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Role of cDNA-Expressed Human Cytochromes P450 in the Metabolism of Diazepam

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ABSTRACT. The metabolic conversion of diazepam (DZ) to temazepam (TMZ, a C3-hydroxylation product of DZ) and *N*-desmethyldiazepam (NDZ, an N1-demethylation product of DZ) was studied using cDNA-expressed human cytochrome P450 (CYP) isozymes 1A2, 2B6, 2C8, 2C9, $2C9_{R144C}$, 2E1, 3A4, and 3A5 and human liver microsomes from five organ donors. Of the CYPs examined, 3A5, 3A4, and 2B6 exhibited the highest enzymatic activities with turnovers ranging from 7.5 to 12.5 nmol of product formed/min/nmol for the total metabolism of DZ, while 2C8, 2C9, and $2C9_{R144C}$ showed lesser and moderate activities. 1A2 and 2E1 produced insignificant amounts of metabolites of DZ. The regioselectivity of CYPs was determined, and 2B6 was found to catalyze exclusively and 2C8, 2C9, and $2C9_{R144C}$ preferentially the N1-demethylation of DZ to form NDZ. 3A4 and 3A5 catalyzed primarily the C3-hydroxylation of DZ, which was more extensive than the N1-demethylation. The ratios of TMZ to NDZ formed in the metabolism of DZ by 3A4 and 3A5 were approximately 4:1. Enzyme kinetic studies indicated that 2B6- and 2C9-catalyzed DZ metabolism followed Michaelis–Menten kinetics, whereas 3A4 and 3A5 displayed atypical and non-linear curves in Lineweaver–Burk plots. Human liver microsomes converted DZ to both TMZ and NDZ at a ratio of 2:1. Our results suggest that hepatic CYP3A, 2C, and 2B6 enzymes have an important role in the metabolism of DZ by human liver. BIOCHEM PHARMACOL **55**;6:889–896, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. diazepam metabolism; human liver microsomes; cDNA expression; cytochrome P450; high-performance liquid chromatography

CYPs¶ play a central role in the oxidative metabolism of a large number of endogenous and exogenous compounds [1, 2]. CYP enzymes are particularly prominent in hepatic tissue, where they catalyze the NADPH-dependent monooxygenation of structurally diverse lipophilic substrates to yield more polar derivatives. DZ (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one) is widely used worldwide as an anxiolytic and hypnotic drug. The major oxidative metabolites of DZ are NDZ (an N1-demethylation product of DZ), TMZ (a C3-hydroxylation product of DZ), and OX (a metabolite formed by either C3-hydroxylation of NDZ or N1-demethylation of TMZ) (Fig. 1) [3–5]. NDZ, TMZ, and OX are all pharmacologically active. By using chemical inhibitors and antibodies to rat cytochrome CYP isozymes, Yasumori *et al.* [6] and Anderson *et al.* [7] reported that CYP2C isozymes catalyze mainly N1-demethylation, whereas CYP3A isozymes catalyze both C3-hydroxylation and N1-demethylation of DZ in human liver microsomes.

Molecular biology techniques have led to the successful cloning and expression of a large number of human CYP isozymes [1, 8, 9]. This has allowed the identification of individual CYP isozymes responsible for the metabolism of a variety of endobiotics and xenobiotics [10–15]. Although the metabolism of DZ was studied thoroughly in the liver microsomes from animals [6, 7, 16], the role of individual human CYPs in the N1-demethylation and/or C3-hydroxylation of DZ, NDZ, and TMZ has not been defined, and the kinetics of major active CYP enzymes for DZ metabolism have not been characterized. In this study, we utilized eight cDNA-expressed human CYP isozymes and human liver microsomes from five individuals to characterize the metabolism of DZ, NDZ, and TMZ.

MATERIALS AND METHODS

Chemicals

DZ was purchased from USP Convention, Inc. NDZ was provided by Hoffmann–La Roche, Inc.; TMZ by the Sandoz

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¶ *Abbreviations:* CYP, cytochrome P450; DZ, diazepam; TMZ, temazepam; NDZ, *N*-desmethyldiazepam; OX, oxazepam; and OQZ, 2-oxoquanzepam.

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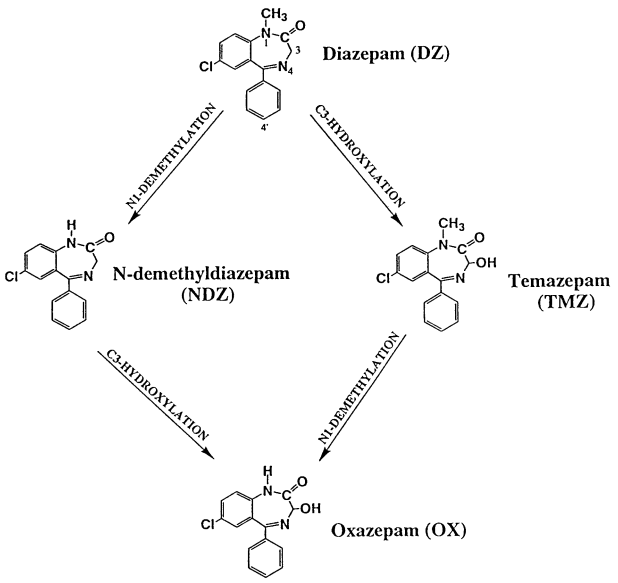

FIG. 1. Metabolic pathways of DZ.

Pharmaceuticals Corp.; and OQZ by the Schering–Plough Corp. NADPH was purchased from Boehringer Mannheim GmbH. HPLC grade acetonitrile, methanol, and dichloromethane were purchased from the Mallinckrodt Specialty Chemicals Co.

High Performance Liquid Chromatography

HPLC was performed on a Hewlett–Packard model 1050 liquid chromatograph equipped with a model 1050 autosampler, a solvent delivery system, and a variable wavelength detector. The system was connected to an HP QS/20 personal computer with HP ChemStation software (Hewlett–Packard Co.). DZ and its metabolites were separated on a Zorbax SB-C18 column (4.6 mm × 15 cm, MAC-MOD Analytical Inc.). The mobile phase was MeCN:MeOH:H_2O (10:40:50, by vol.) at a flow rate of 1 mL/min. The eluent was monitored at 232 nm. Calibration curves of TMZ and NDZ relative to that of a known amount of an internal standard (OQZ) were established by areas under the chromatographic peaks (AUC) at 232 nm. Quantification of TMZ and NDZ formed in the metabolism of DZ was established by their areas under the chromatographic peaks at 232 nm relative to that of OQZ.

Human Liver Microsomes

Human liver specimens, stored at −80° until used, were obtained from organ donors after clinical death. Human liver microsomes were prepared as described by Alvares *et al.* [17]. Microsomal protein and CYP content were determined according to the methods of Lowry *et al.* [18] and Omura and Sato [19], respectively. Information of organ donors and cytochrome CYP content of liver microsomes is shown in Table 1.

Expression of Human CYP Isozymes in Hep G2 Cells

Recombinant vaccinia viruses containing cDNAs encoding the CYPs proteins 1A2, 2B6, 2C8, 2C9, $2C9_{R144C}$, 2E1, 3A4, and 3A5 were constructed as previously described [9, 20]. Human TK^- 143 (thymidine kinase-deficient embryoblast) was used to produce stocks of vaccinia virus coding for a specific CYP. Hep G2 cells were grown to >90% confluency on 175 cm^2 plastic flasks and infected with either the wild-type or the recombinant vaccinia viruses. Cells were harvested 24 hr after infection. For metabolism studies, the cells were thawed, lysed by sonication, and centrifuged for 10 min at 500,000 *g* (Beckman TL 100 ultracentrifuge), and the pellets containing membrane proteins were resuspended in 50 mM potassium phosphate buffer (pH 7.4). The CYP content was determined by Fe^{2+} −CO vs Fe^{2+} difference spectrum [19], and protein concentration was measured by the method of Lowry *et al.* [18].

Metabolism of DZ, NDZ, or TMZ by Human CYP Isozymes

A typical reaction mixture (1 mL) contained 100 nmol of DZ (added in 10 μL methanol), 5–50 pmol CYPs, and 50 μmol phosphate buffer (pH 7.4). The mixture was preincubated for 5 min at 37° in a shaking water bath, the reaction was initiated by adding 1 μmol NADPH, and then the mixture was incubated for up to 20 min. The reaction was terminated by the addition of dichloromethane (4.5 mL) and OQZ (0.5 nmol in 0.1 mL methanol, an internal standard for quantification of metabolite formations). The mixture was vortexed, and centrifuged to separate organic and aqueous phases. The organic phase was evaporated to dryness at ~40° under a gentle stream of nitrogen. The residue was dissolved in 0.1 mL methanol:water (50:50, v/v) for reversed-

TABLE 1. Donor information and CYP content of human liver microsomal preparations

HL No.	Gender	Race*	Age	CYP (nmol/mg protein)	Cause of death
HL2	F	C	46	0.78	Cerebral vascular attack
HL3	M	AA	45	0.41	Subdural hematomas
HL4	M	C	49	0.45	Automobile accident
HL8	M	C	73	0.54	Cerebral vascular attack
HL9	F	C	14	0.88	Respiratory arrest

* C = Caucasian; AA = African-American.

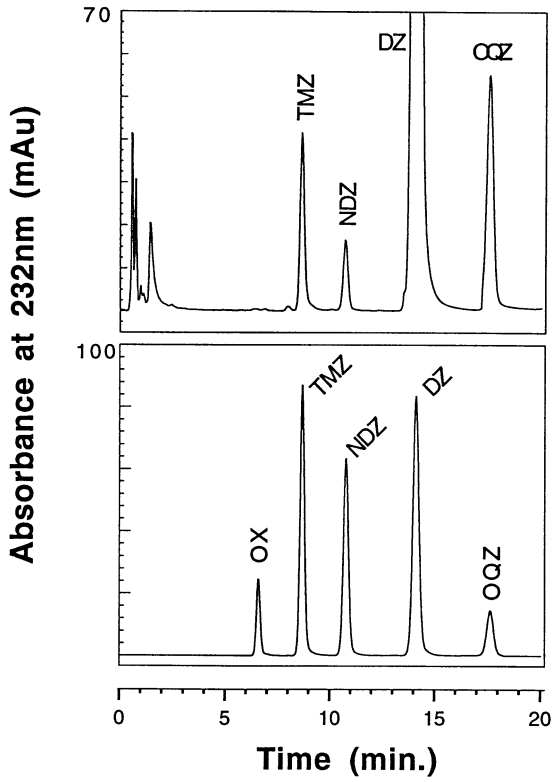

FIG. 2. Reversed-phase HPLC separation of the metabolites formed by the incubation of DZ with CYP3A4 (top panel) and their metabolite standards (bottom panel). OQZ is an internal standard used for quantification purposes. Incubation and chromatographic conditions are described in Materials and Methods.

phase HPLC analysis. The same experimental procedure was employed for the incubation of either NDZ or TMZ.

Enzyme Kinetics

The values of K_m and V_{max} of CYP enzymes were determined to assess the kinetic characteristics of individual CYPs. Either NDZ or TMZ was monitored to determine kinetic parameters (K_m and V_{max}) at a range of DZ concentrations from 10 to 400 μM. Lineweaver–Burk reciprocal plots were performed in Fig. 4. The values of K_m and V_{max} and their standard errors (SE) for individual CYPs were determined according to the statistical method of Cleland as shown in Fig. 4 [21].

RESULTS

Metabolism of DZ by human CYP isozymes

DZ, its metabolites NDZ and TMZ, and the internal standard (OQZ) were separated efficiently by reversed-phase HPLC (Fig. 2). Under the experimental conditions of this study, the formation of OX by various CYP isozymes was insignificant.

Of all the cDNA-expressed CYPs, CYP3A4, 3A5, and 2B6 exhibited the highest enzymatic activities with turnovers ranging from 7.5 to 12.5 nmol product formed/min/nmol in the metabolism of DZ; these activities were 3.6- to 250-fold greater than those obtained with other CYPs (Table 2). CYP2C8, 2C9, and $2C9_{R144C}$ had moderate activity at rates ranging from 0.79 to 2.08 nmol/min/nmol. CYP1A2 and 2E1 did not produce significant amounts of products (≤0.065). CYP2B6, 2C8, 2C9, and $2C9_{R144C}$ exhibited a marked preference for N1-demethylation. The relative N1-demethylation activity was 2B6 ≫ $2C9_{R144C}$ > 2C9 > 2C8. CYP2C8, 2C9, and $2C9_{R144C}$ catalyzed N1-demethylation at rates of 14- to 30-fold higher than rates of C3-hydroxylation. CYP2B6 exclusively catalyzed N1-demethylation with a specific activity of 7.5 nmol/min/nmol. In contrast, CYP3A4 and 3A5 catalyzed C3-hydroxylation at rates 4-fold higher than N1-demethylation. CYP1A2 and 2E1 exhibited weak but measurable activities for DZ metabolism (Table 2). HepG2 cells infected with wild-type vaccinia virus were metabolically inactive.

Metabolism of NDZ and TMZ by Human CYP Isozymes

NDZ and TMZ, the primary metabolites of DZ, are oxidized further to OX, the ultimate derivative that is further

TABLE 2. Metabolism of DZ by cDNA-expressed human CYP isozymes*

CYP	Specific activity (nmol/min/nmol)			Product ratio TMZ/NDZ
	TMZ	NDZ	Total	
1A2	ND	0.065 ± 0.001	0.065	
2B6	ND	7.504 ± 0.053	7.504	
2C8	0.022 ± 0.030	0.763 ± 0.054	0.785	0.03
2C9	0.031 ± 0.008	0.861 ± 0.052	0.892	0.04
$2C9_{R144C}$	0.142 ± 0.006	1.934 ± 0.097	2.076	0.07
2E1	0.010 ± 0.014	0.040 ± 0.001	0.050	
3A4	7.163 ± 0.053	1.829 ± 0.024	8.992	3.92
3A5	9.920 ± 0.397	2.540 ± 0.127	12.460	4.00
Wild-type†	ND	ND		

* DZ (100 nmol in 10 μL methanol) was added to 0.97 mL of incubation mixture containing a phosphate buffer (50 mM, pH 7.4) and 25–50 pmol of CYP. After a preincubation at 37° for 5 min, the reaction was initiated by the addition of NADPH (20 pmol in 20 μL water) and incubated at 37° for 20 min. Specific activities (nmol product formed/min/nmol of P450) are expressed as means ± SD (N = 3). ND = not detected.

† Membrane protein prepared from the Hep G2 cells infected with wild-type vaccinia virus.

TABLE 3. Metabolism of NDZ and TMZ by cDNA-expressed human P450 isozymes*

CYP	Specific activity (nmol/min/nmol)	
	Hydroxylation of NDZ	Demethylation of TMZ
1A2	0.221 ± 0.013	0.003 ± 0.009
2B6	ND	0.392 ± 0.085
2C8	ND	0.154 ± 0.034
2C9	ND	0.199 ± 0.043
$2C9_{R144C}$	ND	0.318 ± 0.053
2E1	ND	0.015 ± 0.001
3A4	2.342 ± 0.150	1.932 ± 0.139
3A5	0.673 ± 0.006	0.447 ± 0.318
Wild-type†	ND	ND

* NDZ (100 nmol in 10 μL methanol) was added to 0.97 mL of incubation mixture containing a phosphate buffer (50 mM, pH 7.4) and 100 pmol of individual CYPs. After preincubation at 37° for 5 min, the reaction was initiated by the addition of NADPH (20 pmol in 20 μL water) and incubated at 37° for 20 min. Specific activities (nmol OX formed/min/nmol of CYP) are expressed as means ± SD (N = 3). ND = not detected.

† Membrane proteins prepared from Hep G2 cells infected with wild-type vaccinia virus.

conjugated and excreted. NDZ is metabolized via C3-hydroxylation to form OX. CYP3A4 and 3A5 were the most active and 1A2 was moderately active in the metabolism of NDZ. Metabolic activities of 2B6, 2C8, 2C9, $2C9_{R144C}$ and 2E1 were not detectable under our assay conditions (Table 3). As compared with DZ, NDZ was a poor substrate for those CYP enzymes that catalyzed C3-hydroxylation.

TMZ is demethylated to form OX. CYP2B6, 2C8, 2C9, $2C9_{R144C}$, 3A4, and 3A5 each had marked activity for N1-demethylation of TMZ (Table 3). The relative magnitude of activity was 3A4 > 3A5 > 2B6 > $2C9_{R144C}$ > 2C9 > 2C8 ≫ 2E1. The enzyme activity for the demethylation of TMZ was ranked in a similar order as that observed using DZ as a substrate except for 2B6 that was the most active in the conversion of DZ to NDZ. Interestingly, the CYP2C9 variant ($2C9_{R144C}$) was more active in the N1-demethylation of DZ and TMZ than wild-type 2C9.

Metabolism of DZ by Human Liver Microsomes

All the human liver microsomes were capable of metabolizing DZ (Table 4). The turnovers for the total metabolism of DZ ranged from 2.3 to 6.6 nmol/min/nmol, and the variation between individuals (N = 5) was 3-fold. The C3-hydroxylation by liver microsomes was approximately 2-fold higher than the N1-demethylation. Although the metabolic pattern appeared similar to that obtained from CYP3A4-catalyzed metabolism of DZ, the ratio of TMZ to NDZ generated by human liver microsomes (ratio = 1.0 to 2.7) was less than that produced by recombinant 3A4/5 (ratio ≈ 4), suggesting that other enzymes, perhaps 2C8/9 and 2B6, that catalyze N1-demethylation may contribute to the overall metabolism of DZ in human liver. Table 4 also shows that the ratio of TMZ/NDZ formed by human liver microsomes increased with substrate concentration.

Enzyme Kinetics

The time-dependent formations of TMZ and NDZ in the metabolism of DZ (0.1 mM) by 3A4 or 3A5 (5 pmol/mL)

TABLE 4. Metabolism of DZ by human liver microsomes*

HLM No.	DZ (μM)	Specific activities (nmol/min/nmol CYP)		Product ratio TMZ/NDZ
		TMZ	NDZ	
HL2	10	1.359 ± 0.222	1.079 ± 0.093	1.26
	25	4.590 ± 0.554	2.027 ± 0.295	2.26
	50	3.096 ± 1.049	1.408 ± 0.475	2.20
	100	2.918 ± 0.000	1.569 ± 0.000	2.86
HL3	10	1.662 ± 0.113	1.460 ± 0.096	1.14
	25	2.012 ± 0.117	1.247 ± 0.090	1.61
	50	2.179 ± 0.095	1.199 ± 0.078	1.86
	100	2.242 ± 0.287	1.184 ± 0.171	1.89
HL4	10	1.525 ± 0.000	1.034 ± 0.000	1.47
	25	1.657 ± 0.075	0.808 ± 0.003	2.05
	50	2.411 ± 0.582	0.955 ± 0.208	2.53
	100	2.028 ± 0.170	0.809 ± 0.208	2.51
HL8	10	1.140 ± 0.052	1.107 ± 0.011	1.03
	25	1.587 ± 0.011	0.898 ± 0.053	1.77
	50	1.879 ± 0.000	0.886 ± 0.050	2.12
	100	1.672 ± 0.228	0.802 ± 0.095	2.08
HL9	10	2.779 ± 0.512	1.586 ± 0.013	1.76
	25	4.192 ± 0.935	1.633 ± 0.281	2.57
	50	4.762 ± 0.937	1.752 ± 0.179	2.72
	100	4.221 ± 0.526	1.672 ± 0.281	2.53

* DZ (100 nmol in 10 μL methanol) was added to 0.97 mL of incubation mixture containing a phosphate buffer (50 mM, pH 7.4) and 25–100 pmol of microsomal CYPs. After a preincubation at 37° for 5 min, the reaction was initiated by the addition of NADPH (20 pmol in 20 μL water) and incubated at 37° for 20 min. Specific activities (nmol product formed/min/nmol of P450) are expressed as means ± SD (N = 3).

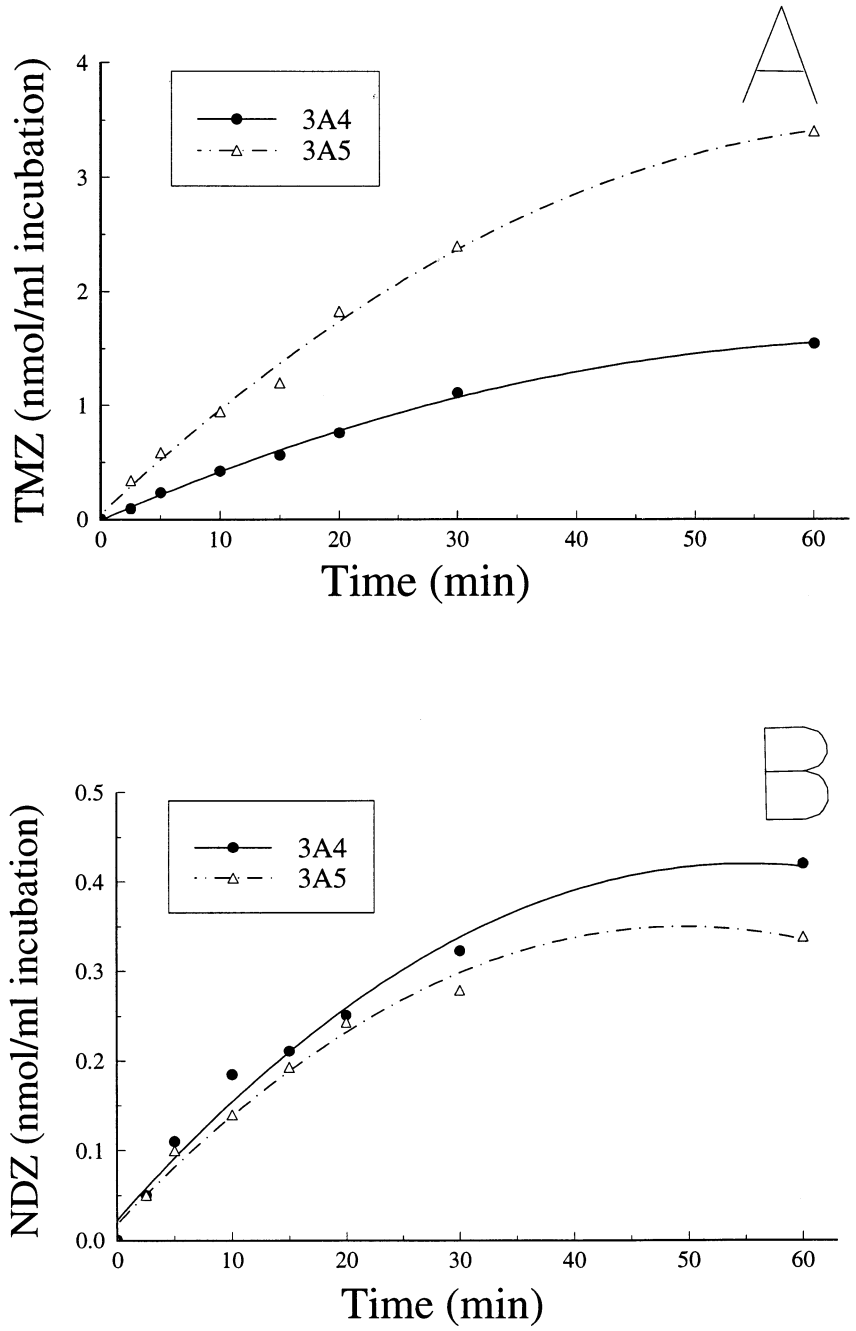

FIG. 3. Time-dependent formations of TMZ and NDZ (nmol formed per mL of incubation mixture) in the metabolism of DZ by CYP3A4 and 3A5. DZ (100 nmol in 10 μL methanol) was added into 1 mL of incubation mixtures, each containing 10 pmol of CYP and other cofactors. Each data point is the mean ± SD (N = 3).

indicated linearity up to 20 min of incubation at 37° (Fig. 3). The relative rate of TMZ formation was 3A5 > 3A4, whereas the relative rate for NDZ formation was 3A4 > 3A5 (Fig. 3). CYP2B6, 2C8, 2C9, and $2C9_{R144C}$ exhibited time-dependent metabolism similar to 3A enzymes (data not shown). Based on these results, an incubation time of 20 min was selected to study the metabolism of DZ. The kinetics of DZ metabolism by 2B6, 2C9, 3A4, and 3A5 were investigated using substrate concentrations ranging from 5 to 400 μM. CYP2B6 and 2C9 activity for the metabolism of DZ followed Michaelis–Menten kinetics, and their Lineweaver–Burk plots were linear (Fig. 4). The values of K_m and V_{max} were 181 μM and 8.5 min^{-1} for 2B6, and 714 μM and 19.7 min^{-1} for 2C9, respectively. However, the Lineweaver–Burk plots of 3A4/5-catalyzed metabolism of DZ were non-linear (Fig. 5), which was consistent with the kinetics for DZ metabolism by human liver microsomes in earlier reports [7, 20], suggesting that 3A enzymes may have more than one affinity site for the substrate.

DISCUSSION

In the present study, the activities of eight cDNA-expressed human cytochrome CYP enzymes for the metabolism of DZ were studied. DZ undergoes mainly C3-hydroxylation by

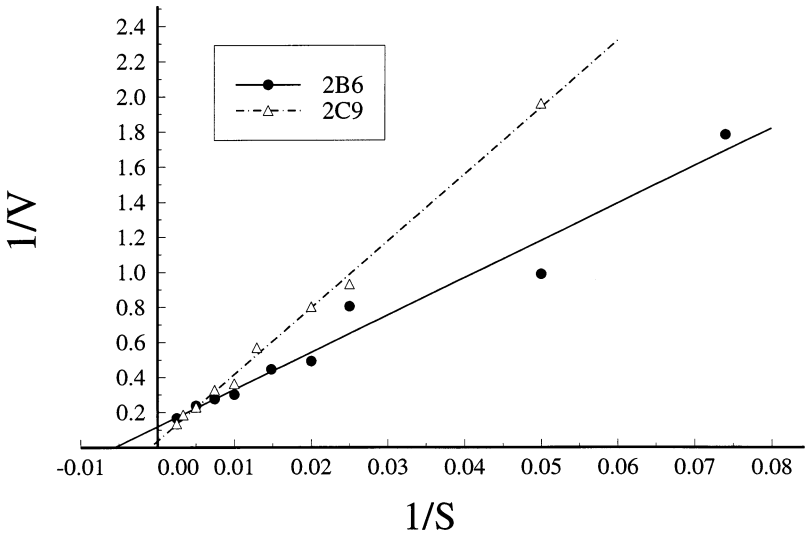

FIG. 4. Kinetics and Lineweaver–Burk plots for 2B6 and 2C9. V = velocity (nmol of NDZ formed/min/nmol of CYP2B6 or 2C9) and S = substrate concentration (μM). K_m and V_{max} are 181 μM (standard error = 22.6) and 8.5 min^{-1} (0.55) for 2B6 and 714 μM (164) and 19.7 min^{-1} (3.3) for 2C9, respectively. Each data point is the mean of triplicates.

CYP3A4 and 3A5 to form TMZ and N1-demethylation by CYP2B6, 2C8, 2C9, 3A4, and 3A5 to form NDZ. CYP1A2 and 2E1 showed weak activities toward DZ. Using either TMZ or NDZ as a substrate, these CYPs also showed similar activities and reaction preferences as that observed with DZ. The results suggest that N1-demethylation and C3-hydroxylation of DZ can be used as molecular probes for specific CYPs in human liver.

CYP2B6 is present in liver at a relatively low level and catalyzes the metabolism of certain carcinogens and drugs such as benzo[*a*]pyrene [15], dibenzo[*a*,*h*]anthracene [22], cyclophosphamide [23], and tamoxifen [24]. N1-Demethylation of DZ by 2B6 was extensive, but C3-hydroxylation was not detectable. Thus, 2B6 is a specific catalyst for N1-demethylation of DZ. CYP2C8 and 2C9 [25, 26] catalyze a variety of substrates such as *S*-mephenytoin, taxol, cyclophosphamide, tolbutamide, (*S*)-warfarin, and phenytoin [27]. We found that they also catalyze the N1-demethylation of DZ and TMZ. The most abundantly expressed CYP isozymes in human liver are the 3A subfamily [26], which is among the most important enzymes in drug metabolism, catalyzing oxidation of over 100 drugs and carcinogens [2, 27]. In the present study, CYP3A4 and 3A5 exhibited the highest activities in total metabolism of DZ, NDZ, and TMZ in which C3-hydroxylation always predominates.

Our study characterized the enzyme kinetic data for the metabolism of DZ using individual human CYPs. Figure 4 shows that the kinetics of 2B6- and 2C9-catalyzed metabolism fit the Michaelis–Menten equation reasonably well, and the K_m values for 2B6 and 2C9 for the N1-demethylation of DZ were 181 and 714 μM, respectively, suggesting that 2B6 had a 4-fold higher affinity than 2C9. Although the V_{max} for 2B6 was 2.3-fold less for the demethylation of DZ than that for 2C9, the activity of 2B6 was approximately 7-fold higher than that observed for 2C9 when 100 μM DZ was used as a substrate. This is probably due to a higher K_m (714 μM) for 2C9 that was not saturated by DZ (100 μM), and therefore the maximal rate for the formation of NDZ by 2C9 could not be achieved because of poor solubility of DZ [28].

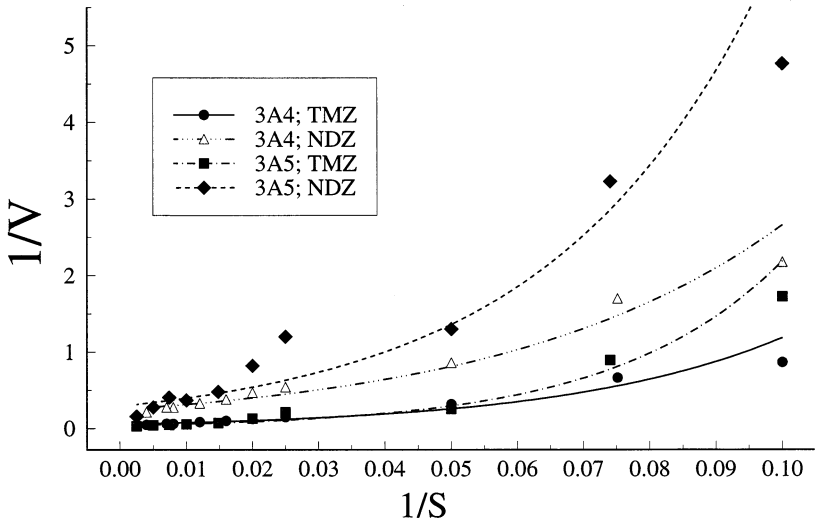

FIG. 5. Kinetics and Lineweaver–Burk plots for 3A4 and 3A5. V = velocity (nmol of NDZ or TMZ formed/min/nmol of CYP3A4 or 3A5) and S = substrate concentration (μM). Each data point is the mean of triplicates.

CYP3A4 and 3A5 exhibited atypical kinetics and non-linear curves in the plots of velocity (1/V) versus substrate concentration (1/S) (Fig. 5), which did not fit the Michaelis–Menten equation. The curved nature of kinetics for 3A4/5 for DZ metabolism was consistent with that obtained from human liver microsomes in previous reports [7, 28]. Thus, it appears that the non-linear kinetics derived from liver microsomes is attributed to 3A4/5. CYP3A4/5 kinetics in the metabolism of DZ suggests that 3A enzymes may possess multiple affinity sites for the substrate, which are expressed by the K_m (higher and low affinities). Due to the limited solubility of DZ, the kinetic characteristics of 3A4/5 cannot be fully established at higher concentrations of DZ. Thus the K_m values for 3A enzymes, particularly for the higher K_m, may not be determined easily.

Human liver microsomes were capable of converting DZ to TMZ and NDZ. However, the ratio of TMZ to NDZ increased with an increase of substrate concentration (Table 4). Since 3A enzymes preferentially catalyzed the C3-hydroxylation of DZ while 2B6 and 2C enzymes almost exclusively catalyzed the N1-demethylation, the change of the TMZ/NDZ ratio thoroughly depends on the kinetic parameters (K_m and V_{max}) of individual CYPs contained in human liver which determine and adjust the rate for the two different reactions with various concentrations of DZ. The relative distribution of various CYPs in liver may not be a determinant in the change of the TMZ/NDZ formed in DZ metabolism when the substrate concentration varies.

In conclusion, we found that human CYP2B6, 2C8, 2C9, $2C9_{R144C}$, 3A4, and 3A5 are capable of oxidizing DZ, TMZ, and NDZ and are selective in two different oxidation reactions. CYP2C and 2B6 isoforms predominate the N1-hydroxylation and the 3A isoform predominates C3-hydroxylation of DZ. The results suggest that these active enzymes are probably the primary catalysts and contribute to the metabolism of DZ in human liver. The quantitative contribution of individual CYPs to the metabolism of DZ is under investigation by an application of inhibitory monoclonal antibodies as probes. In addition, since DZ has a long elimination half-life (50–150 hr) and is excreted only after liver microsomal biotransformation, our study provides the information that care must be taken when DZ is co-administered with other drugs that are also substrates for the enzymes.

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