Diltiazem Inhibition of Cytochrome P-450 3A Activity Is Due To Metabolite Intermediate Complex Formation

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ABSTRACT

Diltiazem (DTZ) N-demethylation occurs by cytochrome P-450 (CYP) 3A based on the following observations: 1) a single enzyme Michaelis-Menten model of metabolite formation, 2) high correlations of DTZ N-demethylation activity to other CYP3A activities, 3) inhibition of DTZ N-demethylation activity by triacetyloleandomycin, and 4) DTZ N-demethylation activity by expressed CYP3A enzymes only. The mean $K_m$ for DTZ N-demethylation in human liver microsomes and expressed CYP3A4($b_1$) were 53 and 16 $\mu$M, respectively. A 30-min preincubation of DTZ in expressed CYPs inhibited CYP3A4($b_1$) by 100%, of which 55% was due to formation of a metabolite intermediate complex (MIC), which is an inactive form of CYP. MIC was observed in human liver microsomes and cDNA-expressed CYP3A only. In experiments to assess simultaneous MIC formation and loss of CYP3A activity, DTZ caused greater than 80% inhibition of midazolam hydroxylation after a 60-min preincubation in human liver microsomes. The rate constants for MIC formation and loss of midazolam hydroxylation activity were equivalent for the line of best fit for both data sets, which illustrates that MIC formation causes the inhibition of CYP3A activity. The mechanistic inhibition was characterized in expressed CYP3A4($b_1$), which exhibited a concentration-dependent formation of MIC by DTZ (1–100 $\mu$M) with an estimated $k_{iact}$ of 0.17 $\text{min}^{-1}$ and $K_I$ of 2.2 $\mu$M. The partition ratio for expressed CYP3A4($b_1$) was substrate concentration dependent and varied from 13 to 86. This study showed that DTZ inhibition of CYP3A substrate metabolism occurs primarily by MIC formation.

Diltiazem (DTZ) is a benzothiazepine calcium channel blocker, which is widely used as a chronic therapy for the treatment of hypertension. N-demethylation of DTZ to N-desmethyldiltiazem (MA) is the primary pathway of elimination in humans, with lesser contributions from O-demethylation and deacetylation (Yeung et al., 1996). Recently, it was reported that MA formation is catalyzed primarily by cytochrome P-450 (CYP) 3A4 in human liver with minor contributions by CYP2C8 and CYP2C9 (Sutton et al., 1997). When DTZ is prescribed for long-term treatment a high potential of undesirable drug interactions occurs with other CYP3A substrates. Examples of compounds that are metabolized by CYP3A and whose elimination is inhibited by DTZ include cyclosporin A, nifedipine, quinidine, midazolam, alfentanil, triazolam, and lovastatin (Toyosaki et al., 1988; Leibbrandt and Day, 1992; Backman et al., 1994; Laganière et al., 1996; Ahonen et al., 1996; Varhe et al., 1996; Azie et al., 1998). The effect of DTZ is to reduce both the first-pass elimination and the systemic clearance of these coadministered drugs. This reduction of hepatic, systemic, CYP3A metabolism occurs in addition to the inhibition of CYP3A in the intestinal wall. Although DTZ is one of the most well established causes of clinically significant drug interactions with CYP3A enzymes, the mechanism of this inhibition is unclear.

Some recent reports have attempted to characterize the mechanism by which DTZ inhibits CYP3A. In vitro studies with rat liver microsomes and human hepatocytes suggested that DTZ inhibition of CYP3A was best described by competitive or noncompetitive inhibition, with $K_S$ ranging from 20 to 50 $\mu$M (Murray and Butler, 1996; Brockmöller et al., 1990). The steady-state plasma concentration of DTZ in humans during chronic DTZ treatment is approximately 0.3 $\mu$M (Yeung et al., 1996) and therefore, significant inhibition of CYP3A by DTZ via competitive or noncompetitive inhibition is not expected. A clinical study suggested that DTZ metab-
olites might cause the observed inhibition, because after 2 weeks of DTZ therapy, the elimination half-life of DTZ was significantly longer than that observed following a single dose and an unexpected accumulation of drug occurred (Montmat and Abernethy, 1987). Subsequently, Sutton et al. (1997) demonstrated that DTZ and its metabolites, MA and \( N,N\)-didesmethyldiltiazem, were competitive inhibitors of CYP3A in human liver microsomes, with \( K_i \)s approaching 2 \( \mu M \) for MA and 0.1 \( \mu M \) for \( N,N\)-didesmethyldiltiazem (Sutton et al., 1997). However, the reported steady-state plasma concentration of MA is 0.15 \( \mu M \) and the concentration of \( N,N\)-didesmethyldiltiazem has not been reported (Yeung et al., 1996). Again, because the plasma levels of the metabolites are much lower than the reported \( K_i \), this would not explain the CYP3A inhibition by DTZ or its metabolites through a reversible mechanism. It remains possible that DTZ and/or a metabolite partition into the tissues that express CYP3A to reach inhibitory levels, but the alternative that inhibition is not simply competitive in nature must also be considered.

An additional inhibitory property exhibited by DTZ is the formation of a metabolite intermediate complex (MIC), a complex of metabolite and CYP, that is catalytically inactive (Bensoussan et al., 1995) and serves to reduce the pool of active CYP (Franklin, 1977). DTZ forms a MIC in vitro and in vivo in dexamethasone- and phenobarbital-induced rat liver microsomes (Bensoussan et al., 1995). To date there has been no demonstration that DTZ forms a MIC with human CYP3A enzymes, and the potential role of this phenomenon in mediating clinical drug interactions has not been evaluated. In this study we demonstrate that DTZ \( N \)-demethylation occurs primarily via CYP3A, show that DTZ forms a MIC with human CYP3A, and report that DTZ inhibition of CYP3A catalysis occurs primarily by MIC, not by reversible inhibition.

**Materials and Methods**

**Chemicals.** \( \alpha \)-Naphthoflavone (\( \alpha \)-NF), caffeine, coumarin, desmethyldiazepam, dextromethorphan, diethylthiocarbamic acid, diltiazem, 7-ethoxycoumarin, 7-ethoxytrifluoromethylcoumarin, 7-hydroxytrifluoromethylcoumarin, 4-methylpyrazole, paraxanthine, potassium ferricyanide, quinicine, resorufin, sodium hydrosulfite, and triacetyleandomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorozoxazone, dextrophan, furafylline, 6-hydroxycoumarin, 7-hydroxycoumarin, 4-hydroxymphenytoin, 4-hydroxytolbutamide, and tolbutamide were purchased from Research Biochemicals International (Natick, MA). Sulfaphenazole and mephenytoin were gifts from CIBA-Geigy (Summit, NJ) and Sandoz (East Hanover, NJ), respectively. \( N \)-desmethyldiltiazem was a gift from Tanabe Seiyaku Co. (Osaka, Japan). Midazolam, 1-hydroxymidazolam, and 4-hydroxymidazolam were a gift from Hoffmann-LaRoche (Nutley, NJ). All HPLC and microsomal preparation supplies were of the highest grade available from standard commercial sources. \( \beta \)-NADP, isocitrate dehydrogenase (Type IV-purified), and isocitrate were purchased from Sigma Chemical Co. Disodium phosphate, magnesium chloride, and sodium phosphate were purchased from Fisher Scientific (Pittsburgh PA). NADPH (98%) was purchased from Boehringer Mannheim (Indianapolis, IN).

**Specimens.** Human adult liver specimens were obtained at surgery in accordance with protocols approved by the appropriate Committee for the Conduct of Human Research (The Medical College of Virginia, Richmond, VA; The Medical College of Wisconsin, Milwaukee, WI; The University of Michigan, Ann Arbor, MI; and Indiana University, Indianapolis, IN). The handling, preparation, and storage of microsomes along with characteristics of the microsomal samples and relative CYP3A levels have been previously described (Gorski et al., 1994a,b). CYP concentrations, if detectable, were quantified in each liver by the method of Omura and Sato (1964). The human liver microsomal samples along with their CYP concentrations are listed below: IUL-2, 0.25; IUL-3, 0.35; IUL-6, 0.31; IUL-10, 0.61; IUL-11, 0.44; and IUL-12, 0.1 nmol CYP/mg protein. The patient whose liver is designated IUL-10 was prescribed rifampin, a known inducer of CYP3A.

**DTZ \( N \)-demethylation in Human Liver Microsomes.** Microsomes from four human livers were used to characterize DTZ \( N \)-demethylation. All microsomal incubations contained the components listed below: 50 \( \mu M \) microsomal protein, 100 \( \mu M \) sodium phosphate buffer (pH = 7.4) containing 5 \( \mu M \) magnesium chloride and up to 1.6 \( mM \) DTZ in a final volume of 500 or 1000 \( \mu L \). The energy for the microsomal oxidation was supported by 1 \( mM \) NADPH or 5 \( mM \) isocitrate, 1 \( U \) isocitrate dehydrogenase, and 1 \( mM \) \( \beta \)-NADP. The reaction mixture was started by adding \( \beta \)-NADP or NADPH, incubated at 37°C for 5 min, unless otherwise noted, and terminated by adding 1000 \( \mu L \) of acetonitrile. The samples were stored at \(-70°C\) until analysis.

**Inhibitor Screen of DTZ \( N \)-demethylation.** The effects of putative inhibitors on MA formation were determined at a DTZ concentration of 0.5 \( mM \) in human liver microsomes (50 \( \mu g \) protein/sample). Each inhibitor was investigated with at least three concentrations. The inhibitor, the specific CYP it affects, and the range of concentrations of each are listed below: \( \alpha \)-NF (1A2), 0.5 to 5 \( \mu M \); furafylline (1A2), 5 to 100 \( \mu M \); coumarin (2A6), 10 to 1000 \( \mu M \); sulfaphenazole (2C9), 5 to 50 \( \mu M \); s-mephenytoin (2C19), 5 to 100 \( \mu M \); quinidine (2D6), 0.5 to 2.5 \( mM \); 4-methylpyrazole (2E1), 25 to 100 \( \mu M \); diethyldithiocarbamate (2E1), 50 to 1000 \( \mu M \); triacetyleandomycin (3A4/5), 20 to 500 \( \mu M \). The inhibitor was assessed to have an effect if a consistent stimulation or inhibition greater than 10% was observed.

**Correlations of Activity with DTZ \( N \)-demethylation.** Twenty-one human liver microsomal preparations (50 \( \mu g \) protein/sample) were incubated with 1 \( mM \) DTZ for 15 min to assess the \( N \)-demethylation activity. Correlations analyses were performed on the \( N \)-demethylation activity and activity of previously characterized CYP pathways: CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 (Wrighton et al., 1993; Hall et al., 1994; Gorski et al., 1994a; Castle et al., 1995).

**DTZ \( N \)-demethylation in cDNA-Expressed CYP.** Microsomes of B-lymphoblastoid cells expressing individual CYPs were employed to further assess the enzyme(s) involved in the biotransformation of DTZ to MA. These microsomes (200 \( \mu g \) /sample) were incubated with 0.5 mM DTZ for 15 min. Additionally, Michaelis-Menten kinetics of DTZ \( N \)-demethylation were performed with microsomes of CYP2A6 and with microsomes from baculovirus-infected insect cells (Superosomes CYP3A4, CYP3A45 (+ b), and CYP3A5). In the latter experiments DTZ (5–500 \( \mu M \)) was incubated with 200 \( \mu g \) of microsomal protein or 40 pmol of CYP for 5 min. An incubation varying time was first performed to establish linear conditions.

**Preincubation of DTZ with Expressed Enzymes.** DTZ (10 \( \mu M \)) was preincubated with individual cDNA-expressed CYPs (Superosomes or B-lymphoblastoid cells; Gentest Corp, Woburn MA), and NADPH for 30 min. Then an aliquot was removed and added to a tube containing the selective substrate for each CYP and NADPH. The selective substrate, concentration of substrate, CYP, and pico moles of CYP used for each treatment were: 1000 \( \mu M \) of caffeine, 50 pmols of CYP1A1 and CYP1A2, 5 \( \mu M \) of 7-ethoxycoumarin, 10 pmols of CYP1B1, 400 \( \mu M \) of coumarin, 27 pmols of CYP2A6, 10 \( \mu M \) of 7-ethoxytrifluoromethyl coumarin, 50 pmols of CYP2B6, 200 \( \mu M \) of tolbutamide, 50 pmols of CYP2C8 and CYP2C9-Arg144, 100 \( \mu M \) of S-mephenytoin, 50 pmols of CYP2C19, 50 \( \mu M \) of midazolam, 20 pmols of CYP3A45 (+ b), CYP3A4, and CYP3A5, 500 \( \mu M \) of lauric acid, and 50 pmols of CYP4A11. The dilution of the aliquot from the preincubation tube with the contents of
the tube containing the selective substrate was 1:10 (v/v) to eliminate competitive inhibition as a possible cause of the inhibitory effect. After a 5- to 15-min incubation, the reaction was terminated and then assayed for activity. A control that contained the selective substrate, cDNA-expressed CYP, and NADPH was incubated for 5 to 15 min. The reaction was terminated and then assayed for activity. The percentage of inhibition was estimated with the following equation: (control activity-treatment activity)/control activity)*100. When the percentage of inhibition by the DTZ (+NADPH) preincubation tube was greater than 30%, additional incubations were performed to resolve the reversible inhibition component underlying the inhibitory effect of DTZ. Thus, DTZ was preincubated with individual cDNA-expressed CYPs in the absence of NADPH for 30 min. Then an aliquot was removed and added to a tube containing the selective substrate for each CYP and NADPH. After a 5- to 15-min incubation, the reaction was terminated and then assayed for activity. The percentage of inhibition of the DTZ (−NADPH) in the preincubation tube was compared with the control that was not preincubated to assess the reversible inhibition.

Metabolite Intermediate Complex Formation. Microsomes from six human livers were used to characterize MIC formation by DTZ. In two of the human liver microsomal preparations DTZ concentrations were varied from 0.1 to 100 μM to determine whether substrate was a limiting factor involved in MIC formation. MIC formation was identified with dual wavelength spectroscopy (Uvikon 933 double beam UVVIS Spectrophotometer; Research Instruments International, San Diego, CA) by scanning from 380 to 500 nm and was quantified by wavelength programming of four wavelengths, selected from the original scans, at 30-s intervals. In each case, the sample cuvette contained protein (usually 500 μg), substrate, and NADPH, whereas the reference cuvette contained protein, substrate vehicle, and NADPH. All MIC formation experiments were initiated by the addition of NADPH and maintained at 37°C. The absorbance difference spectra for the identification of MIC formation were estimated by subtracting the absorbance at 490 nm of the absorbance scan from the difference of the absorbance scan at a given time and a background absorbance scan. The results from the wavelength program runs were estimated by subtracting the absorbance at 490 nm from the absorbance of a given wavelength, usually 452 nm, at a specific time.

Supersomes [CYP2B6, CYP3A4, CYP3A4(+b), and CYP3A5] were used to characterize MIC formation by DTZ. MIC formation was assessed in CYP2B6 because the substrate selectivities of it and CYP3A are similar (Ekins et al., 1999). The CYP concentration was 800 pmol/cuvette for CYP3A4 and CYP3A5 and 200 pmol/cuvette for CYP2B6 and CYP3A4(+b). The procedure to measure MIC formation was the same as that described above for human liver microsomes.

K_{Fe(CN)}_{5}O, a compound that dissociates MIC, was used to dissociate “in vivo”-formed MIC by DTZ. Initially, DTZ (10 μM) was incubated with human liver microsomes and NADPH to allow MIC formation. After 90 min the sample cuvette was split such that half of the contents were placed in a reference cuvette and the other half were used as the sample cuvette. K_{Fe(CN)}_{5}O (10 μM of 5 mM) was added to the reference cuvette and vehicle (10 μL of water) was added to the sample cuvette using the procedure described by Wrighton et al. (1985). The cuvettes were scanned from 380 to 500 nm at 5, 10, and 15 min (Roos et al., 1993), and the absorbance difference spectra were estimated as noted above.

Preincubation of DTZ with Human Liver Microsomes. DTZ (100 μM) or alprazolam (400 μM) were incubated in human liver microsomes with NADPH to simultaneously quantify MIC formation and midazolam hydroxylation inhibition. Alprazolam was selected as the control compound because it is metabolized by CYP3A but does not form a MIC based on preliminary experiments and to assess whether all CYP3A substrates cause an irreversible inhibition. The DTZ concentration was chosen to maximize DTZ in the preincubation. In conjunction with the MIC formation, DTZ or alprazolam were preincubated in human liver microsomes with NADPH at various times (0.5–60 min), whereupon a sample was removed and added to a tube containing midazolam (100 μM) and NADPH. The dilution of the aliquot from the preincubation tube with the contents of the tube containing midazolam was 1:10 (v/v) to eliminate competitive inhibition as a possible cause of the inhibitory effect. After a 5-min incubation, the midazolam metabolism was terminated by the addition of acetonitrile, then the tube was partially submerged into an acetone/dry ice bath for about 10 min to freeze the aqueous matrix. 1’-Hydroxy- and 4-hydroxymidazolam were quantified by gas chromatograph-mass spectrometry as reported previously (Gorski et al., 1998). Gas chromatograph-mass spectrometry was employed in this assay because the HPLC procedure cannot resolve midazolam metabolites from alprazolam metabolites.

Quantification of Enzyme Activity. MA was quantified by HPLC with UV detection (257 nm). The metabolic pathway, UV setting, and selective CYP activity are listed for each CYP sample that was quantified by HPLC: caffeine 3′-N-demethylation (270 nm), CYP1A1 and CYP1A2 (Miners and Birkett, 1996a); tolbutamide 4-hydroxylation (235 nm), CYP2C8 and CYP2C9 (Miners and Birkett, 1996b); mephénytoin 4-hydroxylation (235 nm), CYP2C19 (Wedlund and Wilkinson, 1996); midazolam 1′-hydroxylation and 4-hydroxylation (230 nm), CYP3A4 and CYP3A5 (Gorski et al., 1994b). 7-Ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, and 7-ethoxycoumarin-7-ethoxycoumarin O-deethylation were quantified by fluorescence to assess selective CYP1B1, CYP2A6, and CYP2B6 activities, respectively (Miles et al., 1990; Buters et al., 1993; Crespi et al., 1997). Dextromethorphan O-demethylation was quantified by HPLC with fluorescence detection (excitation 190 nm, emission 310 nm) to assess CYP2D6 activity (Jones et al., 1996). LORUIC acid 12-hydroxylation was quantified by fluorescence derivatization, HPLC, and fluorescence detection (excitation 340 nm, emission 420 nm) to assess CYP4A11 activity (Powell et al., 1996). All substrates and inhibitors were tested for interference before use and no interferences were detected.

Metabolite Intermediate Complex Data Analysis. MIC was quantified from absorbance difference spectra using an extinction coefficient of 65 mM⁻¹ cm⁻¹ (Persing and Franklin, 1982). The pseudo-first-order rate constant for enzyme inactivation, k_{inact}, was estimated from the initial rate of enzyme inactivation (0–5 min) with eq. 1.

\[
E(t) = E_0 \cdot e^{-kt}
\]

where \(E(t)\) is the percentage of active enzyme remaining at time \(t\) and \(E_0\) is 100%. The relationship between \(k\) and inhibitor concentration was fitted to the following equation:

\[
\lambda = \frac{k_{inact}}{K_{i} + [I]}
\]

where \(k_{inact}\) is the rate constant for inactivation, \([I]\) is inhibitor, or inactivator concentration, and \(K_{i}\) is the inactivator concentration that produces half the maximal rate of inactivation, analogous to a \(K_{m}\) (Silverman, 1988). Equation 2 assumes there is negligible change in \([I]\) in the incubation period and that loss of enzyme is due to inactivation. The partition ratio, \(r\), is the number of moles of metabolite formed per mole of enzyme inactivated and is determined from the ratio of \(k_{cat}/k_{inact}\) where \(k_{cat}\) was determined from the ratio of \(V_{max}\) of MA formation to moles of CYP3A (Waley, 1985).

Data Analysis and Statistics. The microsomal activity data represent the mean of duplicate or triplicate assays for every experiment. Untransformed kinetic data were analyzed by nonlinear regression without weighting (WinNonlin v 1.1; SCI Software, Apex, NC). The appropriateness of the fit was determined by the visual inspection of fit and residual patterns, residual sums of squares, and precision of the parameter estimates (Boxenbaum et al., 1974). The correlation coefficient and its corresponding statistical significance were determined by conventional methods (Rohlf and Sokal, 1981).
Results

Characterization of Diltiazem N-demethylation Activity. After identifying linear conditions with respect to time and protein concentration, one enzyme or a family of enzymes with similar $K_m$s appears to be involved in the formation of MA based on Michaelis-Menten plots from four human liver microsomal samples (Fig. 1). The mean $(\pm$ S.D.) $K_m$ of four livers was $53 \pm 22.1$ $\mu$M and the $V_{max}$ ranged from 1,035 to 23,965 pmol/(mg protein $\cdot$ min). The resulting intrinsic clearances ($Cl_{int}$; $V_{max}/K_m$) ranged from 1 to 35 $\mu$l/min.

The maximum rate of MA formation determined at 1000 $\mu$M DTZ was correlated with selective CYP activities in 14 to 21 adult liver microsomal samples described previously. These samples were characterized previously for relative amounts of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 and their form specific activities (Wrighton et al., 1993; Hall et al., 1994; Gorski et al., 1994a; Castle et al., 1995). The formation of MA correlated significantly with CYP3A activity, which included erythromycin N-demethylase activity ($r = 0.88$, $n = 14$, $p < .01$), midazolam 1'-hydroxylase activity ($r = 0.86$, $n = 21$, $p < .01$), midazolam 4-hydroxylase activity ($r = 0.97$, $n = 21$, $p < .01$), dextromethorphan N-demethylase activity ($r = 0.95$, $n = 21$, $p < .01$), and total CYP3A protein ($r = 0.94$, $n = 14$, $p < .01$). There was no significant correlation between the rate of MA formation and previously reported rates of ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, taxol 6a-hydroxylation, phenytoin 4-hydroxylation, S-mephenytoin 4'-hydroxylation, bufuralol 1'-hydroxylation, chlorzoxazone 6-hydroxylation, N-nitrosodimethylamine N-demethylation, and lauric acid 12-hydroxylation, which primarily reflect the activities of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP4A11, respectively (Wrighton et al., 1993; Hall et al., 1994; Castle et al., 1995).

Selective inhibitors were used to further characterize the enzyme(s) responsible for the N-demethylation of DTZ in human liver microsomes. Triacetyloleandomycin was the only compound that strongly inhibited MA formation (Fig. 2). High concentrations of 4-methylpyrazole ($75-100$ $\mu$M) and diethylthiocarbamate ($1000$ $\mu$M) also inhibited MA formation, but these inhibitor concentrations may have resulted in nonselective inhibition, which has been observed previously (Newton et al., 1995). Stimulation of MA formation was observed with $\alpha$-NF and quinidine, which also has been reported previously for CYP3A-mediated catalysis (Newton et al., 1995; J.C.G., D.R.J., M.A.H., S.D.H., S. A. Wrighton, unpublished observations).

Microsomes of human B-lymphoblastoid cells that express human CYPs were used to confirm that CYP3A enzymes primarily catalyze the biotransformation of DTZ to N-demethyl-diltiazem. CYP3A4 exhibited the greatest DTZ N-demethylation activity in these expressed CYPs. The expressed CYP with the DTZ N-demethylase activity relative to CYP3A4 activity in percentages was CYP1A1, 0%; CYP1A2, 0%; CYP2A6, 23%; CYP2B6, 3%; CYP2C9, 1%; CYP2D6, 9%; CYP2E1, 5%; CYP3A4, 100%. Significant activity was associated with CYP2A6 and CYP2D6, but in view of the low relative abundance of these enzymes in vivo, they are unlikely to make a significant contribution to DTZ metabolism.

Enzyme kinetic parameters were estimated in CYP2A6 and CYP3A4 to further investigate the involvement of these expressed systems in DTZ N-demethylation. No detectable formation of MA was observed with CYP2A6. However, MA formation was observed in expressed CYP3A4, CYP3A4(+b), and CYP3A5 (Fig. 3). The estimated $V_{max}$ in CYP3A4(+b) (585 pmol/min) was greater than the $V_{max}$ for CYP3A4 and CYP3A5 (121 and 429 pmol/min, respectively) but less than the $V_{max}$ for any of the human liver microsomal samples. The estimated $K_m$s of CYP3A4(+b) and CYP3A4 were nearly equal, 16 and 17 $\mu$M, respectively, and considerably less than the $K_m$ of CYP3A5 (81 $\mu$M). The calculated $Cl_{int}$ values were 37, 7, and 5 $\mu$l/min for CYP3A4(+b), CYP3A4, and CYP3A5, respectively.

Preincubation of DTZ with Expressed Proteins. To assess the effect of DTZ on selective CYP activity, preincubation experiments (30 min) were performed with cDNA-expressed CYPs (Supersomes Gentest Corp., Woburn MA) and one CYP expressed from B-lymphoblastoid cells. The 30 min preincubation of DTZ with NADPH inhibited the control activity in CYP1B1 by 56%, CYP2A6 by 52%, CYP3A4(+b) by 100%, CYP3A4 by 40%, and CYP3A5 by 59%. Next, the aforementioned CYPs were preincubated with DTZ in the absence of NADPH to determine the reversible inhibitory contribution of DTZ. Only CYP3A4(+b) exhibited a significant mechanistic inhibitory component, which resulted in 55% of the total inhibition. In comparison, CYP1B1, CYP2A6, CYP3A4, and CYP3A5 exhibited less than 5% of the total inhibition as a mechanistic component. The time-dependent inhibition with CYP3A4(+b) may be due to the formation of a MIC with CYP, which would inactivate the enzyme.

MIC Formation by Diltiazem. Triacetyloleandomycin was used initially as the prototypical compound to substantiate measurement of MIC formation in human liver microsomes. After incubation of triacetyloleandomycin with human liver microsomes and NADPH, a peak absorbance difference was observed at 452 nm (Fig. 4A) but not in the absence of NADPH. Additional NADPH, which was supplemented to the sample at 20-min intervals during data collection, and $O_2$ gassing, which was
supplemented to the sample for 5 s at 10-min intervals, had no effect on MIC formation by DTZ (data not shown; Franklin, 1991). After DTZ was incubated with human liver microsomes and NADPH for 90 min (Fig. 4A), the sample containing MIC was split into two cuvettes, one was used as the reference cuvette and the other as the sample cuvette. Then, K₃Fe(CN)₆ was added to the reference cuvette. At 15 min, a peak absorbance difference was detected at 452 nm in the sample cuvette only, indicating a degradation of complex in the reference cuvette (Fig. 4B). The dissociation at 15 min by K₃Fe(CN)₆ was greater than 93%, which substantiates that DTZ forms a MIC in human liver microsomes.

The ability of DTZ to form a MIC was assessed in microsomes of five human livers that contained varying amounts of CYP3A. The magnitude of the MIC increased with time and exhibited a maximum absorbance difference of 0.015 at 452 nm in microsomes of one human liver (Fig. 4A). MIC formation by DTZ (10 mM) measured by continuous monitoring (452–490 nm) from 0 to 80 mins in the same human liver microsomes, although at a decreased total protein concentration, is illustrated in Fig. 5. The estimated λ from eq. 1 and maximum MIC (MIC max) were 0.025 and 0.0068 min⁻¹, respectively. The MIC max is 87% of the total CYP in the sample applying the extinction coefficient of 65 mM⁻¹ cm⁻¹ (Pershing and Franklin, 1982). The MIC max of DTZ was quantified in five of the human liver microsomal samples. Figure 6 illustrates the correlation of maximum DTZ N-demethylation activity (Fig. 6A) or maximum midazolam hydroxylation activity (Fig. 6B) with MIC max (in picomoles) in these microsomal samples (r ≥ 0.99). These data show that MIC formation by DTZ correlates with CYP3A.

Dual wavelength scanning experiments to quantify MIC formation by DTZ were performed with cDNA-expressed CYP2B6, CYP3A4(+b₃), CYP3A4, and CYP3A5 because CYP3A exhibited a mechanistic inhibition component upon preincubation with DTZ (vide supra), and because CYP2B6 shares substrate selectivities with CYP3A. CYP3A4(+b₃) allowed extensive MIC formation (Fig. 7). The magnitude of the MIC increased with time and exhibited a MIC max of 0.0061 at 450 nm. The estimated MIC max was 61% of the total CYP in the sample. The formation of MIC was evident in expressed CYP3A4 and CYP3A5, reaching approximately 10% of the total CYP in 90 mins (Fig. 7). No MIC formation was supported by CYP2B6.

The effect of DTZ concentration (0.1–100 μM) on MIC formation was studied in human liver microsomes and expressed CYP3A4(+b₃). MIC formation by DTZ was quantified by continuous wavelength monitoring and the resulting enzyme inactivation data were fit to eq. 1. There was no evidence of concentration-dependent MIC formation in the human liver microsomal samples. The cDNA expressed CYP3A4(+b₃) illustrated a concentration-dependent formation of MIC by DTZ (1–100 μM) with an estimated k cat/k inact of 0.17 min⁻¹ and K of 2.2 μM (Fig. 8). The partition ratio, k cat/k inact for expressed CYP3A4(+b₃) was 86.

**Preincubation of DTZ with Human Liver Microsomes.** To further understand the role of DTZ MIC formation on CYP3A activity, simultaneous preincubation and MIC formation was studied in human liver microsomes and expressed CYP3A4(+b₃). MIC formation by DTZ was quantified by continuous wavelength monitoring and the resulting enzyme inactivation data were fit to eq. 1. There was no evidence of concentration-dependent MIC formation in the human liver microsomal samples. The cDNA expressed CYP3A4(+b₃) illustrated a concentration-dependent formation of MIC by DTZ (1–100 μM) with an estimated k cat/k inact of 0.17 min⁻¹ and K of 2.2 μM (Fig. 8). The partition ratio, k cat/k inact for expressed CYP3A4(+b₃) was 86.
formation experiments were performed with human liver microsomes. DTZ or alprazolam were preincubated for various times with microsomes containing NADPH, then added to a matrix that contained human liver microsomes, diltiazem, and NADPH, whereas reference cuvette contained human liver microsomes, buffer, and NADPH. Absorbance was monitored from 0 to 90 min. Lines represent change in absorbance difference for scans at 15, 30, 45, 60, 75, and 90 min. B, K₃Fe(CN)₆ dissociation of “in vivo” formed metabolite intermediate complex by diltiazem. After a 90-min incubation of diltiazem (10 μM), human liver microsomes, and NADPH to form metabolite intermediate complex (A), the sample cuvette was split. Half the contents were placed in the reference cuvette and the other half was used as the sample cuvette. Then K₃Fe(CN)₆ was added to the reference cuvette and vehicle was added to the sample cuvette. Absorbance was monitored at 0, 5, 10, and 15 min. Lines represent change in absorbance difference for scans at 5, 10, and 15 min.

Fig. 5. Metabolite intermediate complex formation by diltiazem (10 μM) in human liver microsomes. The points represent continuous scanning measurement of absorbance difference (455–490 nm) over time. The line represents the line of best fit.

**Discussion**

It appears that one family of enzymes, CYP3A, is involved in DTZ N-demethylation based on our observations of 1) a single-enzyme Michaelis-Menten model of metabolite formation (Fig. 1), 2) correlations of DTZ N-demethylation activity to other CYP3A activities (e.g., erythromycin N-demethylation, midazolam 1'-hydroxylation, midazolam 4-hydroxylation, and dextromethorphan N-demethylation), 3) inhibition of DTZ N-demethylation activity by a selective CYP3A inhibitor only (e.g., triacetyleloandamycin; Fig. 2), and 4) DTZ N-demethylation activity by expressed CYP3A4 and CYP3A5 only. These results are similar to those recently reported by Sutton et al. (1997), with the exception that a correlation between DTZ N-demethylation activity and activity of substrates selective for CYP2C8 and CYP2C9 was observed and that DTZ N-demethylation activity by expressed CYP2C8 and CYP2C9 also was observed. Our results did not indicate that CYP2C8 or CYP2C9 make a significant contribution to DTZ N-demethylation. In the initial cDNA-expressed CYP screen, CYP2A6 was implicated as an enzyme that may be involved in the formation of N-desmethyldiltiazem. However, upon further investigation, no detectable N-desmethyldiltiazem was observed by CYP2A6 when DTZ substrate concentrations were increased from 5 to 500 μM. Therefore, our results indicate that DTZ N-demethylation is mediated by CYP3A enzymes only.

A major finding in this report is that DTZ forms a MIC with CYP3A in human liver microsomes. MICs are an inactive form of CYP that serve an inhibitory role in drug metabolism because they remove active CYP from the total CYP pool (Franklin, 1977). DTZ has been reported to form MICs in vitro and in vivo in dexamethasone- and phenobarbitol-induced rat liver microsomes (Bensoussan et al., 1995), but before this study no one had reported MIC formation by DTZ in human liver microsomes or human CYP3A enzymes. Triacetyleloandamycin, a macrolide antibiotic, was used initially as the prototypical compound to substantiate measurement of MIC formation in human liver microsomes (Pershing and Franklin, 1982). After incubation of triacetyleloandamycin with human liver microsomes and NADPH, an absor-
A balance difference was observed at 455 nm, which is characteristic of many MICs (Bensoussan et al., 1995). When DTZ was incubated with human liver microsomes and NADPH, a MIC was detected but not in the absence of NADPH. However, MIC formation by DTZ is small in comparison with metabolism. There is substantial metabolism of DTZ to MA and it is assumed that a metabolic product of MA causes the MIC formation (Bensoussan et al., 1995). Our laboratory interprets the small MIC formation by DTZ to be a result of competitive inhibition of MA metabolism by DTZ. This in turn would inhibit MIC formation.

The importance of CYP3A4 in MIC formation by DTZ was shown in several experiments. First, the correlation of maximum DTZ N-demethylation or midazolam hydroxylation (markers of CYP3A activity) with MICmax by DTZ in human liver microsomal samples was 0.99 (Fig. 6). Second, the preincubation of DTZ with expressed CYP showed a 55% inhibition of CYP3A4(+b5) catalytic activity toward midazolam 1'-hydroxy formation that was a result of some type of inhibition other than reversible inhibition. Third, expressed CYP3A4(+b5) formed MIC in a time-dependent fashion that inactivated greater than 60% of the CYP (Fig. 7). These data illustrate that DTZ forms a MIC in human liver microsomes, that this MIC formation is due to CYP3A4, that MIC formation by DTZ can be reproduced in expressed CYP3A4(+b5), and that a majority of the CYP is inactivated by MIC from DTZ. Even though CYP3A4(+b5) allowed MIC formation, it was not complete (Fig. 7). One possible explanation for this was illustrated by Chiba et al. (1995). They showed that a 30-min preincubation of human liver microsomes with NADPH caused approximately 40% inhibition of activity associated with CYP3A. These data suggest that a preincubation with NADPH will cause enzyme degradation but the mechanism is unknown.

The result of MIC formation, inactive CYP, by DTZ may
lead to a misinterpretation of drug interaction data. For example, it has been assumed that substrates metabolized by the same enzyme, when coadministered, will cause a reversible type of inhibition, usually competitive. In an in vitro study to understand DTZ inhibition, Sutton et al. (1997) suggested that DTZ, MA, and N,N-didesmethyldiltiazem inhibited CYP3A4 in a competitive manner, that metabolites exhibited much lower $K_I$ than the parent compound, and that none of the compounds formed MICs. Steady-state plasma DTZ concentrations are 0.15 μM and N,N-didesmethyldiltiazem concentrations have not been reported (Yeung et al., 1996). Because these concentrations are 100-fold less than the $K_I$ (~50 μM) for reversible inhibition, these results suggest that some type of inhibition is ongoing other than competitive inhibition. Thus, the in vitro data with $K_I$ estimated from reversible inhibition models do not provide a good prediction of in vivo studies. To further understand the mechanism of DTZ inhibition of CYP3A, DTZ was preincubated with NADPH and human liver microsomes, then placed in a tube that contained a CYP3A marker, midazolam. MIC formation by DTZ was monitored over time along with the inhibition of midazolam hydroxylation. The inhibition of midazolam hydroxylation paralleled DTZ MIC formation (Fig. 9) and the line of best fit for each phenomenon had the same rate constant ($\lambda = 0.037$ min$^{-1}$), which provided further evidence that DTZ inhibition of CYP3A4 occurred by MIC formation. These data also showed that MIC formation by DTZ will inhibit CYP3A activity in an irreversible manner and may be accountable for 100% of the inhibition.

The partition ratio, $k_{cat}/k_{inact}$, was 86 for DTZ in expressed CYP3A4, which illustrates, at least for the expressed CYP3A4, that catalysis is a much more prevalent event than inactivation. However, the $K_I$ and $K_m$ values for DTZ were 2.2 and 16 μM, respectively, indicating that the partition ratio is dependent on DTZ concentration. For example, at a DTZ concentration of 0.3 μM, which is the steady-state plasma concentration, the partition ratio ($=(k_{cat} \cdot \text{substrate}/(K_m + \text{substrate})/(k_{inact} \cdot \text{inactivator}/(K_I + \text{inactivator})))$ is 13. This value approximates the partition ratio for other potent inactivators of CYP3A4 in vitro (e.g., gestodene, $r = 9$; ritonavir, 10; and delavirdine, 41; Guengerich, 1990; Koudriakova et al., 1998; Voorman et al., 1998). These results suggest that DTZ may cause significant inhibition due to inactivation of CYP3A when administered clinically. A substrate concentration dependence in partition ratio was also noted for delavirdine. At low concentrations of delavirdine ($<<K_I$ or $K_m$), the partition ratio is 130 assuming a $K_I$ of 21.6 μM and a $K_m$ of 6.8 μM, whereas, at high concentrations the partition ratio is 41 (Voorman et al., 1998). All these data illustrate that $K_m$ and $K_I$ should not be assumed to be equal, and therefore, inactivator concentration affects the partition ratio, which is important for making judgements about relative inactivation potential by CYP3A4 inhibitors.

Multiple studies have shown that DTZ inhibits the metabolism of compounds that are primarily metabolized by CYP3A, specifically, nifedipine, cyclosporin A, triazolam, quinidine, midazolam, alfenantil and lovastatin (Toyosaki et al., 1988; Brockmöller et al., 1990; Varhe et al., 1996; Laganière et al., 1996; Ahonen et al., 1996; Azie et al., 1998).
Most of these studies attributed competitive inhibition as the mechanism of interaction, but the results of our findings suggest that the inhibition may be a result of MIC formation with DTZ. Consistent with this finding, chronic administration of DTZ provided evidence that DTZ affects its own disposition. DTZ exhibited a significantly prolonged half-life after multiple days of dosage and accumulated more than predicted from a single dose (Montamat and Abernethy, 1987). Increasing the dosage of DTZ resulted in an increase in bioavailability (Bianchetti et al., 1991). The area under the plasma concentration time (AUC) of DTZ in a dosing interval at steady state increased significantly compared with a single-dose AUC, indicating an increased bioavailability (Högland and Nilsson, 1989c). Our data suggest that MIC formation by DTZ inhibits the metabolism of parent compound, but more studies are warranted to address this question.

Is MIC formation by DTZ a systemic occurrence or does it also occur in the intestinal wall that also expresses CYP3A4? Data from clinical studies suggest that DTZ inhibition of CYP3A occurs both in the intestinal wall and the liver. DTZ, initially administered orally followed by I.v. administration, caused a 25% increase in i.v. midazolam AUC and a 40% increase in i.v. alfentanil AUC. The interaction involved primarily hepatic metabolism because midazolam and alfentanil were administered i.v. (Ahonen et al., 1996). Conversely, the inhibition of oral lovastatin metabolism by oral DTZ is probably due to inhibition of intestinal wall and hepatic CYP3A. Oral DTZ increased lovastatin oral AUC without a simultaneous change in half-life, which suggests that DTZ inhibition of lovastatin occurs presystemically (Azie et al., 1998). These studies suggest that DTZ inhibition of CYP3A4 occurs at the intestinal wall and hepatic level and may be exploitable drug interaction.

Six human livers were used to study the correlation of maximum DTZ N-demethylation rate with maximum MIC formation. However, one of the livers contained CYP3A5 and was excluded in the correlation (Fig. 6). The DTZ N-demethylation in the liver containing CYP3A5 was greater than the maximum MIC formation when compared with the other five livers. This suggests that the partition ratio for CYP3A5 is higher than CYP3A4. In fact, the estimated partition ratio of CYP3A5, without b0, was 324 in comparison with 70 for CYP3A4, without b0. If CYP3A5 forms more MA than CYP3A4, then these results would agree with data previously published by our laboratory that showed that CYP3A5 favors the formation of 1′-hydroxymidazolam over 4-hydroxymidazolam (Gorski et al., 1994b). Thus, MA formation at high DTZ concentrations (>500 μM) could be used as a marker of significant CYP3A5 expression. Additionally, a liver expressing a high amount of CYP3A5 could be interpreted as a “protective” component because the enzyme forms less MIC with DTZ, which may result in less harmful drug interactions.

This study illustrated that DTZ metabolism to N-demethyl-diltiazem occurs by CYP3A and that DTZ inhibition of CYP3A substrate metabolism occurs primarily by MIC formation, which renders the CYP inactive.

References


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