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REGIOSELECTIVE BIOTRANSFORMATION OF MIDAZOLAM BY MEMBERS OF THE HUMAN CYTOCHROME P450 3A (CYP3A) SUBFAMILY

J. CHRISTOPHER GORSKI,*†‡ STEPHEN D. HALL,† DAVID R. JONES,†
MARK VANDENBRANDEN§ and STEVEN A. WRIGHTON§

*Department of Pharmacy Practice, School of Pharmacy, Purdue University, West Lafayette, IN 47907; †Department of Medicine, School of Medicine, Indiana University, Indianapolis, IN 46202; and §Eli Lilly & Company, Indianapolis, IN 46285, U.S.A.

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Abstract—The capabilities of cytochrome P4503A4 (CYP3A4), CYP3A5, and fetal hepatic microsomes containing CYP3A7 to metabolize midazolam were investigated using human hepatic microsomes and purified CYP3A4 and CYP3A5. Under initial rate conditions and high substrate concentration (400 μM midazolam), variability among eighteen human liver microsomal samples was 30- and 16- fold for 1′- and 4-hydroxylation of midazolam, respectively. Exclusion of two samples isolated from patients previously administered barbiturates reduced the inter-individual variability to 10.5- and 6.0-fold for 1′- and 4-hydroxylation, respectively. Six fetal hepatic microsomal samples showed 10-fold variation in both 1′-hydroxymidazolam and 4-hydroxymidazolam formation rates. The rates of formation of 4-hydroxymidazolam and 1′-hydroxymidazolam from midazolam by adult samples containing only CYP3A4 and by fetal liver samples were highly correlated ($r^2 = 0.99$ and 0.97, $P < 0.01$, respectively). The rates of formation of 1′-hydroxymidazolam and 4-hydroxymidazolam from midazolam (400 μM) by adult samples that contained only CYP3A4 were correlated significantly ($P < 0.01$) with the ability of the samples to N-demethylate erythromycin ($r^2 = 0.95$ and 0.92, respectively), 6β-hydroxylate testosterone ($r^2 = 0.96$ and 0.96, respectively), and the CYP3A4 content of the samples ($r^2 = 0.89$ and 0.86, respectively). Microsomal samples containing CYP3A5 in addition to CYP3A4 exhibited a significantly greater ratio of 1′-hydroxymidazolam to 4-hydroxymidazolam compared with samples containing only CYP3A4 or CYP3A7 ($P < 0.001$). Purified CYP3A5 in a reconstituted system, consisting of dilauroylphosphatidylcholine, cytochrome b_5, and NADPH–cytochrome P450 reductase, and an NADPH-regenerating system displayed a 2-fold greater rate of 1′-hydroxymidazolam formation and a similar rate of 4-hydroxymidazolam formation compared with a reconstituted system with CYP3A4. In conclusion, CYP3A4, CYP3A5, and fetal microsomes containing CYP3A7 catalyze 1′- and 4-hydroxylation of midazolam with the ratio of these metabolites indicative of the CYP3A form.

Key words: cytochrome P450; CYP3A subfamily; midazolam; hydroxylation; human liver microsomes; regioselective

The human CYP3A‖ subfamily has been reported to include four genes, namely CYP3A3, CYP3A4, CYP3A5, and CYP3A7 [1, 2]. CYP3A3 and CYP3A4 are 98% similar in their amino-acid sequence and are thus considered to be indistinguishable using standard separation and immunoidentification techniques [2, 3]. However, the expression of CYP3A3 has not been demonstrated; CYP3A3 was not detected in ten liver samples using PCR techniques with primers specific to CYP3A3¶. This observation suggests that the reported differences in sequence between CYP3A3 and CYP3A4 may be the result of cloning artifacts or that CYP3A3 is a rare allele of CYP3A4. The most abundant cytochrome P450 present in uninduced human adult liver is CYP3A4 [2]. Characteristic biotransformations attributed to CYP3A4 include erythromycin N-demethylation [4], nifedipine oxidation [5], 6β-hydroxylation of testosterone [6], and cyclosporine A metabolism [7].

Another member of this subfamily is CYP3A5, which demonstrates 84% amino-acid sequence similarity with CYP3A4 [1, 2, 8]. A majority of the adult human population does not appear to express CYP3A5, but 20–30% express both CYP3A4 and CYP3A5 [9]. In those individuals expressing this protein, CYP3A5 accounts for 15–30% of the total expressed CYP3A found in hepatic tissue [9]. In limited studies conducted to date, CYP3A5 generally exhibited a lower degree of catalytic capability and different regioselectivity when compared with CYP3A4 [8, 9]. For instance, 6β- and 2β-hydroxylation of testosterone by CYP3A5 occurred at 30 and 24%, respectively, of the rate associated with CYP3A4 [9]. However, when compared with CYP3A4, CYP3A5 exhibited a nearly equivalent ability to metabolize nifedipine [8]. CYP3A5 has a

‡ Corresponding author: J. C. Gorski, Ph.D., Wishard Memorial Hospital, Division of Clinical Pharmacology, OPW 320, 1001 West 10th Street, Indianapolis, IN 46202-2879. Tel. (317) 630-8795; FAX (317) 630-8185.

‖ Abbreviations: CYP3A, cytochrome P4503A; 1′-OHM, 1′-hydroxymidazolam; 4-OHM, 4-hydroxymidazolam; MDZ, midazolam; DLPC, dilauroylphosphatidylcholine; PCR, polymerase chain reaction; and V_{max}, maximum rate of metabolism.

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reduced capability compared with CYP3A4 or appears incapable of metabolizing 7-ethoxycoumarin, benzo[*a*]pyrene, quinidine, and erythromycin [8, 10]. In addition, only one of three primary metabolites formed by CYP3A4 from cyclosporine A was produced by CYP3A5 [8].

The major cytochrome P450 found in the human fetal liver is CYP3A7 [1, 2]. Although limited information is available concerning the metabolic capabilities of this enzyme, CYP3A7 is capable of N-demethylating ethylmorphine, codeine, and dextromethorphan [11, 12], and effects 16 α-hydroxylation of dehydroepiandrosterone [13] and the bioactivation of aflatoxin B_1 [14]. In general, substrate selectivity and enzyme efficiency have not been fully characterized for CYP3A7.

Midazolam is oxidized rapidly *in vitro* by CYP3A4 to two metabolites, 1′-OHM and 4-OHM [15–17]. Based on the high correlation between the *in vitro* formation of these metabolites of MDZ and immunoquantified CYP3A4 levels, antibody recognition, and competitive inhibition by known CYP3A4 substrates, it is clear that CYP3A4 is involved in both reactions [17]. However, as reported by Kronbach *et al.* [17], the correlation between the *in vitro* rates of MDZ 1′- and 4-hydroxylation is attenuated in two of fifteen human liver samples examined. In addition to CYP3A4, these two livers contained a second protein immunochemically related to CYP3A4, which was not identified by Kronbach *et al.* [17]. A similar pattern of immunoreactivity with anti-CYP3A4 antibodies is seen in human hepatic microsomes containing both CYP3A4 and CYP3A5 [9]. Taken together, these observations suggest that CYP3A4 and CYP3A5 exhibit a difference in regioselectivity with respect to the formation of the major metabolites of MDZ. If true, this property of these highly related enzymes could potentially be used to diagnose the presence of the CYP3A5 *in vitro* and *in vivo*. With this goal in mind, in the current study we have undertaken a detailed characterization of the rates of formation of 1′-OHM and 4-OHM by CYP3A4, CYP3A5, and CYP3A7.

MATERIALS AND METHODS

Chemicals and specimens. Midazolam, 1′-hydroxymidazolam, and 4-hydroxymidazolam were gifts of Hoffmann–La Roche (Nutley, NJ and Basel, Switzerland). Isocitrate dehydrogenase (Type IV, porcine), isocitrate, sodium phosphate, magnesium chloride, β-NADP, and flunitrazepam were purchased from the Sigma Chemical Co. (St. Louis, MO). Cytochrome b_5 was a gift from Dr. Richard Okita at Washington State University. HPLC grade methanol, methylene chloride, acetonitrile and pesticide grade cyclohexane were obtained from Fisher Scientific (Pittsburgh, PA).

Human adult and fetal livers were obtained at surgery in accordance with protocols approved by the Committee for the Conduct of Human Research at the institution at which they were received (the Medical College of Virginia, Richmond, VA; the Medical College of Wisconsin, Milwaukee, WI; the University of Michigan, Ann Arbor, MI). All patients had normal bilirubin and transaminase levels. Specimens from the Medical College of Virginia are identified with patient code numbers of HL-30, HL-34, and HL-35. Individual liver specimens received from the Medical College of Wisconsin are coded with letters A through N (e.g. HL-A). One specimen was obtained from the University of Michigan and is coded as UM-11. The ages, genders, smoking habits, drug histories, CYP3A content, and catalytic activities have been reported previously [9, 10]. Fetuses were obtained from therapeutic abortions performed prior to 12 weeks of gestation. The livers were removed, frozen in liquid nitrogen, and stored at −70°. Microsomes from a B-lymphoblastoid cell line expressing CYP3A4, the only CYP3A subfamily member commercially available, were obtained from Gentest (Woburn, MA).

Microsomes were prepared by differential centrifugation and stored at −70° in a 100 mM potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% glycerol, 20 μM butylated hydroxytoluene, and 100 μM phenylmethylsulfonyl fluoride [18]. Protein concentrations were determined colorimetrically by the method of Lowry *et al.* [19]. CYP3A4 and CYP3A5 were purified to a final concentration of 11.1 and 13.7 nmol/mg protein, respectively, according to previously reported methodology [9]. The method of Omura and Sato [20] was used to determine the total P450 concentrations, using an extinction coefficient of 91 mM^{-1} cm^{-1}.

The relative levels of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A, and CYP3A5 in the hepatic samples were determined using immunoblot analysis as previously described [21, 22]. The catalytic activities of CYP1A2, CYP2A6, CYP2CMP, CYP2D6, CYP2E1, and CYP3A were determined by monitoring ethoxyresorufin *O*-deethylase, coumarin 7-hydroxylase, *S*-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, dimethylamine *N*-nitroso *N*-demethylase, and erythromycin *N*-demethylase activity, respectively, as described elsewhere [22].

Midazolam hydroxylase assay. The rates of MDZ hydroxylation by human liver microsomes were determined using a previously published method [23]. Each incubation vessel contained the following: 50 μg of microsomal protein, 100 mM Na_2HPO_4 (pH 7.4) containing 5 mM magnesium chloride, 5 mM isocitrate, 1 U of isocitrate dehydrogenase, and a range of MDZ concentrations up to 400 μM. The total reaction volume was 200 μL. Midazolam was dissolved in acetonitrile and diluted with 100 mM Na_2HPO_4 (pH 7.4) buffer containing magnesium chloride and isocitrate prior to addition to the reaction vessel. The final acetonitrile concentration was 2% of the reaction volume. After a 5-min preincubation at 37°, the reaction was initiated by the addition of β-NADP (final concentration of 1 mM). In some experiments, the duration of incubation was varied while substrate concentration was held constant. After 10 min the reaction was terminated by the addition of 200 μL of methanol containing flunitrazepam as the internal standard, and stored at −70° until analysis. The rate of MDZ hydroxylation by microsomes prepared from B-

lymphoblastoid cells expressing CYP3A4 was determined using 200 μg of microsomal protein for 20 min under the previously described incubation conditions.

To afford direct comparisons with previous studies, the rates of MDZ hydroxylation by purified cytochromes P450 were determined in the same manner as described in those studies [9, 10]. These experiments were performed under the same conditions as those for the human liver microsomes except where noted. Briefly, a 1 μg/μL emulsion of DLPC in deionized water was prepared by sonication at room temperature for 15 min. Purified CYP3A4 or CYP3A5 was reconstituted with NADPH–cytochrome P450 reductase, cytochrome b_5, and DLPC and incubated at room temperature for 15 min. Subsequently, an aliquot containing 40 pmol NADPH–cytochrome P450 reductase, 10 pmol cytochrome b_5, 10 μg DLPC, and 10 pmol CYP3A4 or CYP3A5 was transferred to reaction vessels containing buffer, MDZ (up to 300 μM) and isocitrate dehydrogenase (as described above), and preincubated at 37° for 3–5 min. The reactions were initiated by the addition of β-NADP. After 10 min, the reactions were terminated by the addition of 200 μL of methanol containing internal standard, mixed vigorously, and stored at −70° until analyzed.

HPLC determination of midazolam metabolites. The metabolites of MDZ were determined using a previously published method with some modification [24]. Samples were extracted after the addition of 0.5 mL of 0.2 mM sodium borate (pH 9.6) followed by a 5-mL mixture of cyclohexane:methylene chloride (7:3). Following evaporation of the solvent (≈5 mL), the residue was reconstituted using 100–150 μL of mobile phase [methanol:K_2HPO_4 buffer (pH 7.4):tetrahydrofuran, 52:46:2] and 15–100 μL was injected onto an HPLC column (reverse phase). MDZ and its metabolites were separated with a Beckman Ultrasphere C-18 column (5 μm × 4.6 mm i.d. × 250 mm) and a 2 cm C-18 guard column. The mobile phase was delivered at a flow rate of 1 mL/min, and the eluate was monitored at 230 nm. Peak heights were quantified using a Chromjet (Spectra-Physics, San Jose, CA) integrator. Duplicate standards of known amounts of MDZ, 1′-OHM, and 4-OHM were prepared in reaction buffer and processed as described above. The procedure was used to routinely assay amounts between 10 and 2000 ng for 1′-OHM and 4-OHM. Inter-day variability at 20 ng was 10% and less than 6% at 800 ng for both metabolites.

Analysis of kinetic data and statistics. The data represent the mean of duplicate assays for every experiment. Untransformed kinetic data were analyzed by a nonlinear regression program (PCNONLIN v.4.0, SCI Software, Lexington, KY) assuming single enzyme Michaelis–Menten kinetics. The appropriateness of the fit was determined by visual inspection of residual patterns, residual sums of squares, and precision of the parameter estimates. A weighting factor equal to the reciprocal of the observed data was used.

To examine the consistency of the purified enzyme data and microsomal data, the 1′-OHM to 4-OHM ratios were predicted for each microsomal sample using constants obtained from the reconstitution of the purified CYP3A4 and CYP3A5 and the proportions of CYP3A4 and CYP3A5 determined by immunoquantitation. The following equation was used:

$$v = P \cdot \frac{V_{max_{3A5}} \cdot S}{K_{m_{3A5}} + S} + (1 - P) \cdot \frac{V_{max_{3A4}} \cdot S}{K_{m_{3A4}} + S} \quad (1)$$

where v is the velocity of the reaction; $V_{max_{3A5}}$ or $V_{max_{3A4}}$ is the maximal velocity of 1′-OHM or 4-OHM formation for either CYP3A5 or CYP3A4, respectively; S is the MDZ concentration; $K_{m_{3A5}}$ or $K_{m_{3A4}}$ represents the concentration at half-maximal velocity of 1′-OHM or 4-OHM formation; P represents the proportion of immunoquantified CYP3A identified as CYP3A5; and (1-P) represents the proportion of immunoquantified CYP3A identified as CYP3A4. The predicted metabolite ratio was estimated as the velocity of 1′-OHM formation to the velocity of 4-OHM formation.

The means of the observed metabolite ratios were compared by ANOVA (SAS, V6.04, SAS Institute Inc., Carey, NC) using the Student-Newman-Keuls multiple range test; a difference of $P < 0.05$ was considered significant. The coefficient of determination and its corresponding statistical

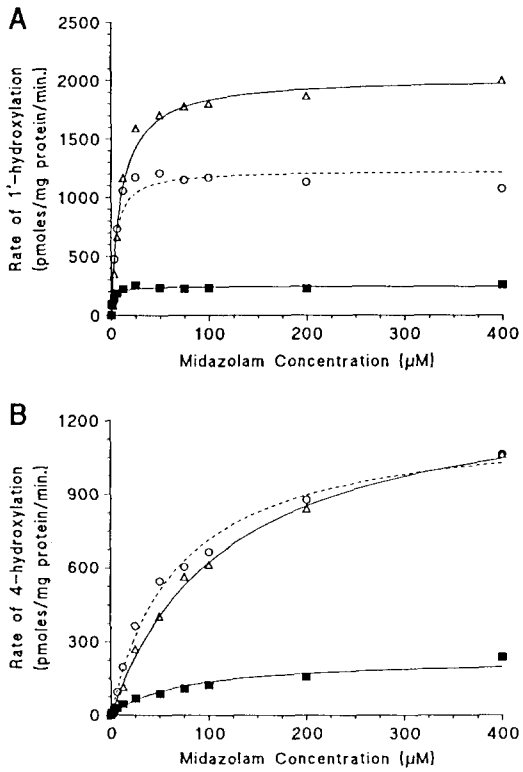

Fig. 1. Effect of substrate concentration on the rates of formation of 1′-hydroxymidazolam (A) and 4-hydroxymidazolam (B) by three human liver microsomal samples, HL-E (△), HL-I (○), and HL-H (■). Lines represent best fit to the Michaelis–Menten equation determined by nonlinear regression.

Table 1. Estimated Michaelis–Menten parameters (±SE*) for the formation of 1′-OHM and 4-OHM by human liver samples

Midazolam	Human liver sample		
	HL-E	HL-I	HL-H
1′-Hydroxylation			
V_{max} (pmol/mg protein/min)	2024 ± 491	1229 ± 44	245 ± 25
K_m (μM)	12.6 ± 1.2	4.7 ± 1.0	2.5 ± 0.5
CL_{int} (mL/min/mg protein)	160.6×10^{-3}	261.5×10^{-3}	98.0×10^{-3}
4-Hydroxylation			
V_{max} (pmol/mg protein/min)	1355 ± 70	1205 ± 45	231 ± 21
K_m (μM)	119.0 ± 14.0	68.9 ± 7.0	71.6 ± 15.0
CL_{int} (mL/min/mg protein)	11.4×10^{-3}	17.5×10^{-3}	3.2×10^{-3}

* Standard error represents the asymptotic standard error of the parameter estimated by nonlinear regression.

significance were determined by conventional methods [25].

RESULTS

Human liver microsomes. The formation of both 1′-OHM and 4-OHM was linear with respect to incubation time for 15 min in the presence of 50 μg (HL-I) of microsomal protein. An incubation time of 10 min was therefore routinely employed to ensure initial rate conditions. A detailed study of the formation of 1′-OHM and 4-OHM as a function of substrate concentration was performed in microsomes from three human livers (HL-E, HL-H, and HL-I). In contrast to the observation of substantial substrate inhibition of 1′-OHM formation noted by Kronbach *et al.* [17], we noted only modest substrate inhibition (Fig. 1). For example, in the most pronounced case, 1′-OHM formation by HL-I exhibited a maximum of 1170 pmol/mg protein/min at a substrate concentration of approximately 25 μM and declined to 1070 at 400 μM (Fig. 1). In view of the modest extent of the latter phenomenon, Michaelis–Menten parameters were estimated using a conventional single enzyme analysis (Table 1). For all MDZ concentrations, there was a preferential formation of 1′-OHM over 4-OHM, as reported previously [16, 17, 23]. The current data indicate that this metabolite ratio primarily reflects the significantly lower K_m for 1′-OHM formation and to a lesser degree a lower V_{max} for 4-OHM formation. The net effect of the latter regioselectivities was that the CL_{int} for 1′-OHM formation was substantially greater than that for 4-OHM (Table 1).

Using MDZ concentrations of 6.25, 75, and 400 μM, the formation of 1′-OHM and 4-OHM was determined in eighteen adult and six fetal liver samples. The rate of 4- and 1′-hydroxylation of MDZ varied considerably in the eighteen adult hepatic samples (range 91–1412 pmol 4-OHM/mg protein/min and 96–2298 pmol 1′-OHM/mg protein/min at 400 μM MDZ, respectively). Fetal liver samples also showed a wide variability in the rate of 4- and 1′-hydroxylation (Table 2). For the adult liver microsomes, the formation of these two metabolites was highly correlated ($r^2 \geq 0.87$, $P < 0.01$) at all substrate concentrations; however, the correlation was improved when samples containing CYP3A5 were excluded ($r^2 \geq 0.99$, $P < 0.01$; Fig. 2). In general, the presence of CYP3A5 was associated with a significantly greater ratio ($P < 0.05$) of 1′-OHM to 4-OHM formation rate at all three substrate concentrations (Fig. 3). Interestingly, this regioselectivity (1′-OHM > 4-OHM formation) was essentially absent for adult microsomes lacking CYP3A5 at 400 μM MDZ but not for those containing CYP3A5. In addition, incubation of MDZ (400 μM) with microsomes prepared from B-lymphoblastoid cells expressing CYP3A4 resulted in a metabolite formation of 40 pmol 1′-OHM and 51 pmol 4-OHM and a metabolite ratio of 0.78, which is consistent with the metabolite ratio observed in microsomes containing only immunodetectable CYP3A4 and reconstituted system containing CYP3A4 (*vide infra*).

For the fetal liver microsomes (Table 2), which contained CYP3A7, there were good correlations between the rates of formation of 1′-OHM and 4-OHM at substrate concentrations of 75 and 400 μM ($r^2 > 0.90$, $P < 0.01$) but not at 6.25 μM ($r^2 = 0.64$, $P > 0.05$). The absolute rates of 1′-OHM and 4-OHM formation by these CYP3A7-containing microsomes were comparable with the rates of formation exhibited by adult samples. However, a different regioselectivity was observed which resulted in a significantly lower 1′-OHM to 4-OHM ratio at 6.25 and 75 μM when compared with adult liver microsomes containing CYP3A4 or CYP3A4 and CYP3A5 (Fig. 3). Microsomes containing CYP3A4, CYP3A4 and CYP3A5, and CYP3A7 appeared to demonstrate distinct regioselectivities in the metabolism of MDZ. It is apparent from Fig. 3 that significant differences were seen in the metabolite ratios at all substrate concentrations. However, based on the 99% confidence intervals (Fig. 3), it would appear that 400 μM MDZ would provide the most robust prediction of CYP3A5 presence in a given microsomal sample. Using this approach, it is reasonable to suggest that metabolite ratios obtained at 400 μM MDZ, which exceed 1.0, would indicate

Table 2. Rate of midazolam metabolite formation (pmol product/mg microsomal protein/min) by human fetal liver microsomal samples containing CYP3A7 at various midazolam concentrations

	6.25 μM MDZ		75 μM MDZ		400 μM MDZ	
Fetal samples	4-OHM	1′-OHM	4-OHM	1′-OHM	4-OHM	1′-OHM
1	20.6	49.6	140.3	208.0	122.8	153.6
2	142.6	27.1	130.9	119.6	137.2	109.5
3	275.1	113.2	752.0	625.8	785.1	591.0
4	56.3	39.9	370.5	271.3	441.9	290.4
5	30.7	23.4	185.5	135.0	203.8	149.5
6	ND*	ND	62.6	39.4	80.7	57.8
Average ± SD	105.1 ± 106.5	50.6 ± 36.5	273.6 ± 256.4	233.0 ± 207.6	295.3 ± 272.2	225.0 ± 195.1

* ND, not detectable.

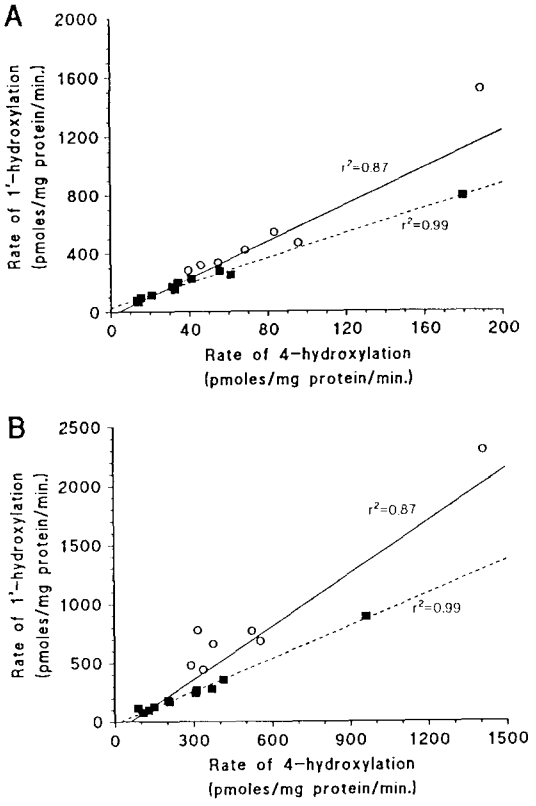

Fig. 2. Correlation between the rates of formation of 4-hydroxymidazolam and 1′-hydroxymidazolam by human adult liver microsomes at low (6.25 μM) (A) and high (400 μM) (B) substrate concentrations. Samples containing only CYP3A4 are designated by closed symbols and those containing CYP3A4 and CYP3A5 by open symbols. Lines of best fit and coefficients of determination (r^2) are given for eleven microsomal samples containing only CYP3A4 by a dashed line and for all eighteen samples by the solid line.

the presence of CYP3A5 because the upper limit of the confidence interval of CYP3A4-only samples is 0.97 while the lower limit of the confidence interval for CYP3A5-containing samples is 1.06.

The rates of formation of 1′-OHM and 4-OHM at a substrate concentration of 400 μM, which approximates the maximal velocities, were employed in a correlation analysis to further define the cytochromes P450 involved in these biotransformations. For the eighteen adult microsomal samples, correlations for the formation rates of 1′-OHM ($r^2 = 0.57$, $P < 0.05$) and 4-OHM ($r^2 = 0.53$, $P < 0.01$) with previously reported total immunoquantified CYP3A [8, 9, 22] were noted (Fig. 4). These relationships improved substantially to 0.89 ($P < 0.01$) and 0.86 ($P < 0.01$), respectively, when the microsomes containing immunoquantifiable CYP3A5 were omitted from the analysis. The correlation of 1′-OHM and 4-OHM formation with erythromycin N-demethylation [22], a prototypical CYP3A biotransformation [4], in fourteen adult liver samples was $r^2 = 0.39$ ($P > 0.05$) and $r^2 = 0.64$ ($P < 0.05$), respectively. When samples containing CYP3A5 in addition to CYP3A4 were excluded from the analysis, the correlation of 1′-OHM and 4-OHM formation with erythromycin N-demethylation improved substantially to $r^2 = 0.95$ ($P < 0.01$) and $r^2 = 0.94$ ($P < 0.01$), respectively (Fig. 5). The 6β-hydroxylation of testosterone is also a characteristic biotransformation of CYP3A enzymes and had been characterized previously in ten of the adult microsomal samples (HL-A to HL-J) [9]. Correlations between the latter biotransformation and 1′-OHM ($r^2 = 0.52$, $P > 0.05$) and 4-OHM ($r^2 = 0.77$, $P < 0.01$) were observed. However, once again the correlation was improved when samples containing only CYP3A4 were employed in the analysis ($r^2 = 0.96$, $P < 0.01$; and 0.96, $P < 0.01$, respectively). The improved correlations in the absence of CYP3A5 were consistent with a different regioselectivity of this enzyme towards MDZ and a lower affinity for erythromycin and testosterone relative to CYP3A4 [9, 10].

With the eighteen adult liver microsomes, there was no significant correlation ($r^2 < 0.5$) between either the formation of 1′-OHM or 4-OHM at 400 μM MDZ concentration and previously

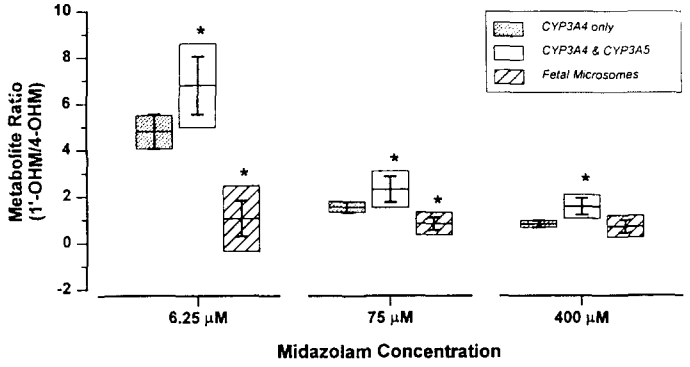

Fig. 3. Comparison of the ratio of the rates of formation of 1'-hydroxymidazolam (1'-OHM) to that of 4-hydroxymidazolam (4-OHM) (metabolite ratio) at three substrate concentrations for human liver microsomes containing only CYP3A4 (N = 11), CYP3A4 and CYP3A5 (N = 7), and CYP3A7 (N = 5 at 6.25 and N = 6 at 75 and 400 μM). Horizontal lines are the mean metabolite ratio and vertical lines represent (±) standard deviation. Boxes represent 99% confidence intervals. Statistically significant differences ($P < 0.05$) from microsomes containing only CYP3A4 are indicated by an asterisk.

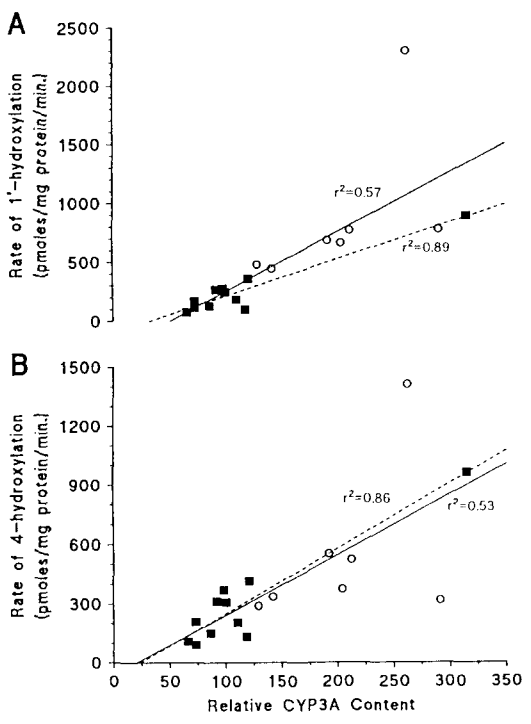

Fig. 4. Correlation between the rates of formation of 1'-hydroxymidazolam (A) and 4-hydroxymidazolam (B) at 400 μM midazolam with immunoquantified levels of CYP3A for eleven human liver microsomal samples containing CYP3A4 only (■) and 7 samples containing CYP3A4 and CYP3A5 (○) [8, 9, 22]. Lines of best fit and coefficients of determination (r^2) are given for eleven microsomal samples containing CYP3A4 by a dashed line and for all eighteen samples by the solid line.

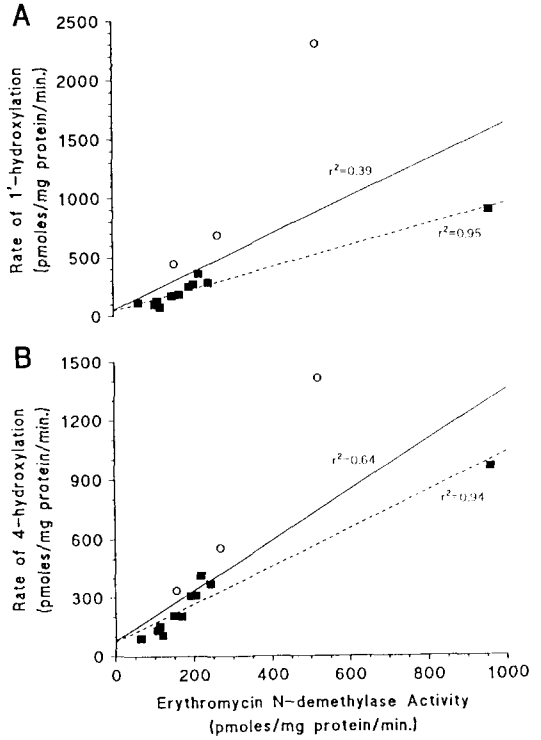

Fig. 5. Correlation between the rates of formation of 1'-hydroxymidazolam (A) and 4-hydroxymidazolam (B) at 400 μM midazolam with erythromycin *N*-demethylase activity [22] for eleven human liver microsomes samples containing CYP3A4 only (■) and three samples containing CYP3A4 and CYP3A5 (○). Lines of best fit and coefficients of determination (r^2) are given for eleven microsomal samples containing CYP3A4 by a dashed line and for all fourteen samples by the solid line.

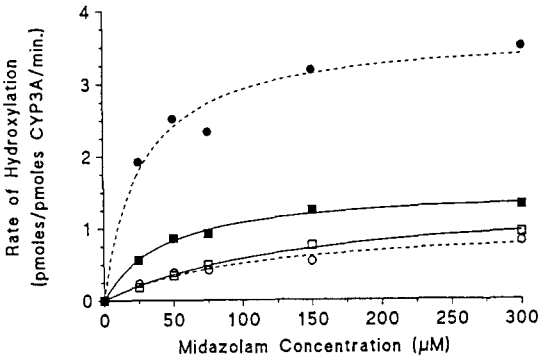

Fig. 6. Effect of substrate concentration on the rates of formation of 1′-hydroxymidazolam (closed symbols) and 4-hydroxymidazolam (open symbols) by purified CYP3A4 (□, ■) or CYP3A5 (○, ●). Lines represent the best fit to the Michaelis–Menten equation determined by nonlinear regression.

determined immunoquantified levels of CYP1A2, CYP2A1, CYP2C8, CYP2C9, CYP2D6, and CYP2E1 [22]. Furthermore, there was also an absence of correlation for either pathway of MDZ metabolism and previously reported ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, *S*-mephenytoin 4-hydroxylation, bufuralol 1′-hydroxylation, and dimethylamine *N*-nitroso N-demethylation which primarily reflect the activities of CYP1A2, CYP2A1, CYP2CMP, CYP2D6 and CYP2E1, respectively [22].

Using a range of MDZ concentrations (up to 300 μM), the rate of formation of 1′-OHM and 4-OHM was examined with purified CYP3A4 and CYP3A5 (Fig. 6), and the estimated Michaelis–Menten parameters are given in Table 3. Both CYP3A4 and CYP3A5 were able to catalyze the formation of 1′-OHM and 4-OHM, and both enzymes preferentially metabolized MDZ to 1′-OHM. The degree of regioselectivity in favor of 1′-OHM was greater for CYP3A5 which reflects the greater V_{max} and lower K_m for this pathway via this enzyme (Table 3). Additionally, purified CYP3A4 and CYP3A5 showed little difference in the intrinsic clearance of midazolam via the 4-hydroxylation pathway (Table 3). However, a 4-fold difference existed in the intrinsic clearance of midazolam via the 1′-hydroxylation pathway (Table 3).

The use of DLPC in the reconstituted enzyme system resulted in readily detectable metabolite formation, and consistent with other reports [3, 5, 9, 21] the reconstituted enzymes exhibited differences in their Michaelis–Menten parameters relative to hepatic microsomes (Table 1 vs Table 3). To address the issue of possible differences in enzyme regioselectivity between the microsomal and the reconstituted environment, the ratio of the rate of formation of 1′-OHM to that of 4-OHM was predicted at three substrate concentrations for each adult microsomal sample using the V_{max} and K_m determined in the reconstitution experiments and the proportion of CYP3A content associated with CYP3A5 (see Materials and Methods, Equation 1). In general, a good agreement between observed and predicted metabolite ratios was noted (Fig. 7), indicating that the regioselectivities of CYP3A4 and CYP3A5 are intrinsic enzymatic properties and are not grossly influenced by the numerous differences between the reconstituted environment and that of human liver microsomes. This was also reflected in the ratio of 1′-OHM to 4-OHM obtained with microsomes from B-lymphoblastoid cells expressing CYP3A4.

DISCUSSION

In this investigation, the effects of the expression of the various members of the human CYP3A subfamily on two hydroxylations of MDZ were examined. The data presented demonstrate that CYP3A4, CYP3A5, and fetal microsomes, which contain CYP3A7, are all capable of hydroxylating MDZ at the 1′- and 4-positions. A good correlation between the rate of MDZ 4-hydroxylation and the rate of MDZ 1′-hydroxylation by human hepatic microsomes suggests that both metabolites are formed by a single enzyme or by a group of enzymes that are closely regulated. This observation is in good agreement with previous findings [16, 17, 19]. In addition, purified CYP3A4

Table 3. Estimated Michaelis–Menten parameters (±SE*) for the formation of 1′-OHM and 4-OHM by purified CYP3A4 and CYP3A5

Midazolam	CYP3A4	CYP3A5
1′-Hydroxylation		
V_{max} (pmol/pmol CYP3A/min)	1.6 ± 0.1	3.7 ± 0.2
K_m (μM)	43.5 ± 4.4	27.1 ± 5.4
CL_{int} (mL/min/pmol CYP3A)	36.8×10^{-6}	136.5×10^{-6}
4-Hydroxylation		
V_{max} (pmol/pmol CYP3A/min)	1.4 ± 0.1	1.1 ± 0.1
K_m (μM)	150.6 ± 16.8	106.0 ± 21.9
CL_{int} (mL/min/pmol CYP3A)	9.3×10^{-6}	10.4×10^{-6}

* Standard error represents the asymptotic standard error of the parameter estimated by nonlinear regression.

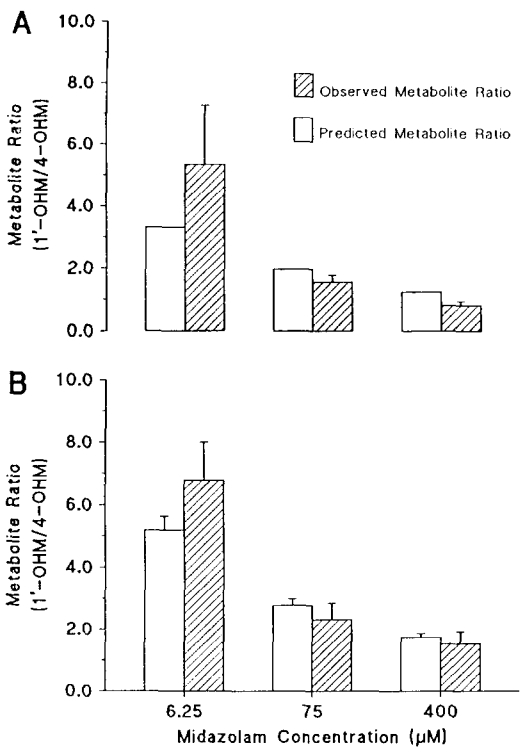

Fig. 7. Comparison of the mean (± SD) observed and predicted ratio of the rates of formation of 1'-hydroxymidazolam to 4-hydroxymidazolam (metabolite ratio) at three substrate concentrations for (A) human liver microsomes containing only CYP3A4 (N = 11) and (B) samples containing CYP3A4 and CYP3A5 (N = 7).

and CYP3A5 in reconstituted systems using DLPC were capable of catalyzing MDZ 1'- and 4-hydroxylations, which is direct evidence that these two CYP3A subfamily members are capable of catalyzing the metabolism of MDZ. Clearly, the use of DLPC as the sole phospholipid in the reconstituted enzyme system does not correspond to the *in vivo* situation. However, this well-defined reconstitution system was chosen to facilitate comparisons with previous work and because DLPC and "natural" lipids or lipid mixtures have resulted in similar catalytic activities for this enzyme family [10, 26]. The good agreement between human microsomes, purified CYP3A4, cDNA expressed CYP3A4, and the prediction by the kinetic model (Equation 1) suggests that either CYP3A3 is not present in these enzyme preparations (*vide supra*) or that it is present but is identical to CYP3A4 in its regioselectivity towards MDZ. In either case, the presence or absence of CYP3A3 does not impact upon the potential use of MDZ as a probe to indicate the presence of CYP3A5 in a given sample.

Interestingly, purified CYP3A5 exhibited a greater efficiency than purified CYP3A4, as reflected in intrinsic clearance (Table 3), in the 1'-hydroxylation of MDZ. These data contrast with previous reports in which the catalytic activity of CYP3A5 was less than (7-ethoxycoumarin, benzo[*a*]pyrene, quinidine, cyclosporine) or at best equal to (nifedipine) the catalytic capability of CYP3A4 [8–10]. Thus, the 1'-hydroxylation of MDZ appears to be the first example of a higher catalytic efficiency of CYP3A5 compared with CYP3A4. The ability of CYP3A4 and CYP3A5 to exhibit this difference in regioselectivities appears to have important consequences for the interpretation of correlations between CYP3A-mediated enzyme activities. For example, our data illustrate that for biotransformations that are minimally dependent on CYP3A5, such as erythromycin N-demethylation and testosterone 6β-hydroxylation, the greatest correlation with MDZ hydroxylation is only apparent for adult microsomal samples devoid of CYP3A5. These findings are of great importance when a correlation between form-specific and unknown biotransformations by microsomes is employed to indicate co-dependence on CYP3A4. The most powerful analysis will result when both substrates are not metabolized by CYP3A5 or microsomes lacking CYP3A5 are employed. In all other cases, the outcome of the correlation approach will reflect the relative dependence of the two biotransformations on CYP3A4 and CYP3A5.

The capability of human fetal hepatic microsomes to catalyze the oxidation of MDZ was also investigated. It was shown that fetal hepatic samples readily hydroxylate MDZ at rates comparable with those observed with samples from adults but with a different regioselectivity. A good correlation between the rate of 1'-OHM formation and the rate of 4-OHM formation was observed in the fetal samples, again suggesting that a single enzyme or a group of closely regulated enzymes are responsible for the hydroxylation of MDZ. Ladona *et al.* [11] demonstrated that MDZ inhibits ethylmorphine and codeine *N*-demethylase activities in fetal liver microsomes, and these biotransformations have been shown to be associated with CYP3A7 via correlation analysis [12]. It is reasonable to suggest, therefore, that CYP3A7 is responsible for the hydroxylation of MDZ in fetal tissue but this was not confirmed due to the scarcity of human fetal liver samples.

Additional stereoselective differences in the catalytic activities of the human members of the CYP3A subfamily may exist due to the prochiral nature of the MDZ molecule at the 4-position. The enantiomers of 4-OHM are readily resolved chromatographically using an α_1-acid glycoprotein column, but the rapid racemization of 4-OHM in aqueous environments prevents characterization of this potential difference in catalytic activity using current techniques.*

For substrates of the CYP3A family of enzymes, there is often considerable variability in pharmacokinetic parameters [27–29]. However, in the case of MDZ, little variability in pharmacokinetic parameters has been noted among normal healthy individuals. For example, Mandema *et al.* [30] found a 2 to 5-fold [coefficient of variation (C.V.) 17–56%] variation in MDZ clearance depending on whether

* Gorski JC and Hall SD, unpublished data.

intravenous or oral administration was considered. With the exception of the microsomes known to be induced, the variability in rates of formation of 4-OHM and 1′-OHM in this study were 6- and 10-fold, respectively (C.V. 47–70%). As expected from the regioselectivities of CYP3A4 and CYP3A5, when the samples containing only CYP3A4 were considered, the variability was reduced to approximately 5-fold for both activities (C.V. 49%). Thus, it appears that, at least in the case of MDZ as compared with most CYP3A substrates, the variabilities in *in vivo* and *in vitro* parameters reflective of metabolic efficiency are comparable. Furthermore, it is possible that the polymorphic distribution of CYP3A5 within normal individuals contributes to the pharmacokinetic variability often observed.

The human CYP3A subfamily is involved in the biotransformation of a number of clinically important pharmacologic agents including cyclosporine A [7], nifedipine [5], diltiazem [31], MDZ [15–17, 23], triazolam [17], quinidine [32], terfenadine [33], lidocaine [34], and erythromycin [4]. Furthermore, differences between CYP3A4 and CYP3A5 in their biotransformation of testosterone, quinidine, erythromycin and ethynylestradiol have been observed *in vitro* [8–10]. Thus, the presence of CYP3A5 in approximately 20–30% of normal individuals [9] may alter the metabolism of these commonly used drugs resulting in clinically significant changes in their pharmacokinetics, pharmacodynamics, and toxicity. Patients expressing CYP3A5 in addition to CYP3A4 may exhibit an increased MDZ clearance *in vivo* or may experience an altered pharmacodynamic profile of MDZ due to increased formation of the active metabolite, 1′-OHM [30, 35]. Hypotheses such as these have not been specifically addressed because the identification of subjects expressing CYP3A5 has not been possible to date. In view of the therapeutic importance and limited therapeutic index (e.g. cyclosporine A, terfenadine) of some CYP3A substrates, there is considerable interest in the development of probe substrates for quantifying CYP3A activity *in vivo*.

One of the earliest attempts to assess *in vivo* CYP3A activity was through the oral administration of nifedipine, an equally good substrate for both CYP3A4 and CYP3A5 [8, 10]. A plot of the frequency distribution of the area under the plasma curve of nifedipine, dehydronifedipine (M-O), or the ratio of the parent drug to metabolite area under the plasma curves resulted in a skewed distribution [27, 36]. This may reflect the finding that approximately 30% of the adult population expresses CYP3A5 in addition to CYP3A4. Although nifedipine may represent the gold standard for assessing CYP3A activity, the routine use of nifedipine is limited by the need for blood samples and secondary metabolism of the M-O metabolite via additional oxidative pathways, such that the urinary excretion of the M-O metabolite may not accurately reflect CYP3A activity. Thus, a more robust *in vivo* probe of CYP3A activity is desired.

Presently, two methods have been prospectively investigated to determine such activity *in vivo*, namely the erythromycin "breath test" and the urinary ratio of 6β-cortisol to free cortisol [29, 37, 38]. The erythromycin breath test involves the measurement of radiolabeled carbon dioxide expired over 1 hr after the intravenous administration of [^{14}C]erythromycin [37]. This breath test correlates with *in vitro* determined erythromycin *N*-demethylase activity [37], immunoquantified amounts of CYP3A [39], and cyclosporine trough concentrations [40]. However, the erythromycin breath test is not suitable for widespread use in its current form primarily due to the use of intravenous radioactive erythromycin. Furthermore, the erythromycin breath test is restricted to the prediction of CYP3A4 activity because erythromycin is apparently not a substrate for CYP3A5 [10]. The latter feature may diminish the power of this breath test to predict cyclosporine trough concentrations since CYP3A5 is capable of metabolizing cyclosporine [40].

A second method of characterizing CYP3A activity *in vivo*, the urinary ratio of 6β-cortisol to free cortisol, has also been used [38]. The major advantage of this method is that no drug is administered. Although it has much potential, this test has not been an adequate estimator of CYP3A activity. Rather, it has produced contradictory results after the administration of inhibitors and inducers [38]. In addition, it is not a good predictor of cyclosporine trough concentrations [40]. At this time, robust methods for determining *in vivo* CYP3A activity are lacking and no method currently used is capable of discriminating between patients expressing CYP3A4 only and those expressing CYP3A5 in addition to CYP3A4. In view of the exclusive metabolism of MDZ via the CYP3A subfamily of enzymes and its potential for discriminating among individuals with and without CYP3A5, MDZ may prove to be a useful phenotypic probe for assessing CYP3A activities *in vivo*. Such an *in vivo* approach may be based on the metabolite ratio in a plasma and/or urine sample following non-sedating doses (approximately 1 mg) of midazolam. Under these conditions plasma concentrations of midazolam would be expected to be low (1 μM or less) relative to the K_m of CYP3A4 or CYP3A5 (Table 3) and, therefore, the relative intrinsic clearances of metabolite formation will determine the observed metabolite ratio. However, the metabolite ratios that would discriminate between the presence or absence of CYP3A5 must also take into account differences in the disposition of 1′-OHM and 4-OHM and will need to be prospectively assessed *in vivo*.

In conclusion, the *in vitro* results demonstrated that MDZ is metabolized by CYP3A4, CYP3A5, and probably CYP3A7. The various human members of the CYP3A subfamily showed regioselective differences in MDZ hydroxylation with the ratio of the rates of metabolite formation indicative of the expressed CYP3A form(s) in a microsomal sample. Future research is planned to explore the potential of using MDZ as an *in vivo* probe for the activity and expression of the human CYP3A subfamily members.

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