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CHARACTERIZATION OF HUMAN CYTOCHROMES P450 INVOLVED IN THEOPHYLLINE 8-HYDROXYLATION

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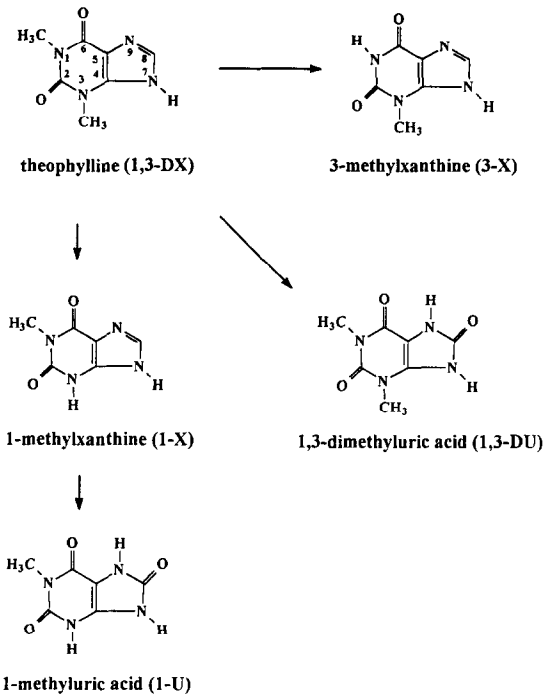
Abstract—Studies were undertaken to determine which human P450 enzymes catalyze the metabolism of theophylline to 1,3-dimethyluric acid (1,3-DU), to facilitate predictions of theophylline drug–drug interactions, and to develop a noninvasive test for human P4501A2. Microsomes from a human cell line transfected individually with human P450 cDNAs for P4501A1, 1A2, 2A6, 2B6, 2C9, 2D6, 2E1, or 3A4 were used to demonstrate that only P4501A2 exhibited catalytic activity for theophylline metabolism to 1,3-DU with high affinity and low capacity (K_m = 0.6 mM, V_{max} = 37.8, pmol/min/mg), while P4502D6, 2E1, and 3A4 (K_m = 14.4, 19.9, and 25.1 mM, respectively, and V_{max} = 219.8, 646.4, and 20.8 pmol/min/mg, respectively) exhibited activities with low affinity and variable capacities. Correlations of rates of theophylline 8-hydroxylation to 1,3-DU with other P450 form-specific activities, in a series of ten human liver microsomal preparations, at 5 and 40 mM theophylline concentrations, revealed that at low concentrations the metabolism was catalyzed primarily by P4501A2, while at high substrate concentrations P4502E1 was primarily responsible for catalysis. The results with individually expressed P450s and hepatic microsomal preparations were consistent, indicating that the former system provides a qualitatively accurate reflection of the function of the heterogeneously expressed liver P450s. At pharmacologic theophylline concentrations achieved *in vivo*, its metabolism must thus be catalyzed primarily by P4501A2.

Key words: theophylline; 1,-3-dimethyluric acid; human P450s; P4501A2; metabolism; liver microsomes

The metabolism of theophylline, a bronchodilating agent, a natural ingredient in tea, and a metabolite of caffeine, is catalyzed primarily by P450 enzymes in humans [1, 2]. The exception is the secondary metabolism of a demethylated product, 1-X§, to the uric acid, 1-U, which is catalyzed by xanthine oxidase (Scheme 1) [2]. Studies have been conducted using human liver microsomal preparations with anti-rat P4501A1/1A2, 3A1/2, 2E1, and 2C13 polyclonal antibodies and microsomes from cell lines expressing transfected human individual P450 cDNAs to investigate the P450 forms catalyzing theophylline 8-hydroxylation to 1,3-DU [3–5]. They confirmed a role for P4501A2 in this metabolism and suggested the participation of P4502E1 and the 3A subfamily. There are, however, several unresolved aspects of the 8-hydroxylation of theophylline, including identification of all of the P450 forms involved, the kinetics of the variously catalyzed reactions, and whether the metabolism will be altered when a multi-enzyme matrix such as that in hepatic microsomes is involved. Formation of 1,3-DU is important since it is the major metabolite of theophylline and primarily determines the clearance of the drug [1].

Demethylation of theophylline to 1-X and 3-X is



Scheme 1. Metabolic pathways of theophylline in humans.

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§ Abbreviations: 1-X, 1-methylxanthine; 1-U, 1-methyluric acid; 3-X, 3-methylxanthine; and 1,3-DU, 1,3-dimethyluric acid.

Table 1. Data on human liver donors

Sample No.	Sex	Age (years)	Race	Smoker	Alcohol use
HBI 2	F	53	Caucasian	Y	N
HBI 3	M	33	Caucasian	Y	Y
HBI 5	M	42	Caucasian	N	N
HBI 6	F	51	Caucasian	Y	Y
HBI 7	M	45	Caucasian	Y	Y
HBI 9	F	11 months	Caucasian	N	N
HBI 10	F	54	Hispanic	N	Y
HBI 11	M	54	African American	N	Y
HBI 12	F	36	Caucasian	?	Y
HBI 13	F	52	Caucasian	Y	Y

catalyzed by P4501A2 and possibly by P4501A1 [3, 4]. Recently, it has been proposed that these demethylations could be used as probes for P4501A1 and 1A2 based on studies with rat liver microsomal preparations and microsomal preparations containing individually expressed human P4501A1 and 1A2 [6].

In this study, we used a group of ten different human liver microsomal preparations, and a series of microsomal preparations from AHH-1 TK +/− cell lines individually transfected with human P450 cDNAs to investigate these aspects of human 8-hydroxylation of theophylline. The application of these two approaches permits evaluation of the capability of individual P450s to metabolize theophylline and of the metabolic capacity of the P450s when occurring together in microsomes. The results provide a basis for assessing potential drug–drug interactions with theophylline, and for the use of theophylline as a noninvasive probe for human P450s.

MATERIALS AND METHODS

Chemicals. Theophylline, 1-X, and 3-X were purchased from the Aldrich Chemical Co. (Milwaukee, WI), and 1,3-DU, 7-(β-hydroxypropyl)-theophylline, NADPH, and Trizma base were purchased from the Sigma Chemical Co. (St. Louis, MO). Magnesium chloride was obtained from the Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade methanol and acetic acid were purchased from Mallinckrodt (Paris, KY).

Microsomes. Microsomal preparations from metabolically competent derivatives of the human AHH-1 TK +/− cell line transfected individually with human P450 cDNAs for P4501A1, P4501A2, P4502A6, P4502B6, P4502C9, P4502D6, P4502E1, or P4503A4, or with the cDNA for epoxide hydrolase, were purchased from the Gentest Co. (Woburn, MA). The parent cell line contained low levels of P4501A1 activity. Control microsomes were from cells transfected with the vector alone. The microsomes contained adequate quantities of NADPH-P450 reductase and cytochrome b_5 for the P450 form-specific metabolic assays. Ten individual human liver microsomal preparations were purchased from Human Biologics, Inc. (Phoenix, AZ). Details of the donors are provided in Table 1.

Theophylline microsomal incubation assays. Rates of formation of 3-X, 1-X, and 1,3-DU from theophylline were proportional to microsomal protein concentrations over the range of 0.1 to 4.0 mg/mL [1], and were linear with time up to at least 30 min [3], and possibly for as long as 180 min [1]. The reaction incubation mixture, in a total volume of 250 μL, contained 1.0 mg microsomal protein, 1.0 mg NADPH, variable amounts of theophylline in 0.05 M Tris buffer, pH 7.4, and 0.015 M magnesium chloride. Reactions were initiated by the addition of an aqueous NADPH solution (20 mg/mL, 50 μL), and were incubated with shaking at 37° for 10–60 min, usually 30 min for reaction rate determinations. Reactions were terminated by quickly cooling the incubation tubes on ice, and cold water (250 μL) was added together with an aqueous solution of the internal standard, 7-(β-hydroxypropyl)theophylline (2 μg/mL). For 1,3-DU analyses, the reaction mixture was clarified by centrifugation for 10 min using a Beckman E microcentrifuge, and the supernatant was filtered through a syringe filter (25 mm, 0.2 μm) into an HPLC injection vial. For analysis of all of the metabolites of theophylline, the reaction mixture was liquid-liquid extracted as previously described [7].

HPLC assays for theophylline metabolites. The metabolites of theophylline were quantified by HPLC using a system from the Waters Co. (Bedford, MA), including a multisolvent delivery unit (Waters 600E), autoinjector (WISP 712), diode array detector (Waters 996), and Millennium 2.00 software package. An Alltima C_{18} 5 μm column (250 mm × 4.6 mm) (Alltech, IL) was used together with mobile phases and gradients modified from those reported by Butler *et al.* [8]. The solvents were 0.045% acetic acid (A) and equal volumes of solvent A and methanol. The typical linear gradients for elution were: 80% A (0 min), 0% A (16 min), 0% A (24 min), 80% A (26–40 min). The flow rate was 1.1 mL/min. Slight variations in the gradients were made based on the column age. Reproducibility of the assay was within 6% (N = 5) for 1,3-DU and less

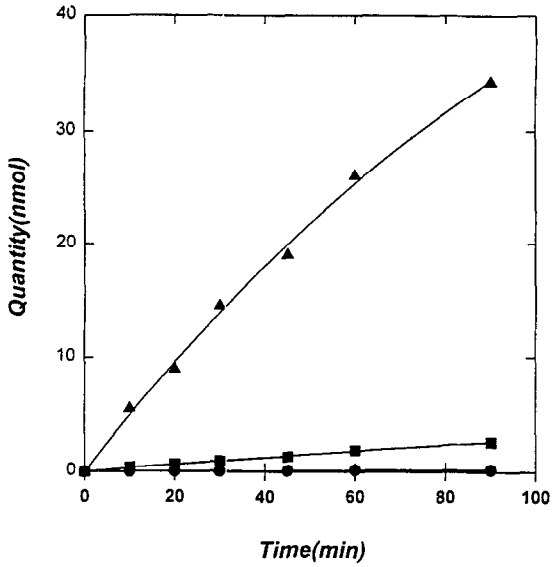

Fig. 1. Time courses for the formation of 1,3-dimethyluric acid catalyzed by human P4501A1 (●), 1A2 (■), and 2E1 (▲) expressed in microsomes from the AHH-1 TK +/− cell line. The microsomal protein concentration was 4 mg/mL. The theophylline concentration was 20 mM. Each point represents a single experiment.

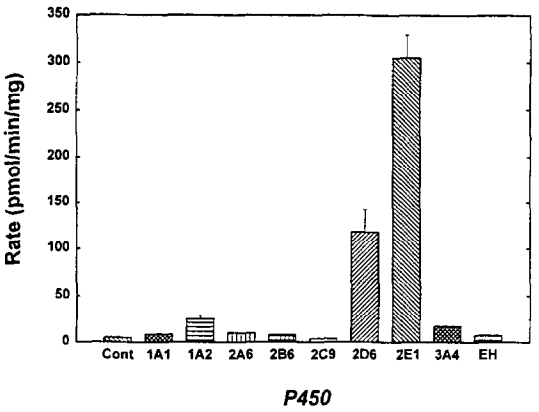

Fig. 2. Rates of formation of 1,3-dimethyluric acid from theophylline catalyzed by human P450s and epoxide hydrolase (EH) expressed in microsomes from the AHH-1 TK +/− cell line. The control microsomes (Cont) were from cells transfected with only the vector. The microsomal protein concentration was 4 mg/mL. Theophylline concentration was 20 mM. Where standard deviations are indicated by error bars, N = 3. Other results are the means of duplicate analyses.

than 1% (N = 5) for 1-X, 3-X, and theophylline. Under the conditions of the assays, the detection limits were 0.5 ng for 1-X, 3-X, and 1,3-DU and 1.0 ng for 1,3-DX and the internal standard.

Data analysis. Apparent K_m and V_{max} values were determined by Eadie–Hofstee plots, with linear regression performed by Sigmaplot and Sigmastat software (Jandel Scientific, San Rafael, CA). Tests of the normality of the data points, the correlations between each specific metabolic activity of the liver microsomes, and the slopes of regression curves were all conducted using this software.

RESULTS

Metabolic studies of NADPH-dependent human liver microsomal metabolism of theophylline were conducted using a substrate concentration of 20 mM, reported to be optimal [3]. Time courses of 1,3-DU formation from theophylline with microsomes containing expressed P4501A1, 1A2, or 2E1 are shown in Fig. 1. Time courses were linear in all cases for 40 min. At a theophylline concentration of 20 mM, microsomal preparations containing expressed P450s catalyzed the formation of 1,3-DU with rates in decreasing order of P4502E1 >> P4502D6 >> P4501A2 > P4503A4 (Fig. 2). P4501A1, 2A6, 2B6, and 2C9 and epoxide hydroxylase catalyzed the reaction at rates that did not exceed that of the control microsomes.

The apparent K_m and V_{max} values for theophylline 8-hydroxylation were determined using a range of substrate concentrations from 0.5 to 40 mM, microsomes containing P4501A2, 2E1, 2D6, or 3A4, and Eadie–Hofstee plots. The results are shown in

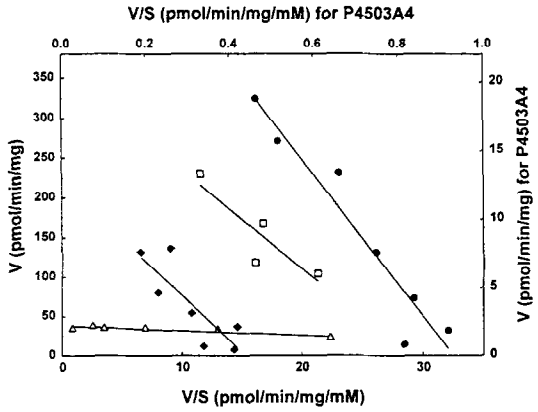

Fig. 3. Eadie–Hofstee plots of the rates of formation of 1,3-dimethyluric acid from theophylline catalyzed by human P4501A2 (△), 2D6 (◆), 2E1 (●), and 3A4 (□) expressed in microsomes from the AHH-1 TK +/− cell line.

Fig. 3, and the calculated values are provided in Table 2. P4501A2 exhibited relatively high affinity, low capacity kinetics, whereas P4502E1 and 2D6 exhibited low affinity, high capacity kinetics, based on V_{max} values being expressed per milligram of microsomal protein. When V_{max} values were based on approximate pmol P450 values, the capacities varied in the order P4502E1 >> 2D6 >>1A2 > 3A4 (Table 2). The relative intrinsic clearances (V_{max}/K_m) were ranked in the order P4501A2 > P4502E1 > P4502D6 > P4503A4. These kinetic data are all based on metabolism by P450s functioning in the

Table 2. Michaelis–Menten kinetic parameters for theophylline 8-hydroxylation catalyzed by microsomes containing individually expressed human P4501A2, 2D6, 2E1, and 3A4*

P450	K_m (mM)	V_{max} (pmol/min/mg)	V_{max}† (pmol/min/pmol P450)	V_{max}/K_m (pmol/min/mg/mM)	V_{max}/K_m (pmol/min/pmol/mM)
P4501A2	0.6	37.8	0.9	63.0	1.5
P4502D6	14.4	219.8	1.4	15.3	0.1
P4502E1	19.9	646.4	12.9	32.5	0.6
P4503A4	25.1	20.8	0.4	0.8	0.0

* Values were calculated by linear regression analysis of Eadie–Hofstee plots of reaction rates.

† Values are approximate and are based on approximate P450 concentrations provided by Gentest (Woburn, MA) of approximately 50 pmol P450/mg protein except for P4502D6, which was approximately 160 pmol/mg protein.

Table 3. Correlation of human liver microsomal theophylline 8-hydroxylation rates with P450 form-specific activities

Theophylline concn	N	Correlation coefficient (r)							
		Total P450 concn	P450 activity*						
			1A2	2A6	2C8/9/10	2C18/19	2D6	2E1	3A3/4
5 mM	9	0.76†	0.78†	0.44	0.33	0.12	0.11	0.32	0.65
40 mM	10	0.72†	0.03	0.24	0.79‡	0.24	0.05	0.99§	0.65†

* Activities used to identify P450 forms were: 1A2, caffeine 3-demethylation; 2A6, coumarin 7-hydroxylation; 2C8/9/10, tolbutamide methyl hydroxylation; 2C18/19, *S*-mephenytoin 4′-hydroxylation; 2D6, dextromethorphan O-demethylation; 2E1, chlorzoxazone 6-hydroxylation; and 3A3/4, testosterone 6β-hydroxylation.

†‡§ Statistically significant correlation: (†) $0.05 > P \geq 0.01$; (‡) $0.01 > P \geq 0.001$; and (§) $P < 0.001$.

absence of other P450 forms, which does not reflect the normal situation in microsomes of most tissues.

To assess which P450s are primarily involved in catalyzing the 8-hydroxylation of theophylline in human tissue, a series of ten human liver microsomal preparations was investigated for rates of 8-hydroxylation of theophylline. Additionally, rates of formation of 3-X and 1-X from theophylline were also determined. The rates of theophylline metabolite formation obtained were correlated against total spectrally determined P450 [9], and the rates of metabolism of other substrates selected to represent specific forms of P450. These analytical data were provided by Human Biologics, Inc. The results of these correlations for 1,3-DU formation are presented in Table 3. Plots of the rates of theophylline 8-hydroxylation for the individual human liver microsomal preparations against the corresponding rates of chlorzoxazone 6-hydroxylation and caffeine 3-demethylation are shown in Fig. 4. These plots provide an assessment of the interindividual variations in theophylline metabolism—at 5 mM theophylline the mean rate of 1,3-DU formation was 13.3 ± 4.9 pmol/min/mg protein (N = 9; range 6 to 22 pmol/min/mg protein) and at 40 mM theophylline the rate was 472.6 ± 306.4 pmol/min/mg protein (N = 10; range 195 to 1250 pmol/min/mg protein). There were significant correlations of 1,3-DU formation rates with caffeine 3-demethylation rates representing P4501A2 activity [10, 11] at the low substrate concentration, and with chlorzoxazone 6-hydroxylation rates representing P4502E1 activity [12], with tolbutamide methyl hydroxylation rates possibly representing combined P4502C8, 2C9, and 2C10 activities [13–15], and with testosterone 6β-hydroxylation rates representing P4503A3/4 activity [16], at high substrate concentrations. The last activity also showed a correlation at the low substrate concentration although it was not statistically significant. At both high and low theophylline concentrations 8-hydroxylation rates correlated with total P450 concentrations, suggesting that the P450s catalyzing the metabolism were possibly major contributors to the total P450 content. No correlations were determined with coumarin 7-hydroxylation rates representing P4502A6 [17], *S*-mephenytoin 4′-hydroxylation rates representing P4502C18/19 [18, 19], and dextromethorphan O-demethylation rates representing P4502D6 activity [20].

Of all of the activities reported by Human Biologics, Inc. for these human liver microsomal preparations, the one that showed the highest correlation with total P450 concentration was P4503A3/4 (Table 4), suggesting that this is the most prominent form present in the human liver microsomal preparations.

The results of correlations of rates of formation of 1-X and 3-X from theophylline by the ten human liver microsomal preparations with the other activities of these preparations are presented in

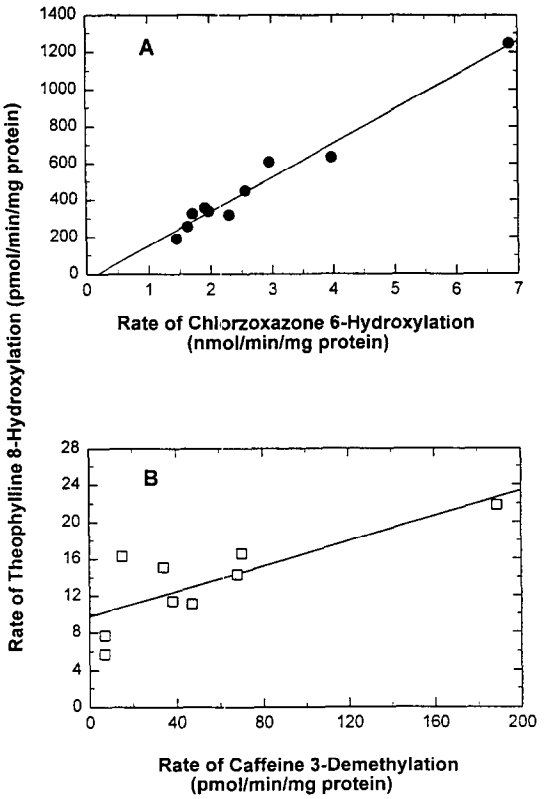

Fig. 4. Rates of theophylline 8-hydroxylation of human liver microsomal preparations plotted against the rates of (A) chlorzoxazone 6-hydroxylation by the same microsomal preparations with theophylline concentration at 40 mM, and (B) caffeine 3-demethylation by the same microsomal preparations with theophylline concentration at 5 mM. Each point represents a single experiment.

Table 5. Formation of 3-X and 1-X at low substrate concentration correlated with P4501A2-related activity, and at high substrate concentration with P4502E1 and 2C8/9/10 activities.

DISCUSSION

An early indication that theophylline 8-hydroxylation is catalyzed by at least two enzymes in humans was provided by observations of biphasic kinetics of 1,3-DU formation with human liver microsomes [21]. The relatively high affinity enzyme was demonstrated to be inducible by polycyclic aromatic hydrocarbons, and was confirmed to be P4501A2 in this and other studies [3–5]. The kinetic studies reported here, with microsomal preparations containing individually expressed forms of human P450, all yielded linear kinetics for 1,3-DU formation, with P4502D6, 2E1, and 3A4 exhibiting K_m values in the range of 14 to 25 mM. Thus, these forms are clearly candidates for the relatively low affinity enzymes catalyzing theophylline 8-hydroxylation. These studies also indicated that P4501A1, 2A6, 2C9, and 2B6 play little or no role in human theophylline 8-hydroxylation.

The results of our studies with individually expressed P450s can be applied to interpretations of our results with human liver microsomal preparations to determine which P450s are involved with theophylline metabolism in humans. Studies were conducted at high and low theophylline concentrations to gain insight into the metabolism by P450s, which function with high and with low affinities for theophylline.

At the low substrate concentration, rates of theophylline 8-hydroxylation by the human liver microsomal preparations correlated significantly with only P4501A2-associated activity. However, a correlation with P4503A4 activity was also observed at this substrate concentration, although it was not statistically significant. The relatively high K_m value for this P450 and the low pharmacologic concentrations of theophylline probably precludes this P450 from contributing to theophylline metabolism *in vivo*. This is consistent with P4501A2 being the only relatively high affinity form, of those tested, to catalyze the 8-hydroxylation of theophylline. In contrast, at the higher substrate concentration, theophylline 8-hydroxylation rates correlated significantly with P4502E1-, 2C8/9/10-, and 3A4-associated activities. It appears that the correlation with P4502C8/9/10 is coincidental—expressed P4502C9, the major tolbutamide methyl hydroxylase of these three forms [13, 21, 22], did not catalyze theophylline metabolism. A possible explanation for these apparently contradictory results is that, in the human liver microsomal samples used, the P4502C8/9/10-associated activity correlated with the P4502E1-associated activity ($r = 0.72$, $0.01 < P < 0.05$). Since P4502E1 clearly catalyzes theophylline 8-hydroxy-

Table 4. Correlation of human liver microsomal total P450 concentrations with P450 form-specific activities

		Correlation coefficient (r)						
		P450 activity*						
	N	1A2	2A6	2C8/9/10	2C18/19	2D6	2E1	3A3/4
P450 concn	10	0.45	0.38	0.71†	0.01	0.15	0.61	0.90‡

* Activities used to identify P450 forms are provided in Table 3.
†,‡ Statistically significant correlation: (†) $0.05 > P \geq 0.01$; and (‡) $P < 0.001$.

Table 5. Correlation of human liver microsomal theophylline 1-demethylation and 3-demethylation rates with P450 form-specific activities

Metabolite	Theophylline concn	N	Total P450 concn	Correlation coefficient (r) P450 activity* 1A2	2A6	2C8/9/10	2C18/19	2D6	2E1	3A3/4
3-X†										
	5 mM	9	0.54	0.95‡	0.36	0.05	0.03	0.24	0.13	0.34
	40 mM	10	0.78‖	0.41	0.10	0.69§	0.27	0.01	0.84‖	0.58
1-X										
	5 mM	10	0.58	0.95‡	0.29	0.11	0.05	0.26	0.15	0.37
	40 mM	10	0.70§	0.22	0.22	0.77§	0.23	0.13	0.92‡	0.56

* Activities used to identify P450 forms are provided in Table 3.
† Abbreviations: 1-X, 1-methylxanthine; and 3-X, 3-methylxanthine.
‡§‖ Statistically significant correlation: (‡) $P < 0.001$; (§) $0.05 > P \geq 0.01$; and (‖) $0.01 > P \geq 0.001$.

lation at high substrate concentrations, based on our studies with expressed P4502E1 and the microsomal preparations, the correlation of this activity level with P4502C8/9/10 activity levels probably explains the apparent correlation of theophylline 8-hydroxylation with P4502C8/9/10 observed in the liver microsomes.

The shift from 8-hydroxylation by microsomal P4501A2 at low theophylline concentrations to P4502E1 at high concentrations is consistent with the kinetic data obtained with the individually expressed enzymes. P4501A2 exhibited high affinity, low capacity kinetics, whereas P4502E1 exhibited low affinity, high capacity kinetics. At low substrate concentrations in the liver microsomal preparations, P4502E1 played a limited role because of its low affinity relative to that of P4501A2, while at high substrate concentrations the low capacity of P4501A2 relative to that of P4502E1 diminished its apparent role and enhanced that of P4502E1. The conformity between our data on theophylline 8-hydroxylation with human liver microsomes and with individually expressed forms of P450 suggests that the latter system accurately reflects the function of liver microsomal P450s.

Both the data from individually expressed P4503A4 and the human liver microsomes suggest that this P450 could play a minor role in theophylline 8-hydroxylation. The kinetic data, which indicate that this enzyme has a low affinity for theophylline and a low capacity for its metabolism, support this conclusion. Moreover, P4503A4 has been reported to be the major component of human hepatic P450 (about 30% of the total P450) [23]—supported by the observation that P4503A4 activity correlates most closely with total P450 in the hepatic microsomal preparations investigated here—and this could enhance the importance of the role of P4503A4 for theophylline clearance. However, it has been demonstrated previously that anti-rat P4503A1/2 antibodies, which cross-react with P4503A3/4, do not inhibit 1,3-DU formation with human liver microsomes [3].

The theophylline 8-hydroxylase activity observed with individually expressed P4502D6, which has relatively low affinity and low capacity when compared with P4502E1 activity, did not correlate with dextromethorphan *O*-demethylase activity in the human liver microsomes at either substrate concentration. It is thus unlikely to play a significant role in theophylline 8-hydroxylation *in vivo*.

The demethylations of theophylline by human liver microsomal preparations mirrored 8-hydroxylation in that at low theophylline concentration both activities correlated significantly with P4501A2-associated activity, whereas at high theophylline concentration both activities correlated with P4502E1-associated activity. In fact all of these activities correlated with each other at both 5 mM ($r = 0.89$ to 0.99) and 40 mM ($r = 0.87$ to 0.97). These results suggest that both demethylations are catalyzed by P4501A2 at low substrate concentrations and by P4502E1 at high substrate concentrations. Such a role for P4502E1 in theophylline metabolism is consistent with its role in caffeine metabolism [4, 24]. Published reports [1, 21] that K_m values for theophylline demethylations are lower than K_m values for theophylline 8-hydroxylation in human liver microsomes suggest that P4501A2 catalysis may favor demethylation over 8-hydroxylation at low theophylline concentrations. While previously published studies with individually expressed P450s did not report any role for P4502E1 in theophylline demethylations [4], it is possible that insufficiently high substrate concentrations were used. Our observations are supported by reports that P4502E1 catalyzes the similar 1- and 7-demethylations of caffeine [4, 24].

In summary, these studies have demonstrated that P4501A2 predominantly catalyzes the metabolism of low concentrations of theophylline to 1,3-DU in human liver microsomes, because of its relatively high substrate affinity. At high substrate concentrations, despite its lower substrate affinity, P4502E1 is primarily responsible for catalysis of theophylline 8-hydroxylation, because of its high capacity for metabolism. However, at the pharmacologic plasma levels achieved *in vivo*, usually less than 100 μM [25] or 50-fold less than the lower concentration in our

assay, theophylline is probably metabolized to 1,3-DU catalyzed predominantly by P4501A2. Thus, theophylline drug–drug interactions could be expected to occur with other drugs that are substrates for P4501A2. Urinary levels of 1,3-DU arising from administered theophylline should thus provide a noninvasive probe of P4501A2 levels in humans.

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