

EIGHT INHIBITORY MONOCLONAL ANTIBODIES DEFINE THE ROLE OF INDIVIDUAL P-450S IN HUMAN LIVER MICROSOMAL DIAZEPAM, 7-ETHOXYCOUMARIN, AND IMIPRAMINE METABOLISM

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ABSTRACT:

Eight inhibitory monoclonal antibodies (MAbs) individually specific to human cytochrome P-450 (P-450) 1A1, 1A2, 2A6, 2B6, 2C subfamily (2C8, 2C9, 2C18 and 2C19), 2D6, 2E1, and 3A4/5 were used to define the role of single P-450s in the metabolism of diazepam (DZ), 7-ethoxycoumarin (7-EC), and imipramine (IMI) in human liver microsomes (HLM). The MAbs were added combinatorially to six HLM samples. With DZ as a substrate, more than 80% of temazepam (TMZ) formation was inhibited in all six samples by the addition of MAb to 3A4/5, indicating an 80% contribution of 3A4/5 to TMZ formation. Nordiazepam formation was inhibited with MAbs to 2B6 (6–23%), 2C subfamily (12–61%) and 3A4/5 (14–45%). The MAbs to 1A1, 1A2, 2A6, 2D6, and 2E1 did not inhibit TMZ or nordiazepam formation; this indicates their noninvolvement in DZ metabolism. The MAb-defined P-450 contribution to 7-EC O-deethylation in six HLM samples was 17 to 60% for 2E1, 15 to 46% for 2A6, and 5 to 22% for 1A2, reflecting the role and variation of each P-450 in this activity. MAbs to 1A1, the 2C subfamily, 2D6, and 3A4/5 did not affect 7-EC metabolism in the HLM samples. IMI is

metabolized mainly to 2-hydroxyimipramine by expressed 2C19 and 2D6, and desipramine (DIM) by expressed 1A2, 2C18, 2C19 and 2D6. Expressed 1A1, 2C9, and 3A4 showed low activities for the formation of DIM. Of six HLM samples, five showed IMI hydroxylation activity (0.35–2.6 nmol/min/nmol P-450) while one (HL43) lacked hydroxylation activity. All six HLM samples showed N-deethylation activity (0.74–1.4 nmol/min/nmol P-450). The MAb-determined contribution of 2D6 and 2C19 to 2-hydroxyimipramine formation ranged from 47 to 90% and from 0 to 49%, respectively, while HL43 did not show 2-hydroxylation. The role of P-450s involved in DIM formation varied for 2C19 (13–50%), 1A2 (23–41%), and 3A4 (8–26%). These studies demonstrate a system for identifying the quantitative metabolic role of single P-450s and their interindividual variability in a tissue containing multiple P-450s. The system using inhibitory MAbs is simple, precise, and applicable to any P-450-mediated catalytic activity including that for drugs, carcinogens, mutagens, toxic chemicals and endobiotics.

Cytochromes P-450 (P-450)¹ are a superfamily of hemoproteins that are responsible for the oxidative, peroxidative, and reductive metabolism of numerous and diverse xenobiotics including drugs, carcinogens, and environmental chemicals, as well as endobiotics including steroids and fatty acids. Human P-450s are present in multiple forms, are heterogeneously distributed in tissues and individuals, and have different substrate and product specificities which are often overlapping (Gonzalez, 1988; Nelson et al., 1996). The metabolism of a substrate in a tissue is a result of the inherent activities of relevant P-450s, the presence of multiple forms of individual P-450, and the specific cellular content of each P-450. The phenotype of an individual with respect to the forms of individual

P-450s and their amounts in human liver and other tissues determines the metabolic rate and pathway of xenobiotic disposition (Wrighton and Stevens, 1992). The interaction and activity of individual P-450s in the metabolism of clinically useful drugs may be crucial factors in defining drug efficacy, drug-drug interaction, and drug toxicity. The immunoblotting antibodies can be used to measure the content of specific P-450 forms in tissues and the substrate specificity of individual P-450s can be measured with cDNA expressed P-450s (Gonzalez et al., 1991b; Guengerich, 1995). These measurements, however, do not determine the contribution of each P-450 to the metabolism or activation of a specific P-450 substrate in a tissue containing multiple P-450s.

Monoclonal antibodies (MAbs) are derived from hybridomas with unlimited growth properties and are of excellent stability. The MAbs are directed to epitopes on the antigen with very high specificity, which is generally greater than the specificity of either polyclonal antibodies or chemical inhibitors. A MAb binding that is specifically and highly inhibitory to a single P-450 can be used to quantify the catalytic role of the P-450 in the metabolism of a substrate in a tissue containing multiple P-450 forms (Gelboin, 1993). The extent of inhibition affected by the MAb measures the contribution of the single target P-450 to the metabolic reaction studied. We have produced

¹ Abbreviations used are: MAb, monoclonal antibody; P-450, cytochrome P-450; DZ, diazepam; 7-EC, 7-ethoxycoumarin; IMI, imipramine; HLM, human liver microsomes; DIM, desipramine; TMZ, temazepam; NDZ, nordiazepam; 2-OH IMI, 2-hydroxyimipramine; HPLC, high-performance liquid chromatography; IB, immunoblot.

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TABLE 1
Specific inhibitory MAbs to human P-450s used in this study

Antihuman P-450*	MAB (ref.)	Ig Subtype	Enzyme-Linked Immunosorbent Assay	I B	% Inhibition	Substrate
1A1	1-7-1	IgG1	+	-	>90	7-ECO, phenanthrene, chlorzoxazone
1A2	26-7-5	IgG1	+	+	>90	7-EC, phenanthrene, IMI, chlorzoxazone
2A6	151-45-4	IgG1	+	+	>90	Coumarin, 4-nitrophenol, phenanthrene, 7-ECO
2B6	49-10-20	IgG2b	+	+	>90	Testosterone, 7-EC, DZ, phenanthrene
2C8/9/18/19	1-68-11	IgM	+	-	>90 (2C9/18/19) >85 (2C8)	DZ, phenanthrene
2D6	512-1-8	IgG1	+	+	>90	Bufuralol, dextromethorphan, IMI
2E1	1-73-18	IgM	+	-	>90	Phenanthrene, <i>p</i> -nitroanisole, toluene, chlorzoxazone, 4-methylanisole
3A4/5	3-29-9	IgM	+	-	>90 (3A4) >60-80 (3A5)	DZ, testosterone, Taxol, cyclosporin, phenanthrene

All MAbs are specific to target P-450s as tested with human P450 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5.

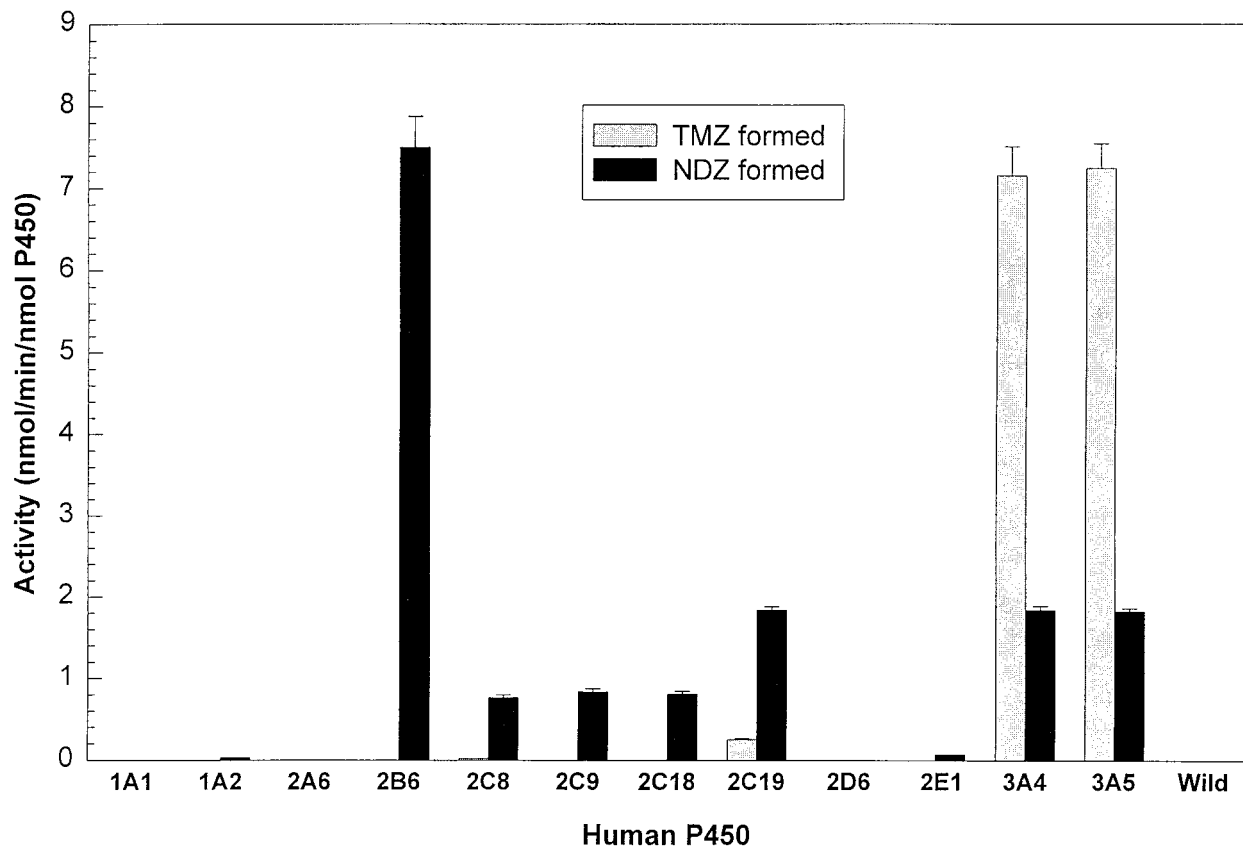


FIG. 1. Metabolism of DZ by cDNA-expressed human P450s.

DZ (200 μM) was added to phosphate buffer (50 mM, pH 7.4) containing 25 pmol of expressed P450. After preincubation at 37°C for 5 min, the reaction was initiated by the addition of NADPH (1 mM) and incubated at 37°C for 20 min. P450 1A2, 2B6, 2C8, 2C9, 2E1, 3A4, and 3A5 were expressed in HepG2 cells by recombinant vaccinia virus, and 1A1, 2A6, 2C18, 2C19, and 2D6 were expressed in Sf9 cells by recombinant baculovirus. Wild indicates the control microsomes from wild-type vaccinia virus infected HepG2 or baculovirus infected Sf9 cells. Data are the means ± S.D. of three incubations.

specific inhibitory MAbs to a number of human P-450s, viz., 1A1 (Park et al., 1982; Yang et al., 1998b), 1A2 (Yang et al., 1998b), 2A6 (Sai et al., 1998), 2B6 (Yang et al., 1998a), 2C subfamily (Gelboin et al., 1997; Park et al., 1989), 2D6 (Gelboin et al., 1997; Krausz et al., 1997), 2E1 (Gelboin et al., 1996), and 3A4/5 (Gelboin et al., 1995). The MAb to the 2C subfamily inhibits the catalytic activities of each of P-450 2C8, 2C9, 2C18, and 2C19. The MAbs used in this study are all inhibitory to at least the 90% level. Thus, these MAbs are central components of the developed system that quantitatively determines the contribution of individual P-450s to drug or carcinogen metabolism in human liver and other tissues. In this study, we have used a combinatorial MAb system to define the quantitative role of each P-450 in the metabolism of diazepam (DZ), 7-ethoxycoumarin (7-EC), and imipramine (IMI) in human liver microsomes.

Materials and Methods

Chemicals. DZ was purchased from USP Convention, Inc. (Rockville, MD). TMZ, nordiazepam (NDZ), and 2-oxoquanzepam were kindly supplied by Dr. Shen K. Yang (Uniformed Services University of the Health Sciences, Bethesda, MD). 7-EC and 7-hydroxycoumarin were purchased from Sigma Chemical Co. (St. Louis, MO). IMI and desipramine (DIM) were purchased from ICN Biomedicals Inc. (Aurora, OH). 2-Hydroxyimipramine (2-OH IMI) was obtained from Novartis Pharma AG (Basel, Switzerland). NADPH was purchased from Boehringer Mannheim (Indianapolis, IN). All reagents were of analytical grade.

Human Liver Microsome (HLM) and cDNA-expressed Human P-450s. Human liver specimens were obtained from organ donors after clinical death (The NCI Cooperative Human Tissue Network, National Institutes of Health, Bethesda, MD) and stored at -80°C until use. Microsomes were prepared as described (Alvares et al., 1970) and microsomal protein (Lowry et al., 1951)

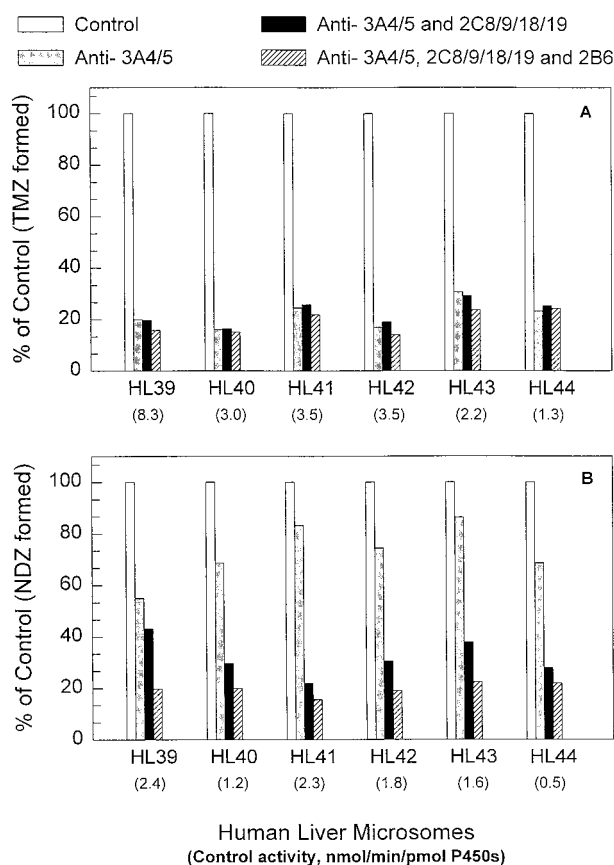


FIG. 2. MAb analysis of individual P450 catalyzed DZ metabolism in HLMs.

A, MAb inhibition of HLM-catalyzed DZ C3-hydroxylation. B, MAb inhibition of HLM-catalyzed DZ N1-demethylation. MAbs were added as indicated to 150 pmol of HLM P450 and preincubated for 5 min at 37°C. The reaction was initiated by adding DZ (200 μ M) and NADPH (1 mM) and terminated by 1 ml of acetone after incubation for 30 min at 37°C. OQZ was used as internal standard. HPLC analysis was the same as described previously (Yang et al., 1998c). Data are the means of duplicate incubations. HyHEL, a MAb to lysozyme, was used as control.

and P-450 content (Omura and Sato, 1964) were measured according to published methods.

Recombinant human P-450 1A2, 2B6, 2C8, 2C9, 2E1, 3A4, and 3A5 were expressed in HepG2 cells using vaccinia virus (Gonzalez et al., 1991a; Yang et al., 1998c). Baculovirus-expressed human P-450 1A1, 1A2, 2A6, 2D6, 2C18, and 2C19 with NADPH P-450 oxidoreductase coexpressed were obtained from Gentest Corporation (Woburn, MA).

Inhibition of P-450s Using MAbs. A typical assay contained 25 pmol of expressed P-450 or 150 to 250 pmol of human liver microsomal P-450s with ascites containing indicated MAbs (about 250 μ g of MAb protein, Table 1) in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4). The mixture was preincubated for 5 min at 37°C and then diluted with the buffer to a 1 ml final volume. Substrates (DZ, 7-EC, and IMI) dissolved in 10 μ l of methanol were added (final concentration 200 μ M), and the reaction was initiated by the addition of NADPH (1 mM) at 37°C. Antilysozyme MAb (HyHEL) with an amount equivalent to MAbs was used as a control for nonspecific binding. Reactions were incubated for 30 min and terminated with 1 ml of acetone. 2-Oxoquanzepam was used as internal standard for DZ metabolism, benzo(a)pyrene *cis*-4,5-diol as internal standard for the metabolism of 7-EC and temazepam (TMZ) as internal standard for IMI metabolism. Samples were extracted twice with 7 ml of dichloromethane and were dried under N₂. The residue was dissolved in mobile phase and immediately analyzed by reversed phased high-pressure liquid chromatography (HPLC). The metabolites formed were identified by comparing their retention times with authentic standards.

HPLC. HPLC was performed using a Hewlett-Packard (HP) model HP1050 liquid chromatography system equipped with a HP model 1050 autosampler, a ternary solvent delivery system, and a diarray detector or multiple wave-

length, which are all controlled by HP ChemStation software (Revision A0.05.01) installed in a Compaq Deskpro 5133 personal computer. HPLC analysis of metabolism of DZ (Yang et al., 1998c) and 7-EC (Yang et al., 1998b) were the same as previously described. The metabolites of IMI were separated according to Nielsen and Brosen (1993) and modified with a Luna C₈ column (4.6 x 250 mm; Phenomenex, Torrance, CA). The mobile phase for separation of IMI and its metabolite were methanol/acetonitrile/20 mM KPi (10:40:50 v/v/v, pH 6.5) to methanol/acetonitrile/20 mM KPi (10:55:35 v/v/v, pH 6.5) by a linear gradient in 25 min. IMI and its metabolites were detected at 250 nm. The metabolites formed were identified by comparing their retention times with authentic standards.

Results

MAb Based Analysis of Individual P-450 Contribution to DZ Metabolism in HLM. We have previously developed a panel of inhibitory MAbs specific to single human P-450 1A1, 1A2, 2A6, 2B6, 2C subfamily, 2D6, 2E1, and 3A4/5 which are referenced in Table 1. These MAbs are highly specific and strongly inhibitory, by more than 85 to 95%, to the target P-450 activity. No cross-reaction with other P-450s by enzyme-linked immunosorbent assay, immunoblot (IB), and inhibition study was observed. A large amount of MAbs was produced from ascites containing 2.5 to 5 mg/ml MAb.

DZ is used worldwide as an anxiolytic/hypnotic drug. DZ is metabolized by C3-hydroxylation to TMZ and by N1-demethylation to NDZ by human P-450s (Yang et al., 1998c). Figure 1 shows the enzyme activities of 12 cDNA-expressed human P-450s in the conversion of DZ to TMZ and NDZ. The P-450 2B6 exhibited the highest enzyme activity for N1-demethylation with a specific activity of 7.5 nmol/min/nmol P-450 using 25 pmol of 2B6/ml incubation mixture. P-450 2C subfamily, 3A4, and 3A5 also exhibited significant N1-demethylation activity. C3-hydroxylation was catalyzed by P-450 3A4 and 3A5, 2C19 exhibited low activity, and no activity was exhibited by the other P-450s examined. The C3-hydroxylation of 3A4/5 was greater than its N1-demethylation activity. However, the individual P-450 activity of DZ metabolism shown in Fig. 1 does not reflect the contribution of each P-450 in human liver. Inhibitory MAbs to P-450s were used to determine the contribution of individual P-450s to TMZ and NDZ formation in human tissues. Six HLM samples showed enzyme activities for C3-hydroxylation ranging from 1.35 to 8.29 nmol/min/nmol P-450 and N1-demethylation from 0.50 to 2.38 (nmol/min/nmol P-450). With the addition of multiple inhibitory MAbs to HLM (Fig. 2A), the formation of TMZ was inhibited by more than 80% by anti-3A4/5 MAb (MAb 3-29-9), whereas the addition of MAb to the 2C subfamily (MAb 1-68-11) did not produce any added inhibition indicating that 2C19 is not significantly involved in C3-hydroxylation. Figure 2B shows the additive effects of inhibitory MAbs on the DZ N1-demethylation of HLM. The addition of each antibody inhibited the NDZ formation to a different extent; this defined the role of each P-450 in DZ-demethylation in the different liver microsome samples. In HL39, 3A4/5 contributed 45% of demethylation activity, 2C subfamily 12%, and 2B6 23%. In HL41, the contribution of 3A4/5 was 17%, 61% for 2C subfamily, and 6% for 2B6. Among the different samples, the range of contribution of 3A4/5 was 14 to 45%, the 2C subfamily 12 to 61%, and 2B6 6 to 23%. No inhibition was observed with the addition of MAbs to 1A1, 1A2, 2A6, 2D6, and 2E1. This indicates that the latter P-450s do not significantly contribute to DZ demethylation. The variation of inhibition between different samples is likely the result of individual variability among liver samples.

MAb Determination of Individual P-450 Contribution to 7-EC Metabolism in HLM. 7-EC *O*-deethylation has been widely used for characterizing P-450 activity in liver microsomes of various animal species including humans. In humans, the major catalysts for 7-EC

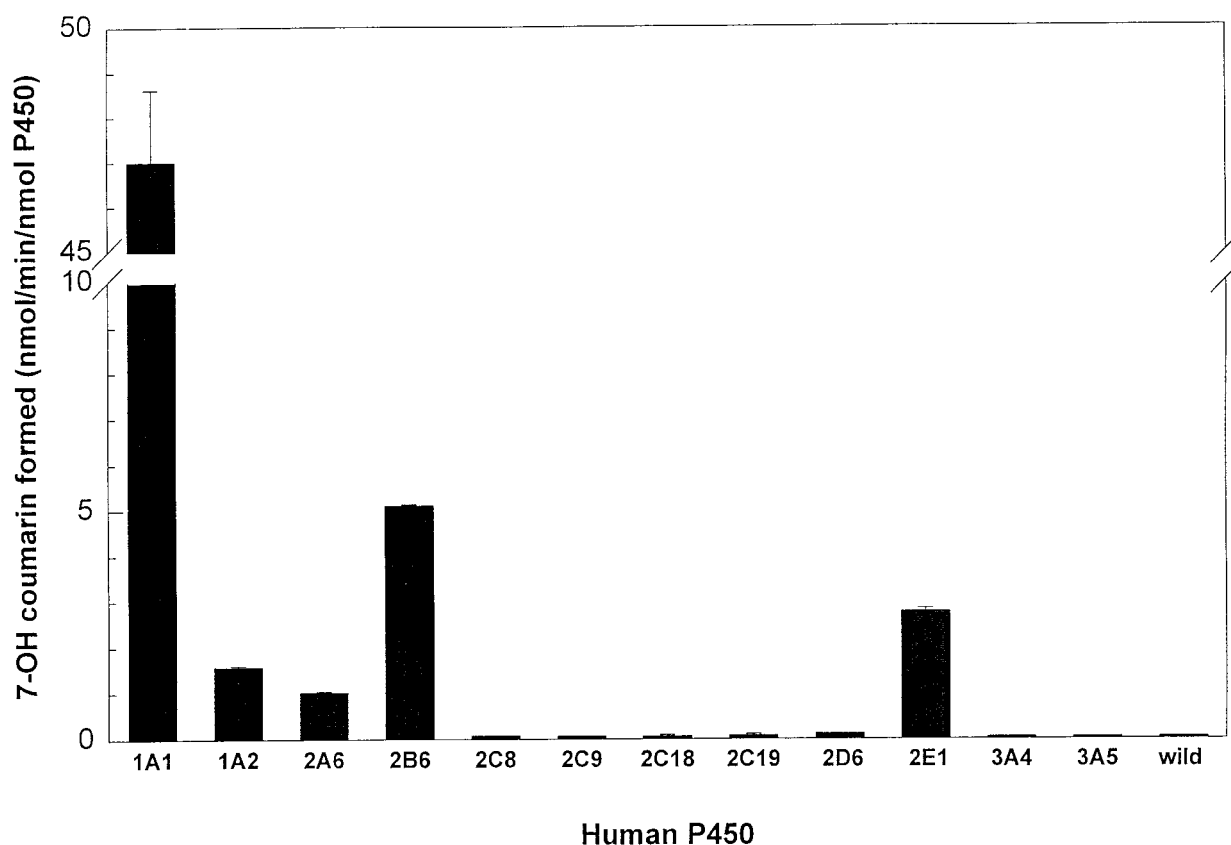


Fig. 3. 7-EC *O*-deethylation by cDNA-expressed human P450s.

7-EC (200 μ M) was added to phosphate buffer (50 mM, pH 7.4) containing 25 pmol of expressed P-450. After preincubation at 37°C for 5 min, the reaction was initiated by the addition of NADPH (1 mM) and incubated at 37°C for 20 min. P450 1A2, 2B6, 2C8, 2C9, 2E1, 3A4, and 3A5 were expressed in HepG2 with vaccinia virus, and 1A1, 2A6, 2C18, 2C19, and 2D6 were expressed in Sf9 cells with baculovirus. Wild indicates the control microsomes from wild-type vaccinia virus infected HepG2 or baculovirus-infected Sf9 cells. Data are the means \pm S.D. of three incubations. HPLC analysis are the same as described previously (Yang et al., 1998b).

O-deethylation are 1A2 and 2E1 (Bayliss et al., 1994; Yamazaki et al., 1996). In this study, we examined the enzyme activity of 12 cDNA expressed human P-450 for 7-EC *O*-deethylation (Fig. 3). Among the P-450 enzymes examined, P-450 1A1 had the highest activity for 7-EC *O*-deethylation, followed by 2B6, 2E1, 1A2, and 2A6. The P-450s 2C subfamily, 2D6, 3A4, and 3A5 exhibited only negligible activity, which was comparable to control values (HepG2 cells infected with wild-type vaccinia virus or Sf9 cells infected with wild-type baculovirus).

Six samples of normal HLMs were used for combinatorial analysis of MAb inhibition of HLM catalyzed 7-EC *O*-deethylation (Fig. 4). The total activity of 7-deethylation varied from 1.34 to 2.51 nmol/min/nmol P-450s in six HLM samples. The activity of individual samples is demonstrated in Fig. 4. With HL40, the addition of anti-2E1 MAb (MAb 1-73-18) inhibited the formation of 7-OH coumarin by 60%, indicating that 60% of the metabolic activity was the result of 2E1. Upon the simultaneous addition of anti-2A6 MAb (MAb151[hyphen45-4]), an additional 15% inhibition of 7-OH EC formation was observed, demonstrating a 15% contribution of 2A6 to the reaction. Simultaneous addition of anti-2B6 and anti-1A2 MAbs showed only a 6% contribution from 2B6 and a 7% contribution from 1A2. In HL44, the contribution of the individual P-450s to 7-OH coumarin formation was 17% for 2E1, 46% for 2A6, 0% for 2B6, and 21.5% for 1A2. The range of contribution among the different HLM samples was 17 to 60% for 2E1, 15 to 46% for 2A6, 0 to 8% for 2B6, and 5 to 22% for 1A2. The addition of MAbs to 1A1, the 2C

subfamily, 2D6, and 3A4/5 to HLM had no effect, indicating that these P-450s do not contribute significantly to 7-EC *O*-deethylation.

An experiment shown in Table 2 compares the inhibition of 7-EC deethylation in HLM by both a single MAb and in the presence of other MAbs. The average difference was 4.4% with a S.D. of 2.2%, indicating that the inhibitory activity of a single MAb is the same regardless whether added singly or with other MAbs. In these additional four HLM samples, the 7-EC-deethylation activity ranged from 1.6 to 4.4 nmol/min/nmol P-450s. The contribution of each P-450 to 7-EC metabolism is shown in Table 2. Together with Fig. 4, the range of activity contribution among the 10 HLM samples was 16 to 60% for 2E1, 11 to 46% for 2A6, 0 to 48% for 2B6, and 5 to 22% for 1A2.

Combinatorial Analysis of IMI Metabolism in HLM Using MAbs. IMI is a tricyclic antidepressant which is widely used in the treatment of depression. The metabolic fate of IMI involves mainly *N*-demethylation to the active metabolite DIM and aromatic hydroxylation to 2-OH IMI. It was reported that P-450 1A2, 2C19, 2D6, and 3A4 catalyze IMI metabolism (Ball et al., 1997; Madsen et al., 1997). Among 12 cDNA-expressed human P-450s, 2C19, 2C18, 2D6, and 1A2 were found to exhibit strong *N*-demethylation activity, and 1A1, 2C9, and 3A4 exhibited low activity (Fig. 5). P-450 2D6 and 2C19 showed high activity for 2-hydroxylation, and 1A1 and 2C18 exhibited low activity. Six HLM samples were used to determine the individual P-450 contribution to IMI metabolism with inhibitory MAbs (Fig. 6). HL39-catalyzed 2-hydroxylation of IMI was inhibited by more than 90% with anti-2D6 antibody, indicating that 2D6 is responsible for more than 90% of 2-hydroxylation in this liver. How-

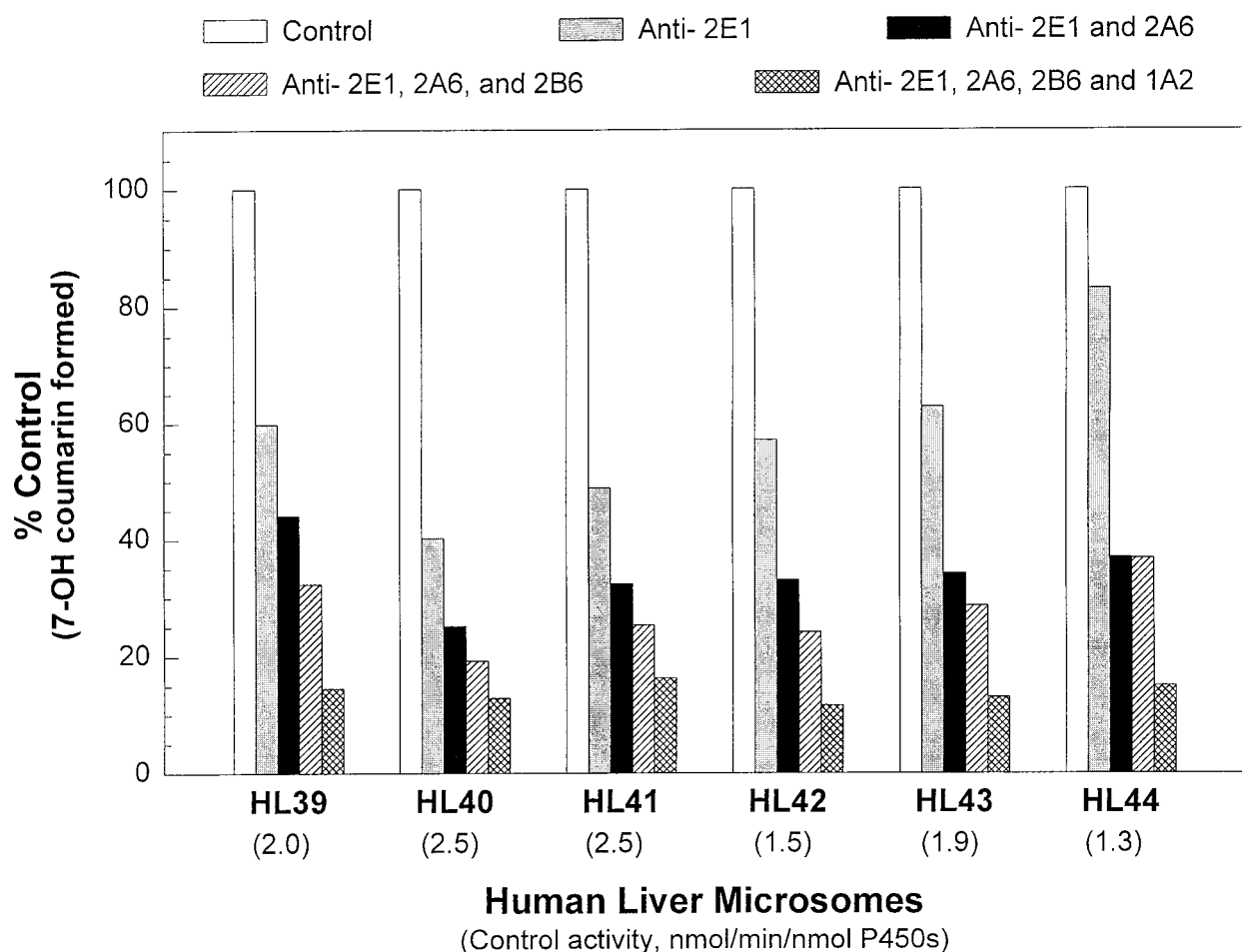


FIG. 4. MAb analysis of individual P-450 catalyzed 7-EC O-deethylation of HLMs.

MABs were added as indicated to 150 pmol of HLM P-450 and preincubated for 5 min at 37°C. The incubation is described in *Materials and Methods* and HPLC analysis is as the same as previous reported (Yang et al., 1998b). Data are the means of duplicate incubations. HyHEL, a MAB to lysozyme, was used as control.

ever, in HL40, anti-2D6 and anti-2C subfamily MABs inhibited 2-hydroxylation of IMI by 47% and 38%, respectively. This demonstrates that both 2D6 and 2C18/19 are major enzymes for the formation of 2-OH IMI in HL40. The cDNA-expressed P-450 2C8 and 2C9 did not show IMI hydroxylation activity (Fig. 5), and thus the MAB 1-68-11 inhibition of IMI hydroxylation in HL40 indicated the contribution of P-450 2C18/19. Furthermore, HL43 did not exhibit 2-hydroxylation activity, indicating a very low content of both 2D6 and 2C19. We further examined the 2D6 content of HL43 with IB analysis with MAB 512-1-8 (anti-2D6) which indicated an absence of 2D6 protein (unpublished data). The results with the human liver specimens showed that the contribution of 2D6 to the hydroxylation ranged widely from 0 to 90%. The 0% reflects an absent 2D6 in the HL43 liver. The range of 2C18/19 to 2-hydroxylation also greatly varied, from 0 to 49%. None of the P-450s 1A2, 2A6, 2B6, 2C8/9, 2E1, and 3A4/5 showed a significant contribution to 2-hydroxylation in six HLM samples (Fig. 6A). Figure 6B shows the contribution of individual P-450s to DIM formation in the six human liver samples. The ranges of contribution of 2C18/19, 1A2, and 3A4 were 13 to 50%, 23 to 41%, and 8 to 26%, respectively. Thus, the major enzymes involved in IMI demethylation were 2C18/19, 1A2, and 3A4. P-450 2C18 content is very low in human liver; therefore, the contribution of 2C18/19 to IMI metabolism is mainly from 2C19. Although the expressed 2D6 exhibited both 2-hydroxylation and demethylation activities, P-450 2D6

TABLE 2

Inhibition of 7-ECO metabolism in HLM by the addition of a single MAB compared to the its inhibitory effects when in combination with other MABs

HLM (activity ^a)	Single MAB		Combinatorial Addition of MABs	
	MAB to P-450	% Inhibition	MAB to P-450	% Inhibition
HL75 (1.6)	Control	0	Control	0
	2E1	26	2E1	26
	2A6	28	2E1 + 2A6	32
	2B6	21	2E1 + 2A6 + 2B6	22
	1A2	22	2E1 + 2A6 + 2B6 + 1A2	18
HL76 (4.6)	Control	0	Control	0
	2E1	16	2E1	16
	2A6	22	2E1 + 2A6	26
	2B6	50	2E1 + 2A6 + 2B6	43
	1A2	14	2E1 + 2A6 + 2B6 + 1A2	11
HL77 (3.1)	Control	0	Control	0
	2E1	57	2E1	57
	2A6	7	2E1 + 2A6	11
	2B6	20	2E1 + 2A6 + 2B6	11
	1A2	10	2E1 + 2A6 + 2B6 + 1A2	8
HL80 (4.4)	Control	0	Control	0
	2E1	22	2E1	22
	2A6	15	2E1 + 2A6	8
	2B6	43	2E1 + 2A6 + 2B6	48
	1A2	10	2E1 + 2A6 + 2B6 + 1A2	7

^a Control activity is presented in nmol 7-OH coumarin formed/min/nmol P-450. Data are means of duplicated incubations.

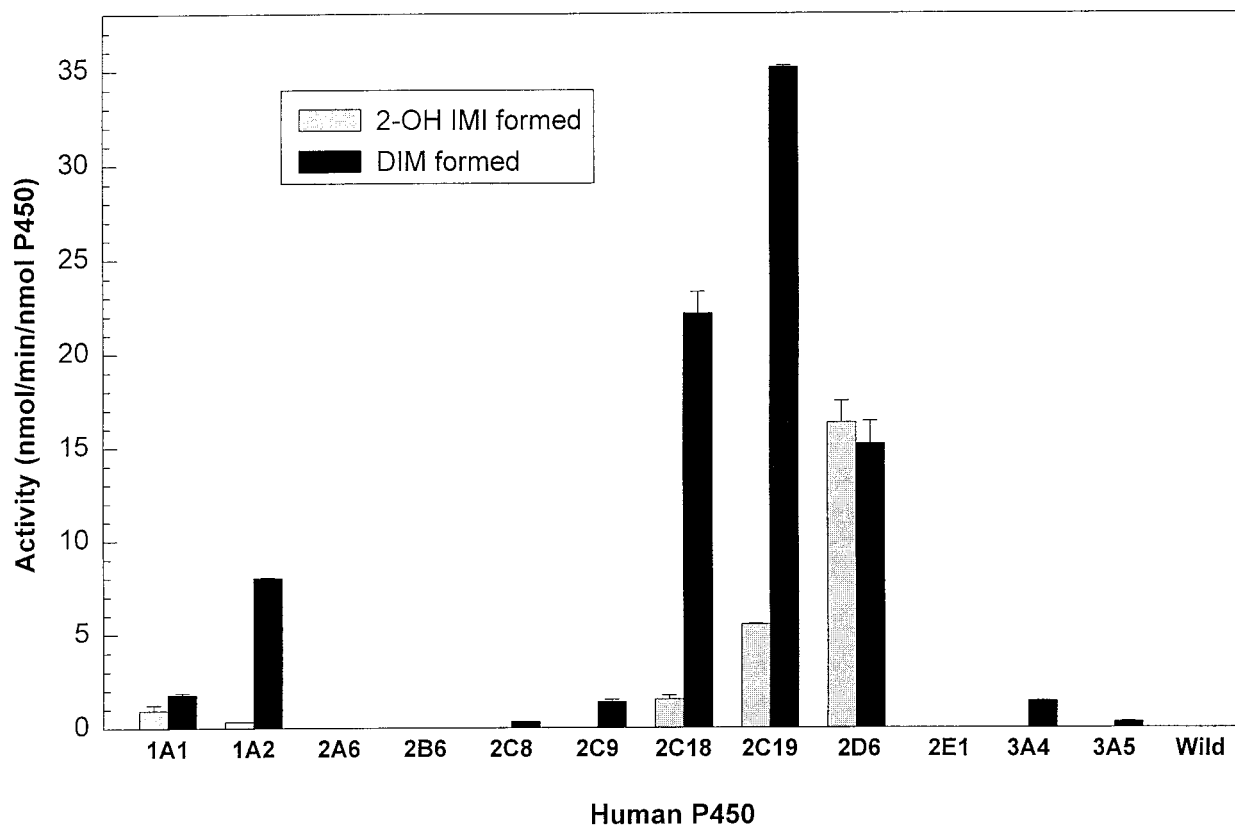


Fig. 5. Metabolism of IMI by cDNA-expressed human P-450s.

IMI (200 μ M) was added to phosphate buffer (50 mM, pH 7.4) containing 25 pmol of expressed P-450. After preincubation at 37°C for 5 min, the reaction was initiated by the addition of NADPH (1 mM) and incubated at 37°C for 20 min. P450 1A2, 2B6, 2C8, 2C9, 2E1, 3A4, and 3A5 were expressed in HepG2 with vaccinia virus, and 1A1, 2A6, 2C18, 2C19, and 2D6 were expressed in Sf9 cells infected with recombinant baculovirus. Wild indicates the control microsomes from wild-type vaccinia virus-infected HepG2 or baculovirus infected Sf9 cell. Data are the means \pm S.D. of three incubations.

did not show a significant contribution to DIM formation in the HLM samples.

Discussion

The multiplicity and heterogeneity of P-450s in human tissues prevent the identification and quantitative analysis of the role of individual P-450s in the metabolism of the numerous P-450 substrates. With a combinatorial method using a panel of eight inhibitory MAb specific to their target P-450s, the current study describes a system using inhibitory MAb to define the role of individual P-450s in the metabolism of DZ, 7-EC, and IMI in human liver.

DZ is *N*-demethylated to NDZ and C3-hydroxylated to TMZ. Many studies in vivo and in vitro have demonstrated that the P-450 2B6 and 2C subfamily are involved in NDZ formation and that 3A4/5 catalyzes TMZ formation (Yasumori et al., 1993; Ono et al., 1996; Yang et al., 1998c). However, cDNA-expressed P-450 activity does not reflect the quantitative role of the individual P-450 in human liver. With the addition of single or multiple MAb to HLM, the role of individual P-450s in different liver samples was defined. P-450 3A4/5 was responsible mainly for the formation of TMZ; none of the other P-450s showed a significant contribution. Therefore, TMZ formation from DZ might be considered a specific reaction for P-450 3A4/5. The range of each P-450 for NDZ formation in six human liver samples was 14 to 45% for 3A4/5, 12 to 61% for the 2C subfamily, and 6 to 23% for 2B6. When the MAb to 3A4/5, 2C subfamily, and 2B6 were all combined, the inhibition of NDZ formation was more than 80%. This indicates that these P-450s are responsible for 80% of DZ *N*-demethylation activity in human liver. Thus, MAb are simple and

precise reagents for the quantitative analysis of an individual P-450 role for drug metabolism in human liver. The percentage of inhibition of each individual P-450 activity in a liver reflects the P-450 contribution to a substrate metabolism in that liver. The variability of percentage inhibition by a single MAb to its target P-450 in different samples is due to the fact that different human samples contain a different and heterogeneous complement of P-450s, some of which may compete with the target P-450 for substrate.

The *O*-deethylation of 7-EC has been widely used as a marker activity for assessing substrate specificities of P-450s. P-450 2E1 and 1A2 have been reported to be principal enzymes involved in 7-EC *O*-deethylation in human liver (Yamazaki et al., 1996). In this study, we examined the roles of individual forms of human P-450s for 7-EC deethylation. Of the 12 cDNA expressed human P-450s, 1A1, 1A2, 2A6, 2B6, 2C19, and 2E1 showed a high *O*-deethylation activity with the rank of 1A1 >> 2B6 > 2E1 > 1A2 > 2A6. In the HLM samples examined, the MAb defined contribution of the individual P-450s to 7-EC *O*-deethylation was 16 to 60% for 2E1, 11 to 46% for 2A6, 0 to 48% for 2B6, and 5 to 22% for 1A2. The total inhibition of HLM by the MAb to the four P-450s was more than 85%, indicating that 2E1, 2A6, 2B6, and 1A2 are responsible for essentially all of *O*-deethylation in human liver. Although 1A1 exhibits the highest *O*-deethylation activity, its content in normal human liver is very low or absent (Guengerich, 1995); thus, it is not a factor in normal HLM metabolism.

IMI metabolism was examined with 12 expressed human P-450s. The activities of the P-450s involved in DIM formation are ranked as 2C19 > 2C18 > 2D6 > 1A2 > 3A4 and 2C9. In HLM samples, DIM

TABLE 3
MAB determined contribution (%) of individual P-450s to the metabolism of seven substrates in HLM

Substrate	Metabolite	HLM Activity*	1A2	2A6	2B6	2C8/9/18/19	2D6	2E1	3A4/5
Bufuralol	1'-OH bufuralol	0.007–0.012	2–25	— ^a	—	24–69	14–76	—	—
Dextromethorphan	Dextrophan	0.058–0.86	—	—	—	nd ^b	50–93	—	—
Phenacetin	Acetaminophen	0.44–2.5	64–84	0–8.8	—	4.6–20 (2C19)	—	—	—
Coumarin	7-OH coumarin	0.29–3.5	—	>90	—	—	—	—	—
7-ECO	7-OH coumarin	1.3–4.4	5–22	11–46	0–48	—	—	16–60	—
DZ	TMZ	1.3–8.3	—	—	—	—	—	—	>80
	NDZ	0.5–2.4	—	—	6–23	12–61	—	—	14–45
IMI	2-OH IMI	0.35–2.6	—	—	—	0–49 (2C18/19)	47–90	—	—
	DIM	0.74–1.4	23–41	—	—	13–50	—	—	8–26

Control HLM activity values are presented in nmol product/min/nmol P-450s.

^a —, contribution less than 4% or not detectable by respective P-450s.

^b nd, not determined; cDNA-expressed 2C8/9/18/19 showed enzyme activity (unpublished data).

formation was mainly catalyzed by 1A2 (23–42%), 2C subfamily (13–50%), and 3A4 (8–26%). P-450 3A4 is the most abundant P-450 in human liver, consisting of about 29% of the total P-450 (Vermeulen, 1996). Thus, 3A4 showed a significant contribution to DIM formation in human liver although expressed 3A4 exhibited a relatively low *N*-demethylation activity. In contrast, 2D6 did not show a contribution to DIM formation in human liver although the expressed 2D6 showed a high *N*-demethylation activity. The percentage of contribution of an individual P-450 to the metabolism of a substrate in

human liver/tissue containing multiple P-450s is the consequence of both the P-450 activity and the other P-450s involved in the metabolism. The P-450 activity is a reflection of both its protein content and the intrinsic activity of the P-450. In HLM, 2D6 is of the relatively low content (about 1.5% of total P-450) and the high content of 1A2 (12.7%), 2C subfamily (18.2%), and 3A4 (28.8%) (Guengerich, 1995; Vermeulen, 1996). The P-450s catalyzing IMI 2-hydroxylation were examined. Their activity is ranked as 2D6 > 2C19 > 2C18. Of the six human liver samples examined, HL43 exhibited *N*-demethylation activity, but did not show detectable 2-hydroxylation activity. This is due to the absence of 2D6 in this liver as determined by IB. In HL39, HL42, and HL44, about 80 to 90% 2-OH IMI formation is a function of 2D6. However, the formation of 2-OH IMI in HL40 and HL41 was contributed by both 2D6 (47% and 43%, respectively) and 2C18/19 (38% and 49%, respectively). The result demonstrates the usefulness of the MABs for defining the individual P-450 contribution to substrate metabolism in an individual tissue such as liver.

Previously, we reported the isolation of MABs to human P-450s 3A4 (Gelboin et al., 1995), 2E1 (Gelboin et al., 1996), 2D6 (Gelboin et al., 1997; Krausz et al., 1997), 2C subfamily (Park et al., 1989; Gelboin et al., 1997), 2B6 (Yang et al., 1998a), 2A6 (Sai et al., 1998), 1A2 (Yang et al., 1998b), and 1A1 (Park et al., 1982; Yang et al., 1998b). With these MABs, we have defined the individual P-450 function in the metabolism of bufuralol, dextromethorphan, phenacetin, and coumarin. Here, MABs we used defined the role of each P-450 in the metabolism of bufuralol, dextromethorphan, phenacetin, and coumarin. Table 3 shows a summary of the MAB-defined quantitative roles of individual P-450s responsible for substrate metabolism in human liver. A complete complement of inhibitory MABs to the major human P-450s would permit the construction of a map of P-450-based metabolism. Another aspect of drug metabolism that can be analyzed with the inhibitory MAB is the metabolic relationship between drugs competitive for the same P-450. The contribution to the metabolism of a single drug or multiple drugs by individual P-450s is important to the understanding the regulation of the rates of drug metabolism, pharmacologic character, and toxicity. In turn, this information can lead to improved control of drug choice, dosage, and efficacy. The system using inhibitory MABs is both simple and precise and applicable to any P-450 mediated catalytic activity which includes that of drugs, carcinogens, mutagens, toxic chemicals, and endobiotics.

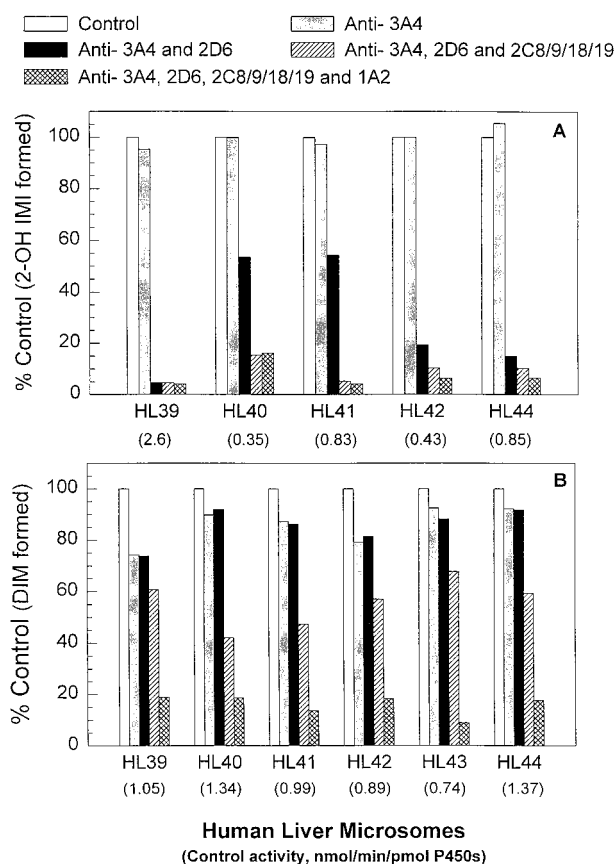


Fig. 6. Analysis of individual P-450 contribution to IMI metabolism of HLMs.

A, MAB inhibition of HLM-catalyzed IMI 2-hydroxylation. B, MAB inhibition of HLM-catalyzed IMI *N*-demethylation. MABs were added as indicated to 200 pmol of HLM P450 and preincubated for 5 min at 37°C. The reaction was initiated by adding IMI (200 μM) and NADPH (1 mM) and terminated by 1 ml of acetone after incubation for 30 min at 37°C. TMZ was used as internal standard. HPLC analysis was described in *Materials and Methods*. Data are the means of duplicate incubations. HyHEL, a MAB to lysozyme, was used as control.

References

- Alvares AV, Schilling G, Garbut A and Kuntzman R (1970) Studies on the hydroxylation of 3,4-benzpyrene by hepatic microsomes: Effect of albumin on the rate of hydroxylation of 3,4-benzpyrene. *Biochem Pharmacol* **19**:1449–1455.
- Ball SE, Ahern D, Scatina J and Kao J (1997) Venlafaxine: In vitro inhibition of CYP2D6 dependent imipramine and desipramine metabolism; comparative studies with selected SSRIs,

- and effects on human hepatic CYP3A4, CYP2C9 and CYP1A2. *Br J Clin Pharmacol* **43**:619–626.
- Bayliss MK, Bell JA, Wilson K and Park GR (1994) 7-Ethoxycoumarin O-deethylase kinetics in isolated rat, dog and human hepatocyte suspensions. *Xenobiotica* **24**:231–241.
- Gelboin HV (1993) Cytochrome P450 and monoclonal antibodies. *Pharmacol Rev* **45**:413–453.
- Gelboin HV, Goldfarb I, Krausz KW, Grogan J, Korzekwa KR, Gonzalez FJ and Shou M (1996) Inhibitory and noninhibitory monoclonal antibodies to human cytochrome P450 2E1. *Chem Res Toxicol* **9**:1023–1030.
- Gelboin HV, Krausz KW, Goldfarb I, Buters JT, Yang SK, Gonzalez FJ, Korzekwa KR and Shou M (1995) Inhibitory and non-inhibitory monoclonal antibodies to human cytochrome P450 3A3/4. *Biochem Pharmacol* **50**:1841–1850.
- Gelboin HV, Krausz KW, Shou M, Gonzalez FJ and Yang TJ (1997) Inhibitory monoclonal antibodies define the role of cytochrome P450 in human tissue: Polymorphically expressed P450 2D6 as paradigm. *Pharmacogenetics* **7**:467–477.
- Gonzalez FJ (1988) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**:243–288.
- Gonzalez FJ, Aoyama T, and Gelboin HV (1991a) Expression of mammalian cytochrome P450 using vaccinia virus. *Methods Enzymol* **206**:85–92.
- Gonzalez FJ, Crespi CL and V, GH (1991b) cDNA-expressed human cytochrome P450s: A new age of molecular toxicology and human risk assessment. *Mutat Res* **247**:113–127.
- Guengerich FP (1995) Human cytochrome P450 enzymes. In *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed) pp 473–535, Plenum Press, New York.
- Krausz KW, Yang TJ, Gonzalez FJ, Shou M and Gelboin HV (1997) Inhibitory monoclonal antibodies to human cytochrome P450 2D6. *Biochem Pharmacol* **54**:15–17.
- Lowry OH, Rosebrough NJ, Farr AL and Randall R (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275.
- Madsen H, Rasmussen BB and Brosten K (1997) Imipramine demethylation in vivo: Impact of CYP1A2, CYP2C19, and CYP3A4. *Clin Pharmacol Ther* **61**:319–324.
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW (1996) P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**:1–42.
- Nielsen KK and Brosten K (1993) High-performance liquid chromatography of imipramine and six metabolites in human plasma and urine. *J Chromatogr* **612**:87–94.
- Omura T and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**:2370–2378.
- Ono S, Hatanaka T, Miyazawa S, Tsutsui M, Aoyama T, Gonzalez FJ and Satoh T (1996) Human liver microsomal diazepam metabolism using cDNA-expressed cytochrome P450s: Role of CYP2B6, 2C19 and the 3A subfamily. *Xenobiotica* **26**:1155–66.
- Park SS, Fujino T, West D, Guengerich FP and Gelboin HV (1982) Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. *Cancer Res* **42**:1798–808.
- Park SS, Waxman DJ, Lapenson DP, Schenkman JB and Gelboin HV (1989) Monoclonal antibodies to rat liver cytochrome P-450 2c/RLM5 that regiospecifically inhibit steroid metabolism. *Biochem Pharmacol* **38**:3067–74.
- Sai Y, Yang TJ, Krausz KW, Gonzalez FJ and Gelboin HV (1998) Inhibitory monoclonal antibodies to cytochrome P450 2A6. *Pharmacogenetics*, in press.
- Vermeulen NPE (1996) Role of metabolism in chemical toxicity. In *Cytochromes P450: metabolic and toxicological aspects* (Ioannides C ed) pp 29–53, CRC Press, New York.
- Wrighton SA and Stevens JC (1992) The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22**:1–21.
- Yamazaki H, Inoue K, Mimura M, Oda Y, Guengerich FP and Shimada T (1996) 7-Ethoxycoumarin O-deethylation catalyzed by cytochromes P450 1A2 and 2E1 in human liver microsomes. *Biochem Pharmacol* **51**:313–9.
- Yang TJ, Krausz KW, Shou M, Yang SK, M., BJT, Gonzalez FJ and Gelboin HV (1998a) An inhibitory monoclonal antibody to human cytochrome P450 2B6. *Biochem Pharmacol* **55**:1633–1640.
- Yang TJ, Sai Y, Krausz KW, Gonzalez FJ and Gelboin HV (1998b) Specific inhibitory monoclonal antibodies to human cytochrome P450 1A2: Analysis of phenacetin O-deethylation in human liver. *Pharmacogenetics* **8**:375–382.
- Yang TJ, Shou M, Karzekwa KR, Gonzalez FJ, Gelboin HV and Yang SK (1998c) Role of cDNA-expressed human cytochromes P450 in the metabolism of diazepam. *Biochemical Pharmacology* **55**:889–896.
- Yasumori T, Nagata K, Yang SK, Chen L, Murayama N, Yamazoe Y and Kato R (1993) Cytochrome P450 mediated metabolism of diazepam in human and rat: Involvement of human CYP2C in N-demethylation in the substrate concentration-dependent manner. *Pharmacogenetics* **3**:291–301.