0026-895X/93/050827-06$03.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 43:827-832

MOL PHARM

Major Pathway of Imipramine Metabolism Is Catalyzed by Cytochromes P-450 1A2 and P-450 3A4 in Human Liver

ANTOINETTE LEMOINE, JEAN CHARLES GAUTIER, DANIEL AZOULAY, LILIANE KIFFEL, CLAIRE BELLOC, F. PETER GUENGERICH, PATRICK MAUREL, PHILIPPE BEAUNE, and JEAN PAUL LEROUX

Unité INSERM 75, CHU NECKER, Paris (A.L., J.C.G., L.K., C.B., P.B., J.P.L.), Service de Chirurgie Hépato-Biliaire, Hôpital Paul Brousse, Villejuif (D.A.), and Unité INSERM 128, Montpellier (P.M.), France, and Department of Biochemistry and Center in Molecular Toxicology (F.P.G.), Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received August 26, 1992; Accepted March 1, 1993

SUMMARY

The metabolism of imipramine by human liver microsomes was examined using a combination of five strategies. Human hepatic microsomes produced *N*-desmethylimipramine (84%), 2-hydroxyimipramine (10%), and 10-hydroxyimipramine (6%). Preincubation of human hepatocytes in culture with β-naphthoflavone and macrolides exclusively induced the formation of desmethylimpramine (552%, $p < 0.05$, and 234%, $p < 0.003$, respectively). Correlations were obtained between rates of imipramine demethylation and cytochrome P-450 (P-450) 1A2 ($r = 0.88$, $p < 0.001$) and P-450 3A ($r = 0.80$, $p < 0.02$) concentrations in human liver microsomal preparations from 13 different subjects. Anti-P-450 1A2 and anti-P-450 3A antibodies selectively inhibited *N*-demethylation (80% and 60%, respectively). *N*-Demethylation was completely inhibited when anti-1A2 and anti-3A were added simultaneously. Kinetic studies with human microsomes confirm the contribution of two different enzymes in the *N*-demethylation. The K_m of 1A2 was similar to the high affinity K_m in human liver microsomes, whereas the K_m of 3A was similar to the low affinity K_m in human liver microsomes. P-450 1A2 was apparently more efficient than 3A4 (lower K_m and higher V_{max}) but was expressed in much lower concentration. Human P-450s 1A2 and 3A4 expressed in yeast efficiently produced desmethylimipramine. These results suggest that P-450 1A2 and P-450 3A4 are the major enzymes involved in imipramine *N*-demethylation in human hepatic microsomes. Similar experiments were conducted using P-450 2D6, and they confirmed that P-450 2D6 catalyzes imipramine 2-hydroxylation. Interindividual variations in 3A4 hepatic content may explain the large variations in imipramine blood levels observed after uniform dosages and thus may explain the variations in clinical efficacy. Caution might be advised in the clinical use of tricyclic antidepressants when drugs are also administered that induce or inhibit P-450s 3A4 and 1A2.

Imipramine is a tricyclic antidepressant that has been widely used for more than 30 years in the treatment of depression. The metabolic fate of imipramine involves mainly *N*-demethylation to the active metabolite desipramine and aromatic hydroxylation to 2-hydroxyimipramine and 10-hydroxyimipramine. These different pathways are catalyzed by several P-450 enzymes in hepatic microsomes (1). *N*-Demethylation is the major pathway leading to desipramine, which is further oxidized to 2-hydroxydesipramine (2).

A major problem in the clinical use of this drug is the large interpatient variability in blood levels for a given dose and the consequences in clinical response and toxicity, particularly cardiotoxicity (3, 4). The observed interindividual variation in the steady state plasma concentration may be due to differences in the concentrations of the different hepatic P-450 enzymes among individuals.

The role of P-450 2D6 in the 2-hydroxylation of imipramine and desipramine may account for some of these interindividual variations. The relationship between imipramine and desipramine metabolism and the sparteine/debrisoquine oxidation polymorphism has been extensively studied *in vivo*. The 2-hydroxylation of imipramine and desipramine is markedly impaired in poor metabolizers. Brosen *et al.* (5) have confirmed *in vitro* that 2-hydroxylation is catalyzed by P-450 2D6, whereas *N*-demethylation and 10-hydroxylation are predominantly catalyzed by other P-450 forms that have not yet been identified.

The *N*-demethylation pathway is not affected by the sparteine/debrisoquine polymorphism and has been reported to be at least partly mediated by a P-450 belonging to the 2C subfamily, according to *in vivo* studies (6). Imipramine demethylation clearance was lower in six poor metabolizers, compared with 16 extensive metabolizers of mephenytoin.

The aim of this study was to identify the hepatic P-450 enzymes involved in the formation of the active metabolite desipramine. Such information may be useful in addressing the variable drug concentrations observed after administration of uniform dosages and in avoiding toxicity or inefficacy when other drugs are administered at the same time.

Materials and Methods

Human livers and hepatocytes. Human livers were obtained, with the agreement of the local ethics committee, from adult kidney

ABBREVIATIONS: P-450, cytochrome P-450; LKM, liver/kidney microsomes.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

transplantation donors without known antecedents. Livers for hepatocyte preparation were obtained at the Saint Eloi Hospital (Montpellier, France). Patient FT4 was a 57-year-old man who underwent left hepatic lobectomy because of metastasis of a colon tumor; he had no medication before surgery and had stopped smoking (15 cigarettes/day) 1 week before entering the hospital. Patient FT15 was a 31-year-old woman who underwent left hepatic lobectomy because of a voluminous adenoma on normal liver. Patient FT43 was a 61-year-old woman who underwent right hepatic lobectomy because of liver metastasis of colorectal cancer; patients FT15 and FT43 had no medication and were nonsmokers.

Preparation of hepatocytes. After washing of the tissue with Eurocollins (2.05 g/liter $NaHCO_3$, 35 g/liter glucose, pH 7.3), hepatocytes were prepared as described before, using collagenase perfusion (7–9). Yield and viability, determined using the Trypan Blue exclusion test, were between 8.2×10^9 and 2.0×10^9 cells and between 78 and 85%, respectively. Four million cells in 3 ml of culture medium were placed in 60-mm plastic dishes that had been precoated with 50 μg of rat tail collagen. The culture medium consisted of a 1:1 mixture of Ham F-12 and Williams E supplemented as recommended by Isom and Georgoff (10). During the first 4 hr, medium was supplemented with 5% fetal calf serum to favor the plating. The medium was then changed and thereafter renewed without serum every 24 hr. Cultures were maintained at 37° in a humidified atmosphere of air and 5% carbon dioxide. Treatments with 50 μM β-naphthoflavone or rifampicin or with 2 mM phenobarbital started 24 hr after plating and lasted for 96 hr. Control cultures received the same volume (3 μl) of the vehicle solvent dimethylsulfoxide.

Preparation of yeast expressing human P-450s. Human P-450 1A1, 1A2, 2C9, 2D6, and 3A4 enzymes were expressed separately in yeast using an adaptation of the procedure described elsewhere, (11–13). P-450 1A1 and 1A2 coding sequences were cloned by polymerase chain reaction from human liver cDNA and were checked by DNA sequencing. The coding sequences of human P-450 2D6 and P-450 2C9 were amplified by polymerase chain reaction from plasmids db1-pUC9 (14) (a generous gift from Dr. U. Meyer, Basel, Switzerland) and MP4-pAAH5 (15), respectively. All sequences were inserted into the yeast expression vector pYeDP60 (16) using classical cloning procedures. The P-450 3A4 coding sequence from plasmid pVNF25 (13) was inserted into vector pYeDP60 by gap repair (13, 17). All these expression vectors were each introduced into the engineered *Saccharomyces cerevisiae* strain W(R), which overexpresses yeast NADPH-P-450 reductase when grown with galactose as carbon source (18). Preparation of culture medium S5 (containing galactose) without adenine, spheroplast preparation by enzymatic digestion of yeast cell walls, and subcellular fractionations were performed as described previously (12, 13).

Microsome preparation and assays. Microsomes from human livers, hepatocytes, and yeast were prepared as described previously (13, 19). P-450 was determined according to the method of Omura and Sato (20) and protein concentrations according to the method of Lowry *et al.* (21).

Imipramine metabolism. [*benzene*-^{3}H]Imipramine hydrochloride was purified by high performance liquid chromatography just before incubation as described previously (22, 23). Imipramine was incubated in a final volume of 1 ml consisting of 100 mM sodium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 20% glycerol, 0.15 mM NADP, and 2.5 mM glucose-6-phosphate. Imipramine and [^{3}H]imipramine (0.5 mM; specific activity, 5 μCi/mmol) were added and incubated for 10 min with 0.3 nmol of microsomal P-450. Kinetic studies were performed using 5×10^{-6} to 5 mM imipramine. The reaction was started by the addition of 5 IU of glucose 6-phosphate dehydrogenase and was stopped by addition of 10 N sodium hydroxide (200 μl). Imipramine and its metabolites were extracted three times with diethylether/dichloromethane (60:40, v/v; 5 ml) and were evaporated to dryness under nitrogen. Residues were subjected to separation by thin layer chromatography on silica plates (Merck), using chloroform/propanol/27% ammonia (50:50:1) as the mobile phase. Quantification of metabolites was achieved by scraping the UV-visualized spots and counting for radioactivity after addition of scintillation cocktail.

Other enzymatic activities. The testosterone 6β-hydroxylase activity was determined by incubating 80 μM testosterone in 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.1 mg/ml NADPH, with 100 μg of microsomal protein, in a final volume of 250 μl. After 15 min of incubation at 37°, the reaction was stopped with 5 μl of trifluoroacetic acid and the organic residue was extracted with dichloromethane. Metabolite separation was achieved by high performance liquid chromatography using a 5-μm C18 column (3 × 0.21 cm), at a flow rate of 1 ml/min (10–40% acetonitrile gradient in water), and was monitored at 254 nm.

7-Ethoxyresorufin *O*-deethylase and 7-methoxyresorufin *O*-demethylase enzymatic activities were measured in yeast microsomes using adaptations of the methods of Lubet *et al.* (24) and Namkung *et al.* (25), respectively.

Immunochemical determination. Human liver microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (26), and Western blotting was performed as described by Towbin *et al.* (27). Proteins were probed with polyclonal antibodies [anti-LKM1 or anti-LKM2 human sera, anti-human P-4501A (PA), or anti-rabbit 3A6]. Anti-rabbit 3A6 antibodies cross-react with human P-450 3A4 (9, 28). Anti-LKM1 and anti-LKM2 human sera are directed to P-450s 2D6 and 2C, respectively (29, 30). The nitrocellulose membranes were scanned for densitometric quantification as described previously (31).

Immunoinhibition of imipramine metabolism. Human microsomes were preincubated at 23° for 10 min with variable amounts of anti-P-450 immunoglobulins [anti-rabbit 1A2 (7) or anti-rabbit 3A6], anti-LKM1 (2D6) or anti-LKM2 (2C) human sera, or polyclonal antibodies directed to purified rat liver P-450 2C9. Tubes were then chilled and all other incubation components were added to carry out the reaction as described above. Results of inhibition are given as percentages of incubations with preimmune immunoglobulins or control serum.

Chemicals. Imipramine and its metabolites (2-hydroxyimipramine, 10-hydroxyimipramine, and desipramine) were kindly provided by Ciba Geigy (Basel, Switzerland) and [^{3}H]imipramine (1.8 TBq/mmol) was from Amersham (Les Ulis, France). All other reagents were of analytical grade (Prolabo, Paris, France).

Results

Imipramine metabolism by human hepatic microsomes. The rate of formation of imipramine metabolites was studied with microsomes from human liver and from hepatocytes in primary culture. Results are summarized in Table 1. The desmethylated derivative was the major metabolite formed by both preparations. Hepatocyte microsomes were prepared from three independent human liver samples treated for 96 hr with different P-450 monooxygenase inducers (Table 2). *N*-Demethylation was increased to a large extent (552%) by β-

TABLE 1

Metabolism of imipramine by human microsomal preparations

Results are expressed as pmol/min/mg of microsomal proteins and are the mean ± standard deviation of different samples (three to five determinations for each preparation). For hepatocytes, each result comes from duplicate experiments; FT4, FT15, and FT43 are independent cultures of hepatocytes isolated from the livers of three subjects.

Reaction	Activity			
	Liver (n = 13)	Hepatocytes		
		FT4	FT15	FT43
	pmol/min/mg of protein			
2-Hydroxylation	68 ± 43	70	61	60
10-Hydroxylation	39 ± 21	20	13	25
N-Demethylation	595 ± 263	100	141	90

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

TABLE 2

Effects of P-450 inducers on metabolism of imipramine by human hepatocytes in culture

Results are expressed as pmol/min/mg of microsomal protein and are the mean ± standard deviation of three independent cultures of hepatocytes. Each culture was divided into control and treated cultures.

Reaction	Activity			
	Control	βNF[a]	PB	RIF
	pmol/min/mg of protein			
2-Hydroxylation	64 ± 6	55 ± 41	46 ± 19	70 ± 5
10-Hydroxylation	19 ± 6	35 ± 7[b]	29 ± 10	35 ± 17
N-Demethylation	110 ± 27	608 ± 209[b]	266 ± 118	257 ± 45[c]

[a] βNF, β-naphthoflavone (50 μM); PB, phenobarbital (2 mM); RIF, rifampicin (50 μM).
[b] $p < 0.05$.
[c] $p < 0.003$, Student *t* test.

naphthoflavone and to a lesser extent (234% and 242%, respectively) by rifampicin and phenobarbital. Induction by β-naphthoflavone and rifampicin was significant ($p < 0.05$ and $p < 0.003$, respectively), whereas induction by phenobarbital was not. No significant induction was observed for the 2-hydroxylation pathway.

Correlation of imipramine metabolism with immunochemically determined concentrations of P-450s 1A2, 2D6, 2C, and 3A. The relative intensity obtained in Western blots for immunochemically determined P-450 enzymes was determined in parallel with the production of imipramine metabolites from 13 subjects analyzed (Fig. 1; Table 3). Significant correlations were obtained only between *N*-demethylation and P-450 1A2 ($r = 0.88$; Fig. 1A) and P-450 3A content ($r = 0.80$, Fig. 1B). 2-Hydroxylation exhibited a significant correlation ($r = 0.77$; Fig. 1C) with hepatic P-450 2D6 content but not with any of the other enzymes tested.

Immunoinhibition of imipramine metabolic activities of human hepatic microsomes by human anti-P-450 1A2, 2D6, and 2C and rabbit anti-3A6 antibodies. The effect of varying concentrations of anti-P-450 sera (human anti-LKM1 and anti-LKM2) and immunoglobulins [anti-rabbit 1A2 and anti-rabbit 3A6 (human 3A4)] on imipramine activities was investigated in microsomes from three human livers (Fig. 2). A marked decrease in *N*-demethylase activity (80% for anti-1A2) was observed with the addition of anti-P-450 1A2 immunoglobulins, whereas no effect was observed on 2-hydroxylation activity, even when the antibody concentration was raised to the level that caused a maximum inhibition of *N*-demethylation.

Increasing amounts of rabbit anti-P-450 3A6 immunoglobulins recognizing human P-450s 3A inhibited *N*-demethylation in a dose-dependent manner, but the extent of inhibition was weaker and did not exceed 60%. A complete inhibition of *N*-demethylation was observed when human anti-P-450 1A2 immunoglobulins and rabbit anti-P-450 3A6 immunoglobulins were added simultaneously (Fig. 2E). 2-Hydroxylation was strongly inhibited (70%) by increasing amounts of anti-P-450 2D6 but not by the other antibodies. Only a weak inhibition (30%) of *N*-demethylation was seen with anti-P-450 2C (anti-LKM2), whereas the same human sera completely inhibited hydroxylation of tienilic acid, a reaction known to be catalyzed by P-450 2C enzymes (30). No inhibition of imipramine metabolism was observed with polyclonal antibodies directed to rat liver P-450 2C11.

Imipramine metabolic activities of yeast-expressed P-450 enzymes. Microsomes prepared from two independent preparations of transfected yeast expressing P-450 1A1, 1A2, 3A4, 2D6, and 2C9 enzymes catalyzed the metabolism of imip-

TABLE 3

Correlation of imipramine metabolism with immunochemically determined concentrations of P-450s 1A2, 2D6, 2C, and 3A

Experiments were performed in triplicate.

Reaction	Correlation			
	1A2	2D6	2C	3A
2-Hydroxylation	0.49	0.77[a]	0.46	0.61
10-Hydroxylation	0.39	0.49	0.54	0.74
N-Demethylation	0.88[b]	0.57	0.59	0.80[a]

[a] $p < 0.02$.
[b] $p < 0.001$.

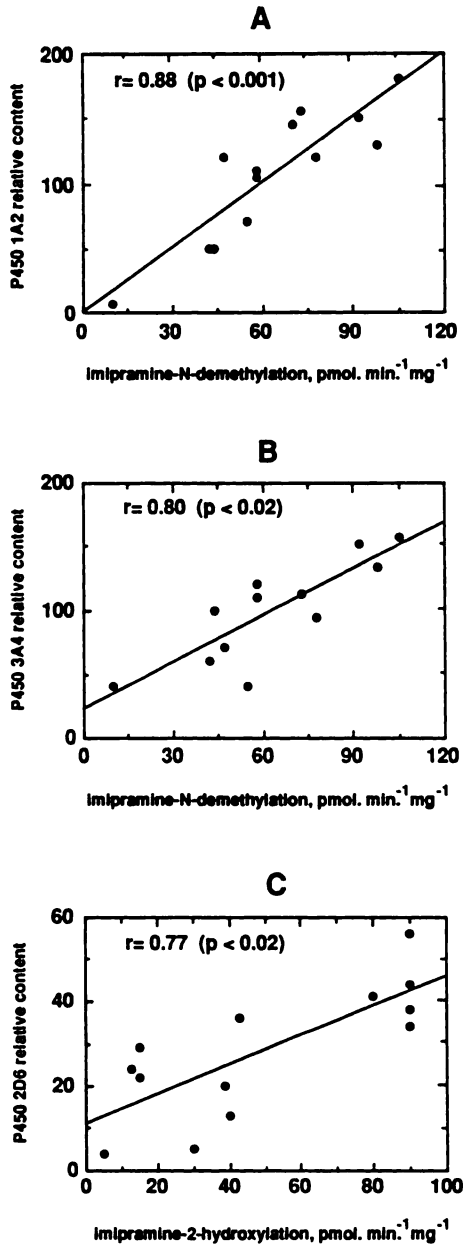

Fig. 1. Relationship between imipramine *N*-demethylation and immunochemically determined amounts of human P-450 1A2 (A) and rabbit P-450 3A6 (B) and between imipramine 2-hydroxylation and P-450 2D6 (C) in microsomes from 13 different human livers.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

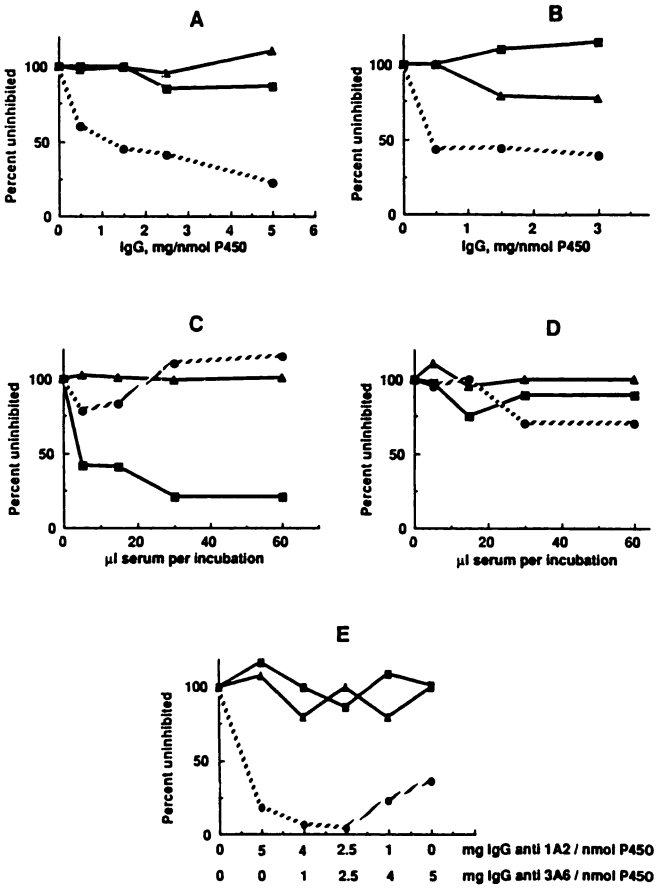

Fig. 2. Effect of immunoglobulins directed to human P-450 1A2 (A) and rabbit P-450 3A6 (B) and sera of patients with autoimmune hepatitis [anti-LKM1 (2D6) (C) and anti-LKM2 (2C) (D)] on imipramine metabolism in human liver microsomes (pool of three different samples). Microsomal proteins (0.3 mg) were preincubated with 0.5–5 mg of protein immunoglobulins or 5–40 µl of serum before incubation with the substrate. E, Immunoinhibition of imipramine metabolism by a total of 5 mg of IgG, consisting of variable amounts of anti-human 1A2 and anti-rabbit 3A6, added simultaneously. All results are the means of duplicate determinations. ●, Desmethylimipramine; ■, 2-hydroxyimipramine; ▲, 10-hydroxyimipramine.

ramine at different rates (Table 4). P-450s 1A2 and 3A4 preferentially catalyzed *N*-demethylation, whereas P-450 2D6 metabolized imipramine to the 2-hydroxy product. P-450 1A1 catalyzed 2- and 10-hydroxylation and *N*-demethylation. Expressed P-450 2C9 did not catalyze any imipramine oxidation, whereas it catalyzed the production of 5-hydroxytienilic acid and the activation of tienilic acid to a reactive metabolite covalently bound to protein (32). Microsomes from control yeast transfected only with the vector itself had no detectable activity. To characterize the recombinant P-450s expressed in yeast, several enzymatic and immunochemical properties were examined and compared with those of the native enzyme. P-450s 1A1 and 1A2 specifically catalyzed ethoxyresorufin *O*-deethylation and methoxyresorufin *O*-deethylation; P-450 3A4 catalyzed testosterone 6β-hydroxylation. The reactivity of the recombinant proteins with the respective antibodies was confirmed by immunoblot analysis (data not shown).

Kinetic studies. In human liver, kinetics were biphasic and allowed calculation, by extrapolation from double-reciprocal plots, of V_{max_2} of 1 nmol/min/nmol of P-450 with a low affinity $K_{m_2} \sim 50$ µM) and V_{max_1} of 0.1 nmol/min/nmol of P-450 with an higher affinity $K_{m1} \sim 5$ µM) (Table 5). For human liver microsomes, in V_{max} nmol of P-450 represented total P-450. For yeast, nmol of P-450 represented specific P-450s. For P-450s expressed in yeast, kinetics were monophasic, with a K_m of about 13 µM and a V_{max} of 4.5 nmol/min/nmol of P-450 for P-450 1A2 and a K_m of about 50 µM and a V_{max} of 1 nmol/min/nmol of P-450 for P-450 3A4.

Discussion

The work presented here indicates that human liver P-450 1A2 and P-450 3A4 have major roles in the *N*-demethylation of the tricyclic antidepressant drug imipramine and confirms the role of P-450 2D6 in 2-hydroxylation. These conclusions resulted from a strategy of five complementary techniques. 1) Induction of imipramine metabolism in microsomes prepared from hepatocytes in culture with inducers of specific P-450s gave useful information on the possible P-450 enzymes implicated. β-Naphthoflavone, rifampicin, and phenobarbital induced imipramine *N*-demethylation, suggesting that enzymes in the P-450 1A and P-450 3A subfamilies could be implicated. The 2-hydroxylation pathway was not increased by any of these treatments. 2) Immunochemically determined levels of P-450s 1A2 and 3A4 were correlated with imipramine *N*-demethylation in human liver ($r = 0.88$ and 0.80, respectively). A good correlation ($r = 0.77$) was also obtained between 2-hydroxylation and P-450 2D6 hepatic content in the same human liver samples, confirming previous results. 3) Kinetic studies with human microsomes confirmed the contribution of two different enzymes in the *N*-demethylation. The K_m for 1A2 was similar to the high affinity K_m in human liver microsomes, whereas the

TABLE 4

Enzymatic activities of human P-450s expressed in yeast

Results are expressed as pmol/pmol of P450/min. Imipramine activities were determined using yeast microsomes from two or three independent preparations. Results are expressed as mean ± standard deviation when three preparations were used. No activity was detected in microsomes from control yeast cells transformed with vector pYeDP60.

Reaction	Activity				
	1A1	1A2	2C9	2D6	3A4
	pmol/pmol of P450/min				
Imipramine 2-hydroxylation	2.6; 1.0	0.3 ± 0.2	<0.1; 0.5	9.7; 8.1	0.2 ± 0.1
Imipramine 10-hydroxylation	3.4; 2.0	0.1 ± 0.1	<0.1; 0.2	3.9; 1.7	0.1 ± 0.0
Imipramine *N*-demethylation	2.1; 0.3	3.6 ± 1.0	<0.1; <0.1	0.4; 2.5	0.9 ± 0.3
Ethoxyresorufin *O*-deethylation	15.5	1.0	<0.1	<0.1	<0.1
Methoxyresorufin *O*-demethylation	8.1	1.5	<0.1	<0.1	<0.1
Testosterone 6β-hydroxylation	1.8	<0.1			0.9

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

TABLE 5

N-Demethylation of imipramine by human liver microsomes and human P450s 1A2 and 3A4 expressed in yeast.

K_{m_1} and K_{m_2} correspond to the high and low affinity Michaelis-Menten constants, respectively, related to the biphasic kinetics found in human liver microsomes; V_{max_1} and V_{max_2} correspond to the maximum rates of metabolism related to the high and low affinity components, respectively. All results are based on experiments that have been carried out in duplicate.

	Human liver microsomes	1A2	3A4
K_{m_1} (μM)	3.3; 6.7	10; 17	
K_{m_2} (μM)	33; 66		40; 66
V_{max_1} (nmol/min/nmol of P450)	0.08; 0.13	3.2; 4.8	
V_{max_2} (nmol/min/nmol of P450)	0.87; 1.2		0.6; 2.0

K_m for 3A was similar to the low affinity K_m in human liver microsomes. P-450 1A2 was apparently more efficient than 3A4 (lower K_m and higher V_{max}) but was expressed in much lower concentration. Therefore, the two enzymes participated actively in the *N*-demethylation of imipramine. 4) In the presence of increasing amounts of anti-P-450 1A2, 80% of the demethylation activity was inhibited in human liver microsomes, without inhibition of the 2- and 10-hydroxylation pathways. The inhibition was similar to that observed for 2-hydroxylation by anti-P-450 2D6. Polyclonal antibodies directed to P-450 3A exclusively inhibited *N*-demethylation, to a lesser extent (60%). *N*-Demethylation was completely inhibited when anti-1A2 and anti-3A immunoglobulins were added simultaneously. The fact that the simultaneous addition of anti-1A and anti-3A led to a 100% inhibition supports our conclusions, i.e., P-450s 1A2 and 3A4 are the main P-450s involved in *N*-demethylation of imipramine. The fact that each of the antibodies inhibited >50% might be reflective of a cross-inhibition with other P-450s. However, in Western blots of human liver microsomes each of the antibodies recognized only a single band. 5) Complementary studies with cloned P-450s expressed in yeast showed that P-450 1A2 and P-450 3A4 were active in demethylating imipramine, with rates of 3.6 ± 1 and 0.9 ± 0.3 pmol/pmol of P-450/min, respectively. P-450 2D6 catalyzed 2-hydroxylation at a rate of 8.9 pmol/pmol of P-450/min, corresponding to 161 pmol/min/mg of microsomal proteins, and did not catalyze imipramine *N*-demethylation. Brosen *et al.* (5) observed the formation of the three primary P-450-dependent metabolites after addition of imipramine (250 μM) to the culture medium of COS-1 cells transfected with P-450 2D6 cDNA. These authors reported that 2-hydroxylated, 10-hydroxylated, and *N*-demethylated metabolites were formed at a rate of 164, 48, and 256 pmol/mg of homogenate proteins/hr, respectively. Although Brosen *et al.* (5) and we found that P-450 2D6 expressed in heterologous systems was able to produce desipramine, it is quite likely that it was not significant in human liver microsomes; no inhibition was observed with anti-LKM1 (Fig. 2). P-450 2D6 is expressed at low levels in human liver microsomes (33), and kinetic data indicated that the K_m is quite high (5). In the same way, Birgersson *et al.* (34) have purified a P-450 (P-450 NT), belonging to the P-450 2D6 subfamily, that is able to catalyze imipramine 2-hydroxylation and debrisoquine 4-hydroxylation and also to demethylate imipramine at a slower rate. On the basis of our results, we cannot conclude that there is a major role for P-450 2C enzymes in imipramine *N*-demethylation, as reported previously by Skjelbo *et al.* (6). We observed a weak inhibition (30%) of *N*-demethylation using two different antibodies characterized to be inhibitory against specific enzymatic activities of the P-450 2C subfamily. Indeed, all data are not supportive of the implication of a P-450 2C; however, this subfamily contains different members, and the possibility has not been excluded that a minor form, such as the one metabolizing (*S*)-mephenytoin, could also produce desipramine. If P-450s 2D6 and 2C catalyze imipramine *N*-demethylation *in vivo*, their contributions to this pathway seem to be minor.

This work shows that each of the five methods used gives useful information, but the conclusions about the identification of the different P-450 enzymes implicated in imipramine metabolism result from comparison of the different information gathered with each technique.

A 16-fold variation was observed in the mean steady state concentrations of imipramine and desmethylimipramine after 100 mg of imipramine/day in 28 sparteine/debrisoquine extensive metabolizers (4). Interindividual variation observed in blood levels of imipramine could be explained not only by the genetic polymorphism of P-450 2D6 but also by the variation observed in P-450 3A4 and 1A2 hepatic content, which is more than 1 order of magnitude and has already been described (35). Because P-450s 1A2 and 3A4 are the major enzymes implicated in the formation of the main active metabolite, desmethylimipramine, care should be taken in the clinical use of imipramine, to ensure efficacy without toxicity, when other drugs that induce or inhibit P-450 1A2 and P-450 3A4 are administered.

Acknowledgments

The authors would like to express their thanks to Dr. J. Domergue for the lobectomies (FT4, FT15, and FT43) and to Dr. J. C. Homberg for human anti-LKM1 and anti-LKM2 sera used in this work.

References

1. Brosen, K., S. V. Otton, and L. F. Gram. Imipramine demethylation and hydroxylation: impact of the sparteine oxidation phenotype. *Clin. Pharmacol. Ther.* **40**:543–549 (1986).
2. Gram, L. F. Metabolism of tricyclic antidepressants. *Dan. Med. Bull.* **21**:218–231 (1974).
3. Glassman, A. H., J. M. Perel, M. Shottak, S. J. Kantor, and J. Fleiss. Clinical implication of imipramine plasma levels for depressive illness. *Arch. Gen. Psychiatry* **34**:17–204 (1977).
4. Brosen, K., R. Klynser, L. F. Gram, S. V. Otton, P. Bech, and L. Bertilsson. Steady-state concentrations of imipramine and its metabolites in relation to the sparteine/debrisoquine polymorphism. *Eur. J. Clin. Pharmacol.* **30**:679–684 (1986).
5. Brosen, K., T. Zeugin, and U. A. Meyer. Role of P450IID6, the target of the sparteine-debrisoquine oxidation polymorphism, in the metabolism of imipramine. *Clin. Pharmacol. Ther.* **49**:609–617 (1991).
6. Skjelbo, E., K. Brosen, J. Hallas, and L. F. Gram. The mephenytoin oxidation polymorphism is partially responsible for the *N*-demehylation of imipramine. *Clin. Pharmacol. Ther.* **49**:18–23 (1991).
7. Diaz, D., I. Fabre, M. Daujat, B. Saint-Aubert, P. Bories, H. Michel, and P. Maurel. Omeprazole is an aryl hydrocarbon-like inducer of human hepatic cytochrome P-450. *Gastroenterology* **99**:737–747 (1990).
8. Pichard, L., I. Fabre, G. Fabre, J. Domergue, B. Saint-Aubert, G. Mourad, and P. Maurel. Cyclosporin A drug interactions: screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metab. Dispos.* **18**:595–606 (1990).
9. Daujat, M., L. Pichard, I. Fabre, T. Pineau, G. Fabre, C. Bonfils, and P. Maurel. Induction protocols for cytochromes P-450 IIIA *in vivo* and in primary cultures of animal and human hepatocytes. *Methods Enzymol.* **206**:345–353 (1991).
10. Isom, H. C., and I. Georgoff. Quantitative assay for albumin-producing liver cells after simian virus 40 transformation of rat hepatocytes maintained in chemically defined medium. *Proc. Natl. Acad. Sci. USA* **81**:6378–6382 (1984).
11. Cullin, C., and D. Pompon. Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3-1 in the yeast *Saccharomyces cerevisiae*. *Gene* **65**:203–217 (1988).
12. Pompon, D. cDNA cloning and functional expression in yeast *Saccharomyces cerevisiae* of β-naphthoflavone-induced rabbit liver cytochromes P-450 LM4 and LM6. *Eur. J. Biochem.* **117**:285–293 (1988).
13. Renaud, J. P., C. Cullin, D. Pompon, P. Beaune, and D. Mansuy. Expression

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

of human liver cytochrome P-450 IIIA in yeast: a functional model for the hepatic enzyme. *Eur. J. Biochem.* **194**:889–896 (1990).
14. Gonzalez, F., F. Vilbois, J. P. Hardwick, O. W. McBride, D. W. Nebert, and U. A. Meyer. Human debrisoquine 4-hydroxylase (P-450 IID1): cDNA and deduced amino acid sequence and assignment of the *cyp* 2D locus to chromosome 22. *Genomics* **2**:174–179 (1988).
15. Srivastava, P. K., C. H. Yun, P. Beaune, C. Ged, and F. P. Guengerich. Separation of human liver microsomal tolbutamide hydroxylase and (*S*)-mephenytoin 4′-hydroxylase cytochrome P-450 enzymes. *Mol. Pharmacol.* **40**:69–79 (1991).
16. Urban, P., C. Cullin, and D. Pompon. Maximizing the expression of mammalian cytochrome P-450 monooxygenase activities in yeast cells. *Biochimie (Paris)* **72**:463–472 (1990).
17. Peyronneau, M., J. P. Renaud, G. Truan, P. Urban, D. Pompon, and D. Mansuy. Optimization of yeast-expressed human liver cytochrome P-450 3A4 catalytic activities by coexpressing NADPH-cytochrome P-450 reductase and cytochrome b_5. *Eur. J. Biochem.* **207**:109–116 (1992).
18. Truan, G., C. Cullin, C. Reisdorf, P. Urban, and D. Pompon. Enhanced *in vivo* monooxygenase activities of mammalian *P*-450 in engineered yeast cells producing high levels of NADPH-*P*-450 reductase and human cytochrome b5. *Gene* **125**:49–55 (1993).
19. Cresteil, T., J. P. Flinois, A. Pfister, and J. P. Leroux. Effect of a microsomal preparation and induction on cytochrome P450 dependent monooxygenase in fetal and neonatal rat. *Biochem. Pharmacol.* **28**:2057–2063 (1979).
20. Omura, T., and R. Sato. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2385 (1964).
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
22. Lemoine, A., D. E. Williams, T. Cresteil, and J. P. Leroux. Hormonal regulation of microsomal flavin-containing monooxygenase: tissue-dependent expression and substrate specificity. *Mol. Pharmacol.* **40**:211–217 (1991).
23. Godbillon, J., and S. Gauron. Determination of clomipramine and imipramine and their mono-demethylated metabolites in human blood or plasma by high-performance liquid chromatography. *J. Chromatogr.* **204**:303–311 (1981).
24. Lubet, R. A., R. T. Mayer, J. W. Cameron, R. W. Nims, M. D. Burke, T. Wolff, and F. P. Guengerich. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. *Arch. Biochem. Biophys.* **238**:43–48 (1985).
25. Namkung, M. J. H., J. Yang, E. Hulla, and R. Juchau. On the substrate specificity of cytochrome P-450IIIA1. *Mol. Pharmacol.* **34**:628–637 (1988).
26. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
27. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
28. Lange, R., C. Larroque, C. Balny, and P. Maurel. Isolation and partial characterization of a rifampicin-induced rabbit liver microsomal cytochrome P-450. *Biochem. Biophys. Res. Commun.* **126**:822–833 (1985).
29. Kiffel, L., J. Loeper, J. C. Homberg, and J. P. Leroux. Human cytochrome P-450 is recognized by anti-liver/kidney-microsome antibodies in autoimmune chronic hepatitis. *Biochem. Biophys. Res. Commun.* **159**:283–289 (1989).
30. Beaune, P., P. M. Dansette, D. Mansuy, L. Kiffel, M. Finick, C. Amar, J. P. Leroux, and J. C. Homberg. Human anti-endoplasmic reticulum auto-antibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P-450 that hydroxylates the drug. *Proc. Natl. Acad. Sci. USA* **84**:551–555 (1987).
31. Beaune, P. H., P. G. Kremers, L. S. Kaminski, J. de Graeve, A. Albert, and F. P. Guengerich. Comparison of monooxygenase activities and cytochrome P-450 isozyme concentrations in human liver. *Drug Metab. Dispos.* **14**:437–442 (1986).
32. Lopez Garcia, P., P. M. Dansette, P. Valadon, C. Amar, P. Beaune, F. P. Guengerich, and D. Mansuy. Human liver P450s expressed in yeast as tools for reactive metabolite formation studies: oxidative activation of tienilic acid by P450 2C9 and P450 2C10. *Eur. J. Biochem.*, **213**:223–232 (1993).
33. Guengerich, F. P., and C. G. Turvy. Comparison of levels of several human cytochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. *Mol. Pharmacol.* **256**:1189–1194 (1991).
34. Birgersson, C., E. T. Morgan, H. Jornvall, and C. Von Bahr. Purification of desmethylimipramine and debrisoquine hydroxylating cytochrome P-450 from human liver. *Biochem. Pharmacol.* **35**:3165–3166 (1986).
35. Guengerich, F. P., M. V. Martin, P. H. Beaune, P. Kremers, P. Wolff, and D. J. Waxman. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **261**:5051–5060 (1986).

Send reprint requests to: Dr. Antoinette Lemoine, Unite INSERM 75, CHU NECKER, 156 rue de Vaugirard, 75730 Paris, Cedex 15, France.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012