280 nm. After 13 min, the mobile phase composition was changed over 1 min to 7.5% methanol, 7.5% acetonitrile, 85% 0.01 M acetic acid, pH 3.5. The mobile phase flow rate was 2.0 ml/min. Retention times for 3MX, 1MX, DMU and theophylline under these conditions were 6.75 min, 8 min, 11.5 min and 16.5 min, respectively. Column effluent was collected in aliquots and counted on a liquid scintillation counter (Beckman Model LS 3801). Chromatography was performed using a Beckman 342 gradient liquid chromatograph modular system, consisting of a model 420 system controller, two model 114 solvent delivery modules, a model 340 organiser, and a model 160 selectable wavelength detector.

Inhibitors. Cimetidine, verapamil, caffeine, dextropropoxyphene, tolbutamide, debrisoquine, phenylbutazone, sulphaphenazole, sulphinpyrazone, propranolol and theobromine were all added to the microsomal system in 0.1 M phosphate buffer pH 7.4 to give final concentrations ranging from 0 to 1000 μM. Mephenytoin, nifedipine and alpha-naphthoflavone were added in 2 μl dimethylsulphoxide (DMSO) to give final concentrations of 0–500 μM, with an equal volume of DMSO being added to the control reactions. Initial screening was performed in at least three livers at two inhibitor concentrations. Inhibitors that significantly reduced activity were fully studied with a range of inhibitor concentrations.

Kinetic analysis. Inhibition constants (K_i) were calculated from Dixon plots of 1/velocity versus inhibitor concentration at the two theophylline concentrations (200 μM and 500 μM). The slopes and intercept of the two lines were calculated by least squares regression analysis with the apparent K_i being calculated from the intercept of the two lines. Two way analysis of variance was used to determine whether the K_i values were statistically different, with a P value < 0.05 being regarded as significant.

Purified enzymes. NADPH–cytochrome P-450 reductase was purified from human liver according to the method of Yasukochi and Masters [15] as described by McManus *et al.* [11].

Rabbit liver cytochrome P-450 Form 4 was purified from microsomes from 3-methylcholanthrene induced rabbits according to the method of Johnson *et al.* [16] as previously described by McManus *et al.* [17]. The specific content of the cytochrome preparation was 12 nmol mg^{-1} and it exhibited a single band on SDS-PAGE.

Preparation of antibodies. Goat polyclonal antibodies to NADPH-cytochrome P-450 reductase and rabbit liver cytochrome P-450 Form 4 were prepared and the IgG fraction isolated as previously described

by McManus *et al.* [11, 18]. A polyclonal antibody to rabbit cytochrome P-450 Form 6 was a gift from Dr. E. F. Johnson (Scripps Clinic and Research Foundation, U.S.A.).

Western blots. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (0.1%) was performed as described by Laemmli [19]. Western blotting for the estimation of human P-450 proteins corresponding to rabbit cytochrome P-450 Forms 4 and 6 was performed as described by Towbin *et al.* [20]. Transfer of proteins from polyacrylamide gels to nitrocellulose paper was accomplished in 16 hr at 30 mA in a "Trans blot Cell" (Bio-Rad, Australia). Following the transfer of proteins, the nitrocellulose sheets were rinsed in 10 mM potassium phosphate containing 0.15 M sodium chloride (PBS; pH 7.4) and then incubated at 37° for 1 hr in a solution containing 3% w/v bovine serum albumin and 5% v/v heat inactivated horse serum to block reactive sites. Blocked sheets were then washed with PBS containing 0.05% v/v Tween 20 (4 changes in 30 min) and incubated with antibody (Form 4, 1:40,000; Form 6, 1:8000) in blocking solution for 2 hr at 37°. Sheets were then washed with PBS and incubated with anti-goat IgG peroxidase conjugate (1:500) in blocking solution for 1 hr at room temperature. Following two further rinses in PBS, the sheets were incubated (5–15 min) in 20 mm imidazole buffer, pH 7.4, containing 0.05% w/v diaminobenzidine tetrahydrochloride and 0.05% v/v hydrogen peroxide to visualise immunoreactive bands. The immunostained nitrocellulose was scanned with a Carmag densitometer and the area of each peak was determined by triangulation.

RESULTS

The polyclonal anti-human cytochrome P-450 reductase antibody inhibited all three theophylline metabolic pathways. Almost complete inhibition was observed at 4 mg of 1 gG (Fig. 1). The results of screening all inhibitors at two concentrations in three to five livers are detailed in Table 2. The inhibitor K_i values are shown in Table 3.

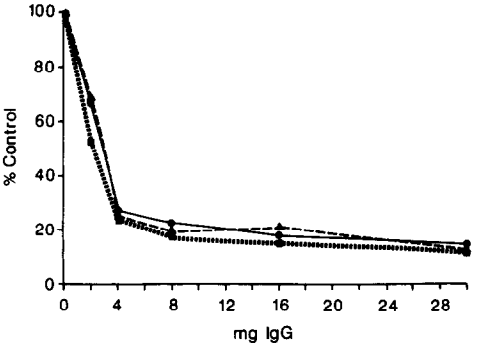

Fig. 1. Effect of anti-human NADPH-cytochrome P-450 reductase on the formation of 3MX (▲), 1MX (●) and DMU (■) in microsomes from human liver F6.

Table 2. Effects of various drugs and chemicals on *in vitro* theophylline metabolism

Inhibitor	Conc. (μM)	Percent of control activity 1DM	3DM	8OH
Tolbutamide	500	96 ± 2	94 ± 1	97 ± 1
	1000*	92 ± 4	90 ± 3	99 ± 6
Phenylbutazone	50	93 ± 6	93 ± 7	92 ± 7
	500	84 ± 3	84 ± 6	84 ± 6
Sulphaphenazole	50	97 ± 4	96 ± 7	97 ± 3
	500	90 ± 3	91 ± 4	91 ± 2
Sulphinpyrazone	50	91 ± 5	92 ± 4	94 ± 3
	500	89 ± 4	87 ± 3	82 ± 10
Mephenytoin	500	87 ± 10	88 ± 5	89 ± 10
	1000*	86 ± 8	83 ± 4	87 ± 12
Debrisoquine	500*	88 ± 6	82 ± 1	90 ± 5
	1000	80 ± 4	75 ± 10	89 ± 5
Theobromine	500	97 ± 2	99 ± 1	97 ± 4
	1000	84 ± 2	88 ± 1	88 ± 1
Caffeine	500	60 ± 2	61 ± 7	78 ± 15
	1000	47 ± 7	48 ± 10	74 ± 25
Verapamil	500	64 ± 20	67 ± 21	65 ± 16
	1000	49 ± 21	53 ± 22	54 ± 13
Dextropropoxyphene	500	68 ± 9	79 ± 4	66 ± 12
	1000	57 ± 18	68 ± 5	58 ± 13
Cimetidine	500	55 ± 8	58 ± 9	61 ± 7
	1000	44 ± 2	46 ± 3	49 ± 1
α-Naphthoflavone	10	4 ± 2	5 ± 3	18 ± 6
	100	19 ± 1	33 ± 3	152 ± 16
Propranolol	100	16 ± 3	16 ± 4	38 ± 6
	500	8 ± 2	9 ± 4	28 ± 5
Nifedipine	125	37 ± 16	47 ± 21	168 ± 106
	500	22 ± 13	35 ± 20	188 ± 157

Data are the mean ± SD of 5 livers, except * where N = 3.

Table 3. Apparent K_i values (mM) for the inhibition of theophylline metabolism by cimetidine, caffeine, verapamil and dextropropoxyphene

Liver	1DM	3DM	8OH
		Cimetidine	
F5	0.49	0.71	0.93
F6	0.70	0.87	1.06
F7	0.64	0.76	1.03
F8	0.57	0.57	0.74
Mean	0.60	0.73	0.94
SD	0.09	0.12	0.14
		Caffeine	
F5	0.75	1.29	2.87
F6	0.48	0.54	1.19
F9	0.86	0.38	2.07
Mean	0.69	0.73	2.04
SD	0.19	0.48	0.84
		Verapamil	
F5	0.33	0.39	1.43
F6	1.12	0.94	1.38
F9	1.80	2.70	1.88
Mean	1.08	1.34	1.56
SD	0.74	1.21	0.28
		Dextropropoxyphene	
F5	0.62	1.57	1.25
F6	1.42	1.78	1.81
F9	0.84	0.96	1.96
Mean	0.96	1.44	1.67
SD	0.41	0.42	0.37

Inhibitor K_i values for the theophylline 1-demethylation (1DM), 3-demethylation (3DM) and 8-hydroxylation (8OH) pathways for cimetidine, caffeine, verapamil and dextropropoxyphene.

Two other methylxanthines were tested as inhibitors. Theophylline metabolism was inhibited by about 20% to 40% by 500 μM caffeine, whereas the same concentration of theobromine had only a minor effect. Even 1000 μM theobromine inhibited by less than 20%. Formal inhibitor studies were therefore performed only with caffeine. Caffeine selectively inhibited theophylline demethylations with the K_i values being 0.69 ± 0.19 mM and 0.73 ± 0.48 mM respectively for 1-demethylation (1DM) and 3-demethylation (3DM) compared with 2.40 ± 0.8 mM for the 8-hydroxylation (8-OH) (ANOVA $P < 0.05$) (Table 3, Fig. 2).

Debrisoquine and mephenytoin, which are both associated with genetic polymorphism and therefore metabolised by distinct forms of cytochrome P-450, showed minimal inhibition of theophylline metabolism at concentrations up to 1000 μM.

Tolbutamide and known inhibitors of tolbutamide hydroxylation *in vivo* (sulphaphenazole, sulphinpyrazone and phenylbutazone) inhibited theophylline metabolism by less than 20% at concentrations of 500 μM.

Cimetidine, verapamil and dextropropoxyphene are compounds which have been reported to decrease theophylline clearance *in vivo*. Each of these compounds inhibited *in vitro* theophylline metabolism (Table 2) and formal *in vitro* inhibition kinetic studies were carried out (Table 3, Fig. 2). The calculated cimetidine K_i values for theophylline 3DM and 1DM were statistically different from that for 8OH (ANOVA $P < 0.05$). Both verapamil and dextropropoxyphene were weak inhibitors of all three theophylline pathways with K_i values greater than 1 mM and statistically there was no significant selective effect on the demethylations when com-

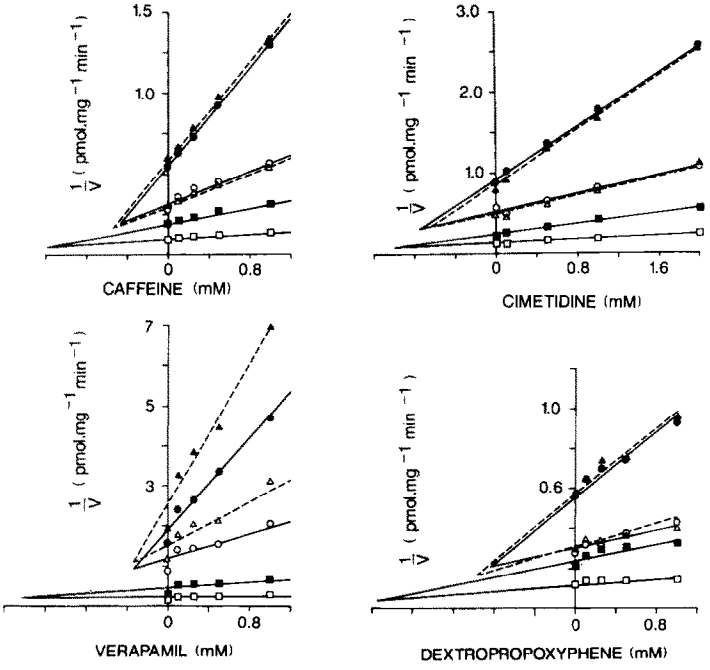

Fig. 2. Dixon plots for the inhibition of theophylline metabolism by caffeine, cimetidine, verapamil and dextropropoxyphene. Squares, triangles and circles show formation of DMU, 3MX and 1MX respectively. Theophylline concentrations were 200 μM (open symbols) and 500 μM (closed symbols).

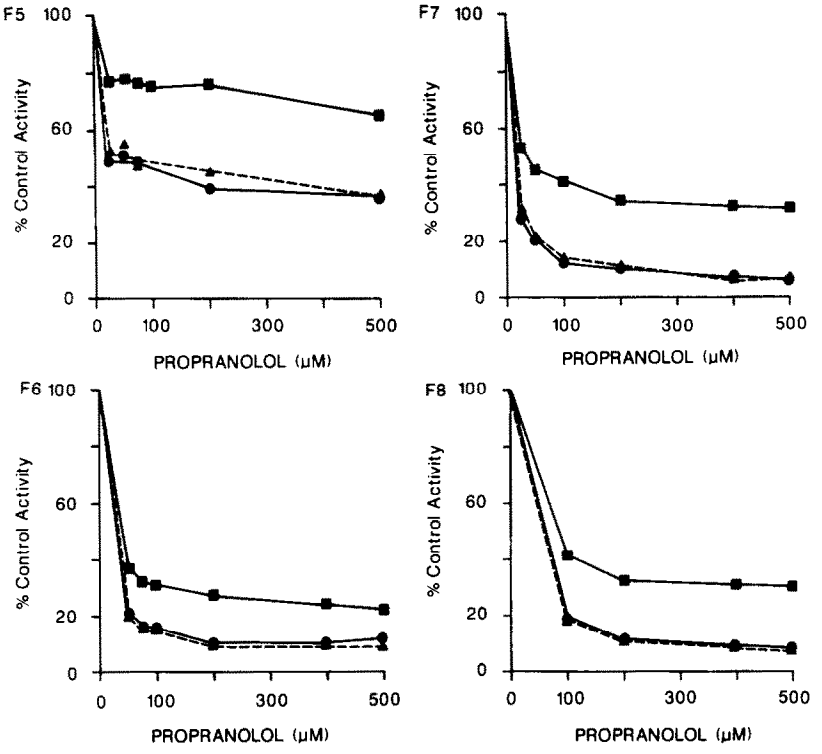

Fig. 3. Effect of propranolol on the formation of 3MX (▲), 1MX (●) and DMU (■) by microsomes from livers F5, F6, F7 and F8. The theophylline concentration was 200 μM.

pared with the 8-hydroxylation pathway (ANOVA $P > 0.05$).

The effects of propranolol, nifedipine and alpha-naphthoflavone were atypical. The data for each liver are therefore presented as percent of control activity versus inhibitor concentration. Propranolol was a potent inhibitor of all three theophylline pathways, causing marked inhibition at concentrations less than 100 μM. Increasing the concentration of propranolol above 100–200 μM caused little further inhibition of theophylline metabolism and a plateau effect occurred. At 500 μM, the IDM, 3DM and 8OH pathways were inhibited to 15 ± 14.7%, 16 ± 14.3% and 37 ± 19.0% (N = 4 livers) of control activity, respectively. There was variation in the degree of inhibition of three pathways with each liver and therefore the individual data for each liver are presented in Fig. 3. Liver F5 was unusual in that all three pathways of theophylline metabolism were inhibited to a lesser extent by propranolol when compared to the other three livers. In liver F5 the demethylations were inhibited to 37% of the control activity and the 8-hydroxylation to 65% of the control activity, compared to 6–10% for the demethylations and 20–30% for the 8-hydroxylation in the other three livers.

Nifedipine at 500 μM inhibited the 1DM and 3DM pathways to 21.8 ± 12.6% and 35.3 ± 19.9% (N = 4 livers) of the control activities respectively. The 8-hydroxylation pathway was activated to a mean of 188.3 ± 156.7% of control activity. The results with nifedipine are similar to those obtained with propranolol in that the effects reached a plateau above 200 μM nifedipine. The livers studied could be divided into two distinct groups based on the inhibitory and activating effects of nifedipine. The livers from the patients who had received dexamethasone (F5 and F7) were very different from the other two livers (F6 and F8) in that IDM was inhibited to 31% and 34%, 3DM was inhibited to 42% and 60% but 8OH was activated to 402% and 209% of control activity. With the other two livers the corresponding values were 9% and 13% for 1DM, 24% and 15% for 3DM and 66% and 76% for 8OH. The effects of nifedipine on *in vitro* theophylline metabolism in the 4 individual livers are presented in Fig. 4.

Alpha-naphthoflavone also exhibited atypical effects in that at low inhibitor concentrations all three pathways of theophylline metabolism were inhibited, but as the concentration of alpha-naphthoflavone increased above 10 μM all three pathways were activated, particularly 8OH. The pattern of inhibition at low alpha-naphthoflavone concentrations was similar to that observed with propranolol in that a plateau effect occurred. The effects of alpha-naphthoflavone on theophylline metabolism with the three livers investigated are presented in Fig. 5.

The immunochemically estimated content, by Western blotting, of the human P-450 orthologues to rabbit Form 4 and Form 6 cytochrome P-450 isozymes were determined in livers F5 to F9. The results, expressed as the mean of four separate determinations, are shown in Table 4. As the extent of cross-reaction of the anti-rabbit isozyme antibodies

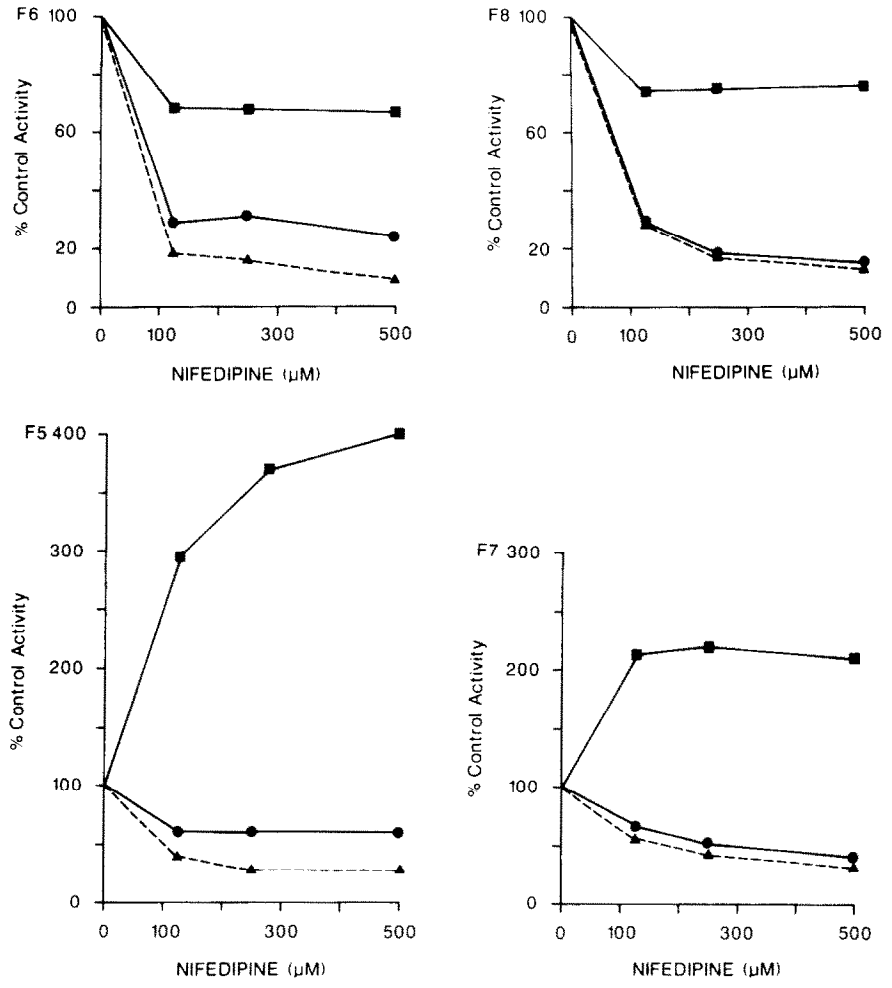

Fig. 4. Effect of nifedipine on the formation of 3MX (▲), 1MX (●) and DMU (■) by microsomes from livers F5, F6, F7 and F8. The theophylline concentration was 200 μM.

with the orthologous human forms is unknown, the values in the table represent minimum contents. The correlations between the contents of the human orthologues to rabbit Forms 4 or 6 and the V_{max} values for each theophylline pathway (14) are shown in Table 5 and indicate that the Form 4 orthologue may be at least partly involved in the *N*-demethylations of theophylline. More individual livers are required to confirm this.

DISCUSSION

The results of this *in vitro* study are consistent with the known *in vivo* theophylline inhibitor interactions and support the hypothesis that at least two isozymes of cytochrome P-450 are involved in theophylline metabolism. The almost complete inhibition of all three theophylline metabolic pathways by the polyclonal antihuman NADPH-cytochrome P-450 reductase antibody provides the first direct biochemical evidence that the cytochrome P-450 system is involved in all three pathways of theophylline metabolism.

The unbound plasma clearance of theophylline *in vivo* has been correlated with those of caffeine ($r = 0.91$) and theobromine ($r = 0.91$) [21]. The high correlation between the clearances of the methylxanthines suggest that theophylline, caffeine and theobromine are either metabolised by the same cytochrome P-450 isozyme(s) or by cytochrome P-450 isozymes under similar regulatory control. The *in vitro* inhibition of theophylline by caffeine is consistent with the *in vivo* data. The lack of significant inhibition by theobromine on *in vitro* theophylline metabolism is an unexpected result. The discrepancy between the *in vivo* and *in vitro* results can possibly be explained by theophylline and theobromine being metabolised by different cytochrome P-450 isozymes which are under similar regulatory control.

Cimetidine differentially inhibits *in vivo* theophylline metabolism, inhibiting the two demethylations to a greater extent than the 8-hydroxylation [5]. The cimetidine K_i values *in vitro* are consistent with the *in vivo* data in that the K_is are lower for the 3DM and 1DM compared with the 8OH. The *in vitro* K_i values for the inhibition of theophylline

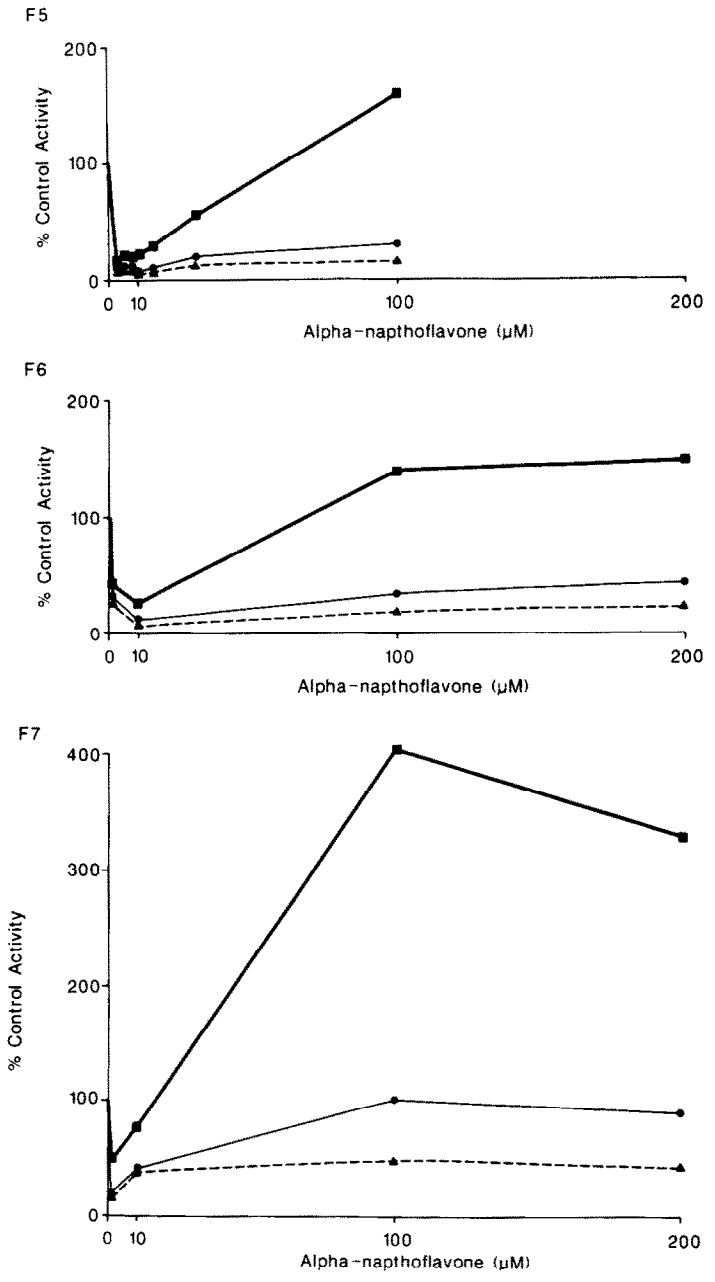

Fig. 5. Effect of alpha-naphthoflavone on 3MX (▲), 1MX (●) and DMU (■) formation by microsomes from livers F5, F6 and F7. The theophylline concentration was 200 μM.

Table 4. Immunochemical estimation of relative contents of cytochrome P-450 Form 4 and 6 orthologues in human liver microsomes

	P-450 content in pmol. mg protein^{-1}		
Liver	Total P-450	Form 4	Form 6
F5	342	1.1 ± 0.1	3.8 ± 0.7
F6	152	8.2 ± 0.2	2.5 ± 1.1
F7	319	4.5 ± 0.6	2.5 ± 0.7
F8	238	6.1 ± 2.0	2.2 ± 0.5
F9	283	9.1 ± 2.5	3.6 ± 0.4

Table 5. Correlation between theophylline V_{max} values and the content of human Form 4 and 6 orthologues

	Spearman rank correlation coefficient (r_s)	
Formation of:	Form 4 orthologue	Form 6 orthologue
3MX	0.9	−0.4
1MX	1.0	−0.3
DMU	−0.1	0.6

Critical value of the Spearman rank correlation coefficient for $P < 0.05$ is 0.99 for $N = 5$.

metabolism by cimetidine are similar to the values reported for other substrates [22]. Cimetidine plasma concentrations *in vivo* are in the range of 3–10 μM [23] which is lower than the reported *in vitro* K_i values. Jensen and Gugler [22] suggested that the formation of a cimetidine–cytochrome P-450 complex possibly explained the discrepancy between the *in vivo* plasma concentrations and the *in vitro* K_i values because pre-incubation of cimetidine with microsomes gave lower K_i values for 7-ethoxycoumarin deethylase activity. However, pre-incubation of microsomes with cimetidine for 30 min before the addition of theophylline did not alter the cimetidine K_i values for the three theophylline pathways (data not shown).

Verapamil *in vivo* decreases the clearance of carbamazepine [24] and antipyrine [25]. There is one case report of verapamil inhibiting theophylline clearance resulting in toxic theophylline plasma concentrations [26]. To date no *in vivo* theophylline verapamil interaction study has been reported, but on the basis of the *in vitro* data we would predict that verapamil will be a weak non-selective inhibitor of theophylline metabolism.

Dextropropoxyphene is a selective inhibitor of the cytochrome P-450 system. Dextropropoxyphene decreases the clearance of carbamazepine by 40–61% [27, 28] and alprazolam clearance by 38% [29]. Smaller reductions in clearance have been observed with phenytoin (14%) and phenobarbitone (20%) by Hansen *et al.* [27]. Dextropropoxyphene treatment had only minor effects on theophylline clearance *in vivo*, consistent with the high K_i values for dextropropoxyphene inhibition of theophylline metabolism *in vitro*.

The lack of any significant effect of debrisoquine on the *in vitro* metabolism of theophylline is consistent with the *in vivo* observation that debrisoquine polymorphism is not correlated with theophylline metabolism. Different cytochrome P-450 isozymes are involved in the metabolism of theophylline [10] compared with debrisoquine.

Mephenytoin is another drug for which genetic polymorphism has been demonstrated [30]. As yet there has been no *in vivo* study investigating the relationship between theophylline metabolism and mephenytoin polymorphism. The *in vitro* results suggest that the P-450 isozyme involved in mephenytoin metabolism are different from the P-450 isozymes involved in theophylline metabolism.

The atypical effects on theophylline metabolism of propranolol, nifedipine and alpha-naphthoflavone provided further strong evidence for the involvement of at least two different P-450 isozymes in theophylline metabolism. Low concentrations of propranolol and alpha-naphthoflavone almost completely inhibited the *N*-demethylations, but left about 40% of the 8-hydroxylation activity intact. At high concentrations, alpha-naphthoflavone stimulated the 8-hydroxylation activity to above control values. On the basis of these results, it is likely that 90% of the *N*-demethylations, but only 50–60% of the 8-hydroxylation activities are carried out by one P-450 isozyme which is inhibited by cimetidine, caffeine, propranolol and alpha-naphthoflavone. The remaining 8-hydroxylation activity is catalysed by another P-450 isozyme which performs less than 10% of the *N*-demethylation activity, is less or not inhibited by the above compounds, and is activated by alpha-naphthoflavone. The effect of nifedipine in livers F6 and F8 is qualitatively consistent with the results with propranolol and alpha-naphthoflavone. In the two livers F5 and F7 obtained from patients treated with dexamethasone, nifedipine stimulated theophylline 8-hydroxylation.

The lack of any *in vitro* interaction between tolbutamide, sulphinpyrazone, sulphaphenazole and phenylbutazone and theophylline metabolism are also consistent with the *in vivo* data. The *in vivo* data suggest that the P-450 isozymes involved in theophylline metabolism are different from those involved in tolbutamide metabolism in man, as sulphinpyrazone strongly inhibits tolbutamide hydroxylation [31] but induces theophylline metabolism [8]. Sulphaphenazole [32] inhibits tolbutamide but not theophylline metabolism *in vivo*.

The lack of inhibition of one substrate by another is reasonable evidence that different enzymes are involved in the metabolism of the two compounds. The corollary that reciprocal inhibition implies metabolism by the same isozyme(s) is not necessarily correct. Although quinidine is a potent inhibitor of debrisoquine hydroxylation it is not metabolised by the cytochrome P-450 isozyme responsible for debrisoquine 4-hydroxylation [33]. It is therefore important not to over interpret the results of *in vivo* and *in vitro* inhibition studies.

The correlations between the content of a Form 4 orthologue in human microsomes and theophylline *N*-demethylation activities suggests that this isozyme may be involved in theophylline *N*-demethylation in man. Studies with larger numbers of individual livers will be required to confirm this suggestion which is, however, consistent with the induction of theophylline metabolism *in vivo* in man by cigarette smoking [7].

Overall the *in vitro* results are qualitatively similar to the *in vivo* data and support the hypothesis that at least two isozymes of cytochrome P-450 are involved in theophylline metabolism. The *in vitro* K_i values for the demethylations are similar and tend to differ from the K_i value for the 8OH. This is consistent with one isozyme of cytochrome P-450 being involved with the demethylations and a different isozyme involved with the 8-hydroxylation.

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