Midazolam and Triazolam Biotransformation in Mouse and Human Liver Microsomes: Relative Contribution of CYP3A and CYP2C Isoforms

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ABSTRACT

Midazolam (MDZ) and triazolam (TRZ) hydroxylation, reactions considered to be cytochrome P-4503A (CYP3A)-mediated in humans, were examined in mouse and human liver microsomes. In both species, α- and 4-hydroxy metabolites were the principal products. Western blotting with anti-CYP3A1 antibody detected a single band of immunoreactive protein in both human and mouse samples: 0.45 ± 0.12 and 2.02 ± 0.24 pmol/mg protein (mean ± S.E., n = 3), respectively. Ketoconazole potently inhibited MDZ and TRZ metabolite formation in human liver microsomes (IC50 range, 0.038–0.049 μM). Ketoconazole also inhibited the formation of both TRZ metabolites and of 4-OH-MDZ formation in mouse liver microsomes (IC50 range, 0.0076–0.025 μM). However, ketoconazole (10 μM) did not produce 50% inhibition of α-OH-MDZ formation in mouse liver microsomes. Anti-CYP3A1 antibodies produced concentration-dependent inhibition of MDZ and TRZ metabolite formation in human liver microsomes and of TRZ metabolite and 4-OH-MDZ formation in mouse liver microsomes to less than 20% of control values but reduced α-OH-MDZ formation to only 66% of control values in mouse liver microsomes. Anti-CYP2C11 antibodies inhibited α-OH-MDZ metabolite formation in a concentration-dependent manner to 58% of control values in mouse liver microsomes but did not inhibit 4-OH-MDZ formation. Thus, TRZ hydroxylation appears to be CYP3A specific in mice and humans. α-Hydroxylation of MDZ has a major CYP2C component in addition to CYP3A in mice, demonstrating that metabolic profiles of drugs in animals cannot be assumed to reflect human metabolic patterns, even with closely related substrates.

Species differences are known to exist for cytochrome P-450 (CYP) content, activity, and protein sequence (Abel et al., 1993; Echizen et al., 1994; Sharer et al., 1995; Ghosal et al., 1996; Chauret et al., 1997; Court et al., 1997; Guengerich, 1997; Tomlinson et al., 1997; Zhao and Ishizaki, 1997; Higashikawa et al., 1999; Whalen et al., 1999). Nonetheless, studies in animals are often undertaken to provide possible clinical insight without complete validation of the human relevance. Rodents have historically been used as in vivo metabolism models due to their relatively low expense and minimal husbandry needs. The use of mice in models of disease and therapeutics has grown; the National Cancer Institute continues with both the Mouse Cancer Genome Anatomy Project and the Mouse Models of Human Cancer Consortium. In addition, genetic knockout mice have become available in recent years and are the only knockout models currently available to study drug metabolism and disposition. Some examples of knockouts used in recent drug metabolism studies include the aryl hydrocarbon receptor, specific CYP isoforms, and P-glycoprotein (Valentine et al., 1996; Diliberto et al., 1997; Nebert and Duffy, 1997; Perloff et al., 1999). Little work has been done, however, to validate mouse CYP isoform-specific index reactions and their relevance to humans. A comparison of normal mouse and human drug metabolism is necessary if data from knockout mice or therapeutic interventions in mouse models of disease are to be extrapolated to humans.

Of particular interest in humans is CYP3A4, which is the most abundant CYP isoform and is thought to metabolize more than 50% of drugs used in clinical practice (Benet et al., 1996; Maurel, 1996). Of the three CYP3A isoforms isolated in mice, CYP3A11 is the most comparable to CYP3A4, having 76% amino acid homology (Yanagimoto et al., 1992). Few studies have compared human and mouse CYP3A-mediated reactions. Some putative human CYP3A substrates have been tested in mice with varying results regarding CYP3A specificity and kinetic profile in relation to humans (Abel et
at 37°C, reactions were stopped by cooling on ice and the addition of microsomal protein. After 5 min (MDZ) or 20 min (TRZ) incubation with human and mouse microsomes was linear with respect to incubation time and protein, respectively. Formation of metabolites with human and mouse microsomes was probed with the secondary antibody, donkey anti-rabbit Ig-horseradish peroxidase, and 3% dry milk for 1 h, incubated with polyclonal rabbit anti-CYP3A1 cDNA-expressed CYP3A4 (Genetest) was used to generate calibration standards at concentrations of 0.375, 0.75, 1.5, and 3.0 pmol/µl of 10 mM phosphate buffer, pH adjusted to 7.4 with NaOH, with a flow rate of 1.4 ml/min. The analytical column (30 cm × 3.9 mm) was a stainless steel reverse phase C-18 microBondapak (Waters Associates, Milford, MA). Column effluent was monitored by ultraviolet absorbance at 220 nm.

**Quantitative Western Blotting.** Western blot procedures were similar to those previously described (Perloff et al., 1999). Microsomal protein was separated from SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels. Sample wells were loaded with 0.4 µg of mouse or 1.2 µg of human liver microsomal protein. Human cDNA-expressed CYP3A4 (Genetest) was used to generate calibration standards at concentrations of 0.375, 0.75, 1.5, and 3.0 pmol/well. Samples were run at 80 V for 2 h in 25 mM Tris buffer/0.2 M glycine/0.1% SDS buffer (pH 8.3). Samples were then transferred to Immobilon-P (PVDF membrane; Millipore, Bedford, MA) for 1 h at 10 V in 25 mM Tris buffer/20% methanol. Blots were blocked with 3% dry milk for 1 h, incubated with polyclonal rabbit anti-CYP3A1 (1:3000) for 1 h, and then reblocked for 30 min. Blots were then probed with the secondary antibody, donkey anti-rabbit Ig-horseradish peroxidase to yield final concentrations of 50 and 250 µM TRZ and 10 µg of microsomal protein were preincubated for 30 min at 37°C with 0, 25, 50, or 100 µg of serum protein from immunized rabbits or goats or the same quantities of appropriate control preimmune serum. Incubations were initiated by the addition of cofactor solution and stopped after 5 (MDZ) or 20 (TRZ) min with cooling and acetonitrile as described above. Controls with no inhibitor were performed at the beginning and end of each inhibition experiment. Metabolite formation is expressed as a percentage of control, based on metabolite to internal standard peak height ratio, in the inhibition studies and IC50 analysis.

**Chemical Inhibition.** Inhibition studies with ketoconazole (0–10 μM), chloramphenicol (100 µM), α-naphthoflavone (5 µM), omeprazole (10 µM), orphenadrine (200 µM), quinidine (5 µM), quinine (5 µM), and sulfaphenazole (20 µM) were performed by coaddition to MDZ or TRZ incubates. Chemical inhibition incubations were performed as described earlier. Mechanism-based inhibitors were preincubated (standard duration) with microsomes and cofactor for 2 min (8-methoxypsoralen, 2.5 µM), 15 min (diethyldithiocarbamate, 20 and 100 µM), or 20 min (troleandomycin, 500 µM) before the addition of MDZ. The incubation was then stopped 5 min after the addition of MDZ by cooling and acetonitrile as described above.

**Materials.** MDZ, TRZ, α-OH-MDZ, 4-OH-MDZ, α-OH-TRZ, and 4-OH-TRZ were kindly provided by their pharmaceutical manufacturers or purchased from Ultrafine Chemicals (Oxford, England). Ketoconazole was a gift from Janssen Pharmaceutica N.V. (Beerse, Belgium). Other chemical inhibitors and reaction cofactors (NADP+, DL-isocitric acid, magnesium chloride, isocitric dehydrogenase, and potassium phosphate buffer solutions) were obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibody to rat CYP3A1 and control serum developed in rabbit were obtained from Xenotech (Phoenix, AZ). Polyclonal antibody to rat CYP2C11, CYP2E1, and control serum developed in goat were obtained from Gentest (Woburn, MA).

**Microsomal Preparation.** Liver samples from human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA) or by the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis). Three human livers, characterized as high CYP3A metabolizers from a library of livers, were used for all studies. Mouse livers were taken from three male Crl:CD-1(ICR)BR (CD1) mice (8–10 weeks old; Charles River Laboratories, MA). Mice had been maintained on a 12-h light/dark cycle and provided food and water on an ad libitum basis. All microsomes were prepared using standard techniques (von Molkte et al., 1993). In brief, microsomes were prepared through ultracentrifugation; microsomal pellets were resuspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −80°C until use. Total protein concentration was determined by a bicinchoninic acid protein assay (BCA assay; Pierce Chemical, Rockford, IL) with BSA as a standard (Smith et al., 1985).

**Incubation.** Incubation mixtures contained 50 mM phosphate buffer, 5 mM Mg2+, 0.5 mM NADP+, and an isocitrate/isocitric dehydrogenase regenerating system. For inhibition studies, MDZ and TRZ in methanol solution were aliquoted into incubation tubes containing 20% glycerol and stored at 80°C until use. Total protein in reaction mixtures was determined based on calibration curves constructed from a series of standards containing varying known amounts of metabolite standards together with internal standard.

**Reaction velocities were calculated in units of nanomoles of product formed per minute per milligram of microsomal protein.** Control incubations with no cofactor, no protein, and/or no MDZ or TRZ substrate were performed concurrently to validate CYP-dependent metabolism. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards.

**Chemical Inhibition.** Inhibition studies with ketoconazole (0–10 µM), α-naphthoflavone (5 µM), orphenadrine (200 µM), quinidine (5 µM), quinine (5 µM), and sulfaphenazole (20 µM) were performed by coaddition to MDZ or TRZ incubates. Chemical inhibition incubations were performed as described earlier. Mechanism-based inhibitors were preincubated (standard duration) with microsomes and cofactor for 2 min (8-methoxypsoralen, 2.5 µM), 15 min (diethyldithiocarbamate, 20 and 100 µM), or 20 min (troleandomycin, 500 µM) before the addition of MDZ. The incubation was then stopped 5 min after the addition of MDZ by cooling and acetonitrile as described above. Controls with no inhibitor were performed at the beginning and end of each inhibition experiment. Metabolite formation is expressed as a percentage of control, based on metabolite to internal standard peak height ratio, in the inhibition studies and IC50 analysis. **Immunoinhibition.** Serum from goats or rabbits containing polyclonal anti-CYP antibodies has previously been shown to inhibit the activity of human CYPs in a concentration-dependent manner (Kronbach et al., 1989; Ghosal et al., 1996; Schmider et al., 1996; von Molkte et al., 1996b). Initially, antisera to CYPs 3A1, 2B1, 2C11, and 2E1 was tested for activity of each antibody against MDZ hydroxylation in mouse and human liver microsomes at a single polyclonal serum-to-microsomal protein ratio (10:1). Polyclonal antibody that inhibited MDZ metabolism formation was then tested at several polyclonal serum-to-microsomal protein ratios (2.5:1, 5:1, and 10:1) to demonstrate a concentration-dependent response. In preliminary experiments, 20:1 ratios were also tested, demonstrating minimal increase in inhibition over 10:1 ratios. In each experiment, 50 µM MDZ or 250 µM TRZ and 10 µg of microsomal protein were preincubated for 30 min at 37°C with 0, 25, 50, or 100 µg of serum protein from immunized rabbits or goats or the same quantities of appropriate control preimmune serum. Incubations were initiated by the addition of cofactor solution and stopped after 5 (MDZ) or 20 (TRZ) min with cooling and acetonitrile as described above.

**HPLC.** For MDZ and TRZ and their metabolites, HPLC mobile phase consisted of 200 ml of acetonitrile, 350 ml of methanol, and 450 ml of 10 mM phosphate buffer, pH adjusted to 7.4 with NaOH, with a flow rate of 1.4 ml/min. The analytical column (30 cm × 3.9 mm) was a stainless steel reverse phase C-18 microBondapak (Waters Associates, Milford, MA). Column effluent was monitored by ultraviolet absorbance at 220 nm.
ish peroxidase (1:6000) for 1 h. Super Signal Cl-HRP Substrate System (Pierce Chemical) was used to activate the horseradish peroxidase signal. Blots were then exposed to radiographic film. Quantification of protein was completed via computer image analysis (Image Pro Plus; Media Cybernetics). A standard curve of pixel area density versus picomoles of CYP3A4 was created and fit to the equation: 

\[ y = \frac{V_{\text{max}} \cdot S}{S + K_{m} (1 + I/K_i)} \]

Data Analysis. Kinetic parameters for MDZ and TRZ biotransformation without the addition of inhibitors were determined through nonlinear least-squares regression analysis of untransformed data. In both mouse and human liver microsomes, 4-OH-MDZ formation data were fit to a substrate activation (Hill) model, and \( \alpha \)-OH-MDZ formation data were fit to a model incorporating Michaelis-Menten kinetics with uncompetitive substrate inhibition (Segel, 1975; von Moltke et al., 1996b; Perloff et al., 1999). In human liver microsomes, 4-OH-TRZ formation data were fit to a substrate activation (Hill) model, and \( \alpha \)-OH-TRZ formation data were fit to a Michaelis-Menten model (Segel, 1975; von Moltke et al., 1996b). In mouse liver microsomes, 4-OH-TRZ formation data were fit to a Michaelis-Menten model, and \( \alpha \)-OH-TRZ formation data were fit to a Michaelis-Menten kinetics model with uncompetitive substrate inhibition (Segel, 1975). The parameters that were estimated are \( V_{\text{max}} \), the maximum reaction velocity (uninhibited); \( K_{m} \), the substrate concentration corresponding to 50% \( V_{\text{max}} \); and \( K_i \), the uncompetitive substrate inhibition constant.

Data for reaction velocities with the coaddition of ketoconazole were evaluated using double reciprocal (Lineweaver-Burke) plots. Based on Lineweaver-Burke plots (Segel, 1975; von Moltke et al., 1996b) and nonlinear regression goodness-of-fit analysis, inhibition of metabolite formation was analyzed by 1) a competitive inhibition model, 2) a mixed competitive-noncompetitive inhibition model, or 3) a competitive model with uncompetitive substrate inhibition:

\[ V = \frac{V_{\text{max}} \cdot S}{S + K_{m} (1 + I/K_i)} \]

\[ V = \frac{V_{\text{max}} \cdot S}{K_m (1 + I/K_i) + S [1 + (I/\alpha K_i)]} \]

\[ V = \frac{V_{\text{max}} \cdot S}{K_m (1 + I/K_i) + S [1 + (S/K_i)]} \]

where \( I \) is the concentration of ketoconazole, and \( K_i \) is the inhibition constant. \( V_{\text{max}}, K_{m}, \) and \( K_i \) were determined from these equations using nonlinear regression analysis. \( S \) is substrate concentration, \( V \) is the reaction velocity in presence of the ketoconazole, and \( \alpha \) is a factor that is greater than or equal to 1 and represents the "mix" of inhibition mechanism between competitive and noncompetitive.

Fig. 1. Rates of formation of \( \alpha \)-OH-MDZ, 4-OH-MDZ, \( \alpha \)-OH-TRZ, and 4-OH-TRZ in relation to concentrations of the substrates, MDZ and TRZ, by microsomal preparations of a representative mouse (A and C) and human (B and D) sample. Note that the y-axis in D is not the same as in A–C. Units for \( V_{\text{max}} \) and \( K_{m} \) are nmol/min/mg protein and \( \mu \text{M} \), respectively. Lines represent functions determined by nonlinear least-squares regression analysis.

\[ y = m(x) + b \]
strate concentration in the presence of varying inhibitor concentrations (Venkatakrishnan et al., 1998b; von Moltke et al., 1998).

\( K_i \) and kinetic values in the presence of 2.5 \( \mu \)M ketoconazole are presented as mean \( \pm \) S.D. (\( n = 2 \)). All other values that represent more than one individual trial were performed using three different liver samples (\( n = 3 \)) and are presented as mean \( \pm \) S.E. Each data point represents the mean of duplicate incubations.

Results

MDZ and TRZ Biotransformation In Vitro. Kinetic parameters for MDZ hydroxylation in the mouse samples were \( V_{max} = 1.43 \pm 0.07 \) (mean \( \pm \) S.E., \( n = 3 \)) and 0.60 \( \pm \) 0.05 nmol/min/mg protein and \( K_m = 3.28 \pm 0.59 \) and 20.4 \( \pm \) 1.75 \( \mu \)M for \( \alpha-OH-MDZ \) and 4-OH-MDZ formation, respectively (Fig. 1A). Substrate inhibition (\( K_i = 405 \pm 17 \) \( \mu \)M) was evident in the kinetics of MDZ \( \alpha \)-hydroxylation. Kinetic parameters for MDZ hydroxylation in human samples were \( V_{max} = 2.43 \pm 0.31 \) and 2.44 \( \pm \) 0.54 nmol/min/mg protein and \( K_m = 6.35 \pm 1.26 \) and 56.7 \( \pm \) 8.8 \( \mu \)M for \( \alpha-OH-MDZ \) and 4-OH-MDZ formation, respectively (Fig. 1B). \( \alpha-OH-MDZ \) formation demonstrated substrate inhibition (\( K_i = 675 \pm 63 \) \( \mu \)M). MDZ \( \alpha \)-hydroxylation accounted for a greater percentage of estimated intrinsic clearance (\( V_{max}/K_m \)) than 4-hydroxylation in both the mouse and human samples (93.7 \( \pm \) 0.8% and 90.0 \( \pm \) 1.7%, respectively). In incubations performed at MDZ concentrations below 1 \( \mu \)M, the \( \alpha-OH-MDZ \) metabolite accounted for 91.3 and 89.4% of detectable metabolite formed in mouse and human liver microsomes, respectively (data not shown). Submicromolar concentrations (0–1 \( \mu \)M = 0–341.7 \( \mu \)M) correspond to human plasma levels at an ED_{50} for MDZ in various clinical effect models (Laurijssens and Greenblatt, 1996). This, along with estimated intrinsic clearance data, indicates \( \alpha-OH-MDZ \)-formation is the principal MDZ clearance pathway.

Kinetic parameters for TRZ hydroxylation in the mouse samples were \( V_{max} = 1.93 \pm 0.15 \) and 2.07 \( \pm \) 0.13 nmol/min/mg protein and \( K_m = 28.0 \pm 1.2 \) and 234 \( \pm \) 25 \( \mu \)M for \( \alpha-OH-TRZ \) and 4-OH-TRZ formation, respectively (Fig. 1C). Substrate inhibition (\( K_i = 1618 \pm 281 \) \( \mu \)M) was evident in the kinetics of TRZ \( \alpha \)-hydroxylation. Mean kinetic parameters for TRZ hydroxylation in human samples were \( V_{max} = 8.25 \pm 0.69 \) and 15.7 \( \pm \) 1.93 nmol/min/mg protein and \( K_m = 89.2 \pm 2.3 \) and 265.6 \( \pm \) 1.2 \( \mu \)M for \( \alpha-OH-TRZ \) and 4-OH-TRZ formation, respectively (Fig. 1D). TRZ \( \alpha \)-hydroxylation accounted for a greater percentage (88.6 \( \pm \) 3.2%) of estimated intrinsic clearance (\( V_{max}/K_m \)) than 4-hydroxylation (11.4 \( \pm \) 0.5%) in the mouse samples. However, in the human samples, intrinsic clearance values for the \( \alpha \)-hydroxylation pathway (61.0 \( \pm \) 6.3%) and the 4-hydroxylation pathway (39.0 \( \pm \) 4.9%) were similar.

Immunoreactive CYP3A Protein Quantification. Western blotting detected a single protein band for both human and mouse samples (Fig. 2A). Blots were then scanned and quantified by computer image analysis. A standard curve was plotted using known amounts of CYP3A4 (Fig. 2B) and used for calibration. The mean quantity of immunoreactive CYP3A protein was 2.02 \( \pm \) 0.24 (mean \( \pm \) S.E., \( n = 3 \)) pmol/mg protein in mice and 0.45 \( \pm \) 0.12 pmol/mg in humans (Fig. 2C).

Inhibition of MDZ and TRZ Biotransformation In Vitro with Ketoconazole

Ketoconazole IC_{50} determinations were performed at substrate concentrations (50 \( \mu \)M MDZ and 250 \( \mu \)M TRZ) near or exceeding the \( K_m \) values. In mouse liver microsomes, ketoconazole potently inhibited 4-OH-MDZ formation (IC_{50} = 0.025 \( \pm \) 0.001 \( \mu \)M, mean \( \pm \) S.E., \( n = 3 \)) but did not produce 50% inhibition of \( \alpha-OH-MDZ \)-formation, with reaction velocity reduced to 54% of control values at 10 \( \mu \)M ketoconazole (Fig. 3A). However, ketoconazole was a potent inhibitor of both 4-OH-MDZ and \( \alpha-OH-MDZ \)-formation in human liver microsomes, with IC_{50} values of 0.038 \( \pm \) 0.013 and 0.044 \( \pm \) 0.014 \( \mu \)M, respectively (Fig. 3B). TRZ metabolite formation in mouse and human liver mi-
crosomes was highly sensitive to ketoconazole inhibition. IC₅₀ values were 0.0076 ± 0.0034 and 0.012 ± 0.002 μM for 4-OH-TRZ and α-OH-TRZ formation, respectively, in mouse liver microsomes (Fig. 3C) and 0.049 ± 0.021 and 0.049 ± 0.020 μM in human liver microsomes (Fig. 3D).

Table 1 shows Ki values determined using inhibition models that were consistent with Lineweaver-Burke plots and represented the “best fit,” with theoretical equations demonstrating r² values of greater than 0.82 in all cases. Ketoconazole Ki values for α-OH-MDZ formation were approximately 25 times higher than Ki values for 4-OH-MDZ formation in mouse liver microsomes. Ki values for α-OH-MDZ formation
were more than 300 times higher in the mouse compared with human liver microsomes, whereas values for 4-OH-MDZ were similar between species (Table 1). In inhibition experiments using 2.5 μM ketoconazole, MDZ hydroxylation was nearly eliminated in the human liver microsomes (Fig. 5A). In mouse liver microsomes, α-OH-MDZ formation (Fig. 4A) remained a significant metabolic pathway in the presence of 2.5 μM ketoconazole [apparent $V_{\text{max}}$ value of 1.81 ± 0.270 nmol/min/mg protein (mean ± S.E., n = 3) and a $K_m$ value of 8.99 ± 0.025 μM that can be attributed to CYP2C based on antibody inhibition studies. 4-OH-MDZ, α-OH-TRZ, and 4-OH-TRZ formation was greatly reduced with 2.5 μM ketoconazole (B–D), eliminating more than 90% of estimated intrinsic clearance. Lines represent functions determined by nonlinear least-squares regression analysis.

![Graphs](image)

**Fig. 4.** Metabolite formation rates in relation to concentrations of the substrates MDZ and TRZ by microsomal preparations of a representative mouse sample with and without the coaddition of 2.5 μM ketoconazole. α-OH-MDZ formation kinetics in the presence of 2.5 μM ketoconazole (A) demonstrates activity with a $V_{\text{max}}$ value of 1.81 ± 0.270 nmol/min/mg protein (mean ± S.E., n = 3) and a $K_m$ value of 8.99 ± 0.025 μM that can be attributed to CYP2C based on antibody inhibition studies. 4-OH-MDZ, α-OH-TRZ, and 4-OH-TRZ formation was greatly reduced with 2.5 μM ketoconazole (B–D), eliminating more than 90% of estimated intrinsic clearance. Lines represent functions determined by nonlinear least-squares regression analysis.

Inhibition of MDZ Biotransformation In Vitro with Various Chemical Inhibitors

Only the compounds previously established as CYP3A inhibitors reduced 4-OH-MDZ and α-OH-MDZ formation to less than 75% of control values in both mouse (pooled samples) and human liver microsomes (Table 2). Diethyldithiocarbamate (DDC) caused little inhibition of MDZ metabolite formation at 20 μM but nearly eliminated MDZ hydroxylation at 100 μM. Ketoconazole (2.5 μM) reduced 4-OH-MDZ and α-OH-MDZ metabolite formation to 11 and 52% of control values, respectively, in mouse liver microsomes and to less than 10% in human liver microsomes. Troleandomycin (500 μM) reduced 4-OH-MDZ and α-OH-MDZ metabolite formation to 46 and 66% of control values in mouse liver
microsomes and 10 and 13% of control values in human liver microsomes, respectively.

Inhibition of MDZ Biotransformation by Polyclonal Antibody. MDZ metabolism was also investigated by coincubating polyclonal antibody serum to various CYPs with liver microsomes (10:1 protein ratios). These specific antibodies have previously been shown to have inhibition profiles against CYP index reactions (Schmider et al., 1996). In pooled human liver microsomes, minimal inhibition was produced by serum containing anti-CYP2B1, -CYP2C11, and -CYP2E1 antibodies and control preimmune serum. Serum containing anti-CYP3A1 antibodies reduced 4-OH-MDZ and \( \alpha \)-OH-MDZ metabolite formation to approximately 15% of control values in human liver microsomes (Fig. 6A). In mouse liver microsomes, minimal inhibition was produced by serum containing anti-CYP2B1 and -CYP2E1 antibodies and control preimmune serum. Serum containing anti-CYP3A1 antibodies reduced 4-OH-MDZ and \( \alpha \)-OH-MDZ metabolite formation to 17 and 67% of control values in mouse liver microsomes, respectively. Serum containing anti-CYP2C11 antibodies inhibited \( \alpha \)-OH-MDZ metabolite formation to 59% of control values but did not inhibit 4-OH-MDZ formation in mouse liver microsomes (Fig. 6B).

In immunoinhibition concentration-response experiments, anti-CYP3A1 reduced all MDZ and TRZ metabolite formation in mouse and human liver microsomes in a concentration-dependent manner (less than 20% of control values at the 10:1 ratios), with the exception of \( \alpha \)-OH-MDZ formation.

**TABLE 2**

Effect of various chemical inhibitors on MDZ metabolite formation in mouse and human liver microsomes

Experiments were performed with a fixed concentration of MDZ (50 \( \mu \)M) using pooled mouse and human liver microsomes. Each value represents the mean of duplicate incubations. In no case did the duplicates differ by greater than 10%.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mouse Microsomes</th>
<th>Human Microsomes</th>
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<tbody>
<tr>
<td></td>
<td>( \alpha )-OH-MDZ</td>
<td>4-OH-MDZ</td>
</tr>
<tr>
<td>Troleandomycin*</td>
<td>500 ( \mu )M</td>
<td>66.0</td>
</tr>
<tr>
<td>Ketocazole</td>
<td>2.5 ( \mu )M</td>
<td>58.3</td>
</tr>
<tr>
<td>( \alpha )-Naphthoflavone</td>
<td>5 ( \mu )M</td>
<td>5.7</td>
</tr>
<tr>
<td>8-Methoxyxpsoralen*</td>
<td>2.5 ( \mu )M</td>
<td>74.9</td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>200 ( \mu )M</td>
<td>67.5</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>20 ( \mu )M</td>
<td>100.8</td>
</tr>
<tr>
<td>Omeprazole</td>
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<td>Quinine</td>
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<td>Quinidine</td>
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<td>103.6</td>
</tr>
<tr>
<td>DDC</td>
<td>20 ( \mu )M</td>
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</tr>
<tr>
<td></td>
<td>100 ( \mu )M</td>
<td>15.1</td>
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<tr>
<td>Chloramphenicol</td>
<td>100 ( \mu )M</td>
<td>74.8</td>
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*Mechanism-based inhibitors that were preincubated with microsomes and cofactor before addition of MDZ; all other inhibitors were coincubated with MDZ.
in mouse liver microsomes, which was only reduced to 66% of control values. No significant inhibition was produced by preimmune control serum (Fig. 7). Anti-CYP2C11 antibodies caused no reduction of MDZ or TRZ metabolite formation in mouse and human liver microsomes, with the exception of α-OH-MDZ formation in mouse liver microsomes, which was inhibited in a concentration-dependent manner (58% of control values at 10:1 ratios). No significant inhibition was produced by preimmune control serum (Fig. 8).

Discussion

Species differences in major metabolites produced, CYPs involved, $V_{\text{max}}$, and $K_m$ are common for many CYP substrates (Abel et al., 1993; Echizen et al., 1994; Sharer et al., 1995; Chauret et al., 1997; Court et al., 1997; Tomlinson et al., 1997; Eagling et al., 1998; Whalen et al., 1999). However, there are similarities in the mouse and human microsomal biotransformation of MDZ. The same two major metabolites are produced by each species (α-OH-MDZ and 4-OH-MDZ) but with notable differences in kinetic parameters for metabolite formation ($V_{\text{max}}$ and $K_m$). However, the net estimated intrinsic clearance via hydroxylation is very similar. It should be noted that the human liver samples used in this study were known to have high CYP3A content. Since it is recognized that human CYP3A content and activity can vary greatly from person to person (Shimada et al., 1994), $V_{\text{max}}$ and intrinsic clearance values obtained in this study may be higher than the mean values obtained from unselected liver samples. In any case, in vitro data presented here suggest that α-hydroxylation is the predominant mode of MDZ clearance in mice and humans. In vivo data have confirmed these results in humans, where α-OH-MDZ represented the majority of metabolite recovered in plasma and urine after MDZ administration (Heizmann and Ziegler, 1981).

In previous in vitro studies, α- and 4-hydroxylation of the benzodiazepines TRZ and alprazolam were highly sensitive to CYP3A inhibitors in both mice (Fahey et al., 1998; Warrington et al., 2000) and humans (von Moltke et al., 1996a; Schmider et al., 1996; Venkatakrishnan et al., 1998a). Despite the structural similarity of MDZ to TRZ and alprazolam, ketoconazole failed to inhibit MDZ α-hydroxylation more than 40% at concentrations (<2.5 μM) that are considered to be CYP3A specific in humans (Newton et al., 1995; Eagling et al., 1998). TRZ hydroxylation was potently inhib-
Ketoconazole. Although ketoconazole $K_i$ values for $\alpha$-OH-TRZ formation in mouse liver microsomes were higher than those in humans, similar IC$_{50}$ values were obtained at TRZ concentrations of 250 $\mu$M in both species. The reasons for this inconsistency are not established. Nonetheless, net intrinsic clearance of TRZ in the presence of 2.5 $\mu$M ketoconazole was less than 10% of control values in mouse and human liver microsomes, suggesting predominant CYP3A dependence in both species. This was verified in immunoinhibition studies in which anti-CYP3A1 antibodies reduced TRZ hydroxylation by 80% in both mouse and human liver microsomes.

Hydroxylation of TRZ in mouse and human samples was mainly CYP3A-dependent, although differences in enzyme efficiency and capacity were apparent. Net estimated intrinsic clearance via hydroxylation, however, was similar. $V_{\text{max}}$ for the formation of TRZ metabolites was much higher in human liver microsomes than the mouse, even though mouse liver microsomes had four times the amount of CYP3A immunoreactive protein. The antibody used in Western blotting was generated against rat CYP3A1, which has greater homology with mouse CYP3A than human (Yanagimoto et al., 1992). Therefore, differences across species in quantities of immunoreactive protein may not reflect corresponding quantitative differences in functionally active protein. However, the same anti-rat CYP3A1 antibody inhibited CYP3A-mediated metabolism equally in the mouse and human (Fig. 7). This suggests equal CYP affinity with respect to inhibition. Although inhibition studies and Western blotting were performed with the same antibody, binding site and affinity may greatly differ between the two assays. Furthermore, Western blotting was calibrated to human cDNA expressed standard (mouse cDNA expressed CYPs are not com-

![Fig. 7. Inhibition of formation of $\alpha$-OH-MDZ, 4-OH-MDZ, $\alpha$-OH-TRZ, and 4-OH-TRZ in mouse and human liver microsomes by rabbit serum containing polyclonal antibody against rat CYP3A1. Samples contained 50 $\mu$M MDZ (A and B) or 250 $\mu$M TRZ (C) and 10 $\mu$g of microsomal protein. Inhibition of metabolite formation is expressed as a ratio (in percent) of velocity with coaddition of 25, 50, or 100 $\mu$g of antiserum protein relative to the velocity with no antiserum added. Control incubations were performed with the addition of equivalent amounts of preimmune serum. Experiments were performed using pooled microsomes from three human and three mouse liver microsomal samples. Each value represents the mean of duplicate determinations, which did not differ by more than 10%.

![Fig. 8. Inhibition of formation $\alpha$-OH-MDZ and 4-OH-MDZ (A) and $\alpha$-OH-TRZ and 4-OH-TRZ (B) in mouse and human liver microsomes by goat serum containing polyclonal antibody against rat CYP2C11. Samples contained 50 $\mu$M MDZ or 250 $\mu$M TRZ and 10 $\mu$g of microsomal protein. Inhibition of metabolite formation is expressed as a ratio (in percent) of velocity with coaddition of 25, 50, or 100 $\mu$g of antiserum protein relative to the velocity with no antiserum added. Control incubations were performed with the addition of equivalent amounts of preimmune serum. Experiments were performed using pooled microsomes from three human and three mouse liver microsomal samples. Each value represents the mean of duplicate determinations, which did not differ by more than 10%.}
Inhibition profiles revealed that a major component of α-OH-MDZ formation was not mediated by CYP3A in the mouse. Ketoconazole failed to eliminate α-OH-MDZ formation in mouse liver microsomes. Troleandomycin, a relatively specific mechanism-based CYP inhibitor in humans, is considered a global inhibitor of CYP3A in mouse liver microsomes. Immunoinhibition studies confirmed CYP3A dependence for MDZ hydroxylation in mice, whereas in rats, a significant percentage (>40%) of α-OH-MDZ formation can be attributed to CYP2C.

Clinical benzodiazepine agonist effects of MDZ occur at submicromolar plasma concentrations (Laurijssens and Greenblatt, 1996), and at these levels, clearance would be predominantly attributable to the α-OH-MDZ pathway. This report demonstrates that two pathways (CYP3A and CYP2C) contribute to biotransformation of MDZ in the mouse, while only CYP3A is responsible for biotransformation in the human. This suggests that MDZ clearance is not a specific CYP3A index reaction in mice and may reflect that metabolic profiles of drugs in animals cannot be assumed to reflect human metabolic patterns. Animal studies involving metabolism and disposition, as well as therapeutic models of disease, must be designed and interpreted with an understanding of these possible differences.


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