

SEQUENTIAL METABOLISM IS RESPONSIBLE FOR DILTIAZEM INDUCED TIME-DEPENDENT LOSS OF CYP3A

Ping Zhao*, Caroline A. Lee and Kent L. Kunze

Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development
La Jolla Laboratories (P.Z. and C.A.L.) and Department of Medicinal Chemistry, School
of Pharmacy, University of Washington (K.L.K.)

Running title: CYP3A inactivation by diltiazem metabolites

Corresponding author: Ping Zhao, PhD

Number of text pages: 22

Number of Figures: 4

Number of references: 26

Word counts:

Abstract: 248

Introduction: 425

Results: (including in-text citations) 897

Discussion: (including in-text citations) 1743

Abbreviations:

CYP3A: cytochrome P450 3A; HLM: human liver microsomes; f_c : free fraction of inactivator/inhibitor in hepatocyte incubation; I_u : free inactivator/inhibitor concentration in hepatocyte incubation medium; $[I]_{u,ave}$, microsomal inactivator concentration corrected for both %Free and metabolic consumption by time-averaged factor; K_i : reversible inhibition constant; k_{inact} : maximum inactivation rate constant; K_I : apparent inactivation constant; k_{inact} : maximum inactivation rate constant; $K_{I,u,ave}$: K_I obtained using $[I]_{u,ave}$; λ : apparent inactivation rate constant; MA: N-desmethyl diltiazem; MD: N,N-didesmethyl diltiazem; PGRD: Pfizer Global Research and Development; TDI: time-dependent inactivation

ABSTRACT

Kinetic parameters (k_{inact} and K_I) obtained in microsomes are often used to predict time-dependent inactivation (TDI). We previously reported that microsomal inactivation kinetic parameters of diltiazem under-predicted CYP3A inactivation in hepatocytes. In this study, we evaluated the contributions of inactivation and reversible inhibition of CYP3A by diltiazem and its N-desmethyl- and N,N-didesmethyl- metabolites (MA and MD, respectively). In human liver microsomes, MA was a more potent time-dependent inactivator of CYP3A than its parent drug, with apparent k_{inact} approximately four-fold higher than diltiazem at microsomal protein concentration of 0.2 mg/mL. MD did not inactivate CYP3A. Inactivation of CYP3A by diltiazem was dependent on microsomal protein concentration (25, 36, and 41% decrease in CYP3A activity at 0.2, 0.4, and 0.8 mg/mL microsomal protein, respectively, incubated with 10 μM diltiazem over 20 min), whereas inactivation by MA did not seem to be protein concentration-dependent. MA and MD were reversible inhibitors of CYP3A with competitive K_i values of 2.7 and 0.2 μM , respectively. In cryopreserved hepatocytes incubated with diltiazem, time-dependent loss of CYP3A was accompanied by increased formation of MA and MD, with the MA level similar to its K_I at higher diltiazem concentrations. In addition, the metabolites appeared to be accumulated inside the cells. In summary, time-dependent CYP3A inactivation by MA seems to be the major contributor responsible for the loss of CYP3A in HLM and human hepatocytes incubated with diltiazem. These findings suggest that prediction of CYP3A loss based solely on microsomal inactivation parameters of parent drug may be inadequate.

Introduction

The calcium channel blocker diltiazem is extensively metabolized via multiple pathways including N-demethylation, O-demethylation, and deacetylation (Figure 1). The demethylation pathways are mediated by cytochrome P450 (CYP) enzymes primarily in the liver, whereas deacetylation is believed to be carried out by esterases in multiple tissues (Homsy, 1995, Sutton, 1997). N-desmethyl diltiazem (MA) is the major metabolite which undergoes further N-demethylation process to form N,N-didesmethyl diltiazem (MD). It has been shown that diltiazem caused clinically significant drug-drug interactions by decreasing the elimination of substrates through inhibition of CYP3A (Jones and Morris, 2002, Jerling, 2005). The causes of inhibition have been attributed to both diltiazem and its metabolites. In human liver microsomes (HLM), MA and MD appeared to competitively inhibit testosterone 6- β hydroxylation with inhibition constants (K_i) of approximately 2 and 0.1 μ M (Sutton et al, 1997). However, reversible inhibition may not sufficiently explain the observed drug interactions given that plasma concentrations of diltiazem and its metabolites are lower than their respective K_i values. Time dependent inactivation (TDI) was also observed in HLM for both diltiazem (Jones et al, 1999, Dai et al, 2003) and MA (Mathew et al, 2000) and has been suggested to be responsible for the clinically relevant drug interactions. To date, comprehensive study has not been performed to elucidate the contribution of different mechanism (inhibition versus inactivation) by diltiazem and its oxidative metabolites to diltiazem-induced pharmacokinetic drug interaction caused by diltiazem.

In a previous report, we evaluated time-dependent CYP3A inactivation by compounds exhibiting this property in cryopreserved human hepatocytes (Zhao et al,

2005). Hepatocytes can serve as an intact system to test the predictability of parameters obtained in HLM, a much simpler system. A large discrepancy was found to exist between the inactivation predicted by directly applying HLM generated parameters and that observed in hepatocytes. The discrepancy between the two *in vitro* systems that differ greatly in complexity suggests that there is some risk of misinterpretation of *in vivo* outcomes predicted from HLM parameters. Among the inactivators tested, diltiazem appeared to be more potent in hepatocytes than expected from the microsomal kinetic parameters. The discrepancy is not likely due to nonspecific binding, metabolic consumption or efflux of inhibitor, since these factors result in less potent inhibition in hepatocytes (Zhao, 2005).

The objectives of this study were 2-fold: i) to reassess the inhibition and inactivation of CYP3A by diltiazem, MA, and MD and identify the major contributor(s) of diltiazem-induced loss of CYP3A, and ii) to evaluate the impact of sequential metabolism on the prediction of drug interaction using microsomal parameters obtained for parent compound.

Materials and Methods

Materials. Cryopreserved human hepatocytes and hepatocyte thawing medium were purchased from InVitro Technologies (Baltimore, MD). Pooled human liver microsomes from 59 individuals were obtained from a Pfizer Global R&D (PGRD, a division of Pfizer Inc) in-house supply. HEPES was purchased from Invitrogen Life Technologies (Carlsbad, CA). MA and MD are generous gifts from Tanabe Seiyaku Co., Ltd. (Osaka, Japan). All other reagents were from Sigma Co. (St Louis, MO) unless otherwise stated.

Microsomal Studies

TDI of CYP3A in HLM. The main body of this study used 1'-hydroxylation of midazolam as the probe reaction for CYP3A in both HLM and cryopreserved human hepatocytes. TDI by MA in HLM was performed according to methods described elsewhere (Ito et al, 1998). Briefly, the inactivator (MA) was incubated at varying concentrations with HLM in potassium phosphate buffer (0.1 M, PH 7.4) at 37°C for 5 minutes prior to the addition of NADPH. The final concentrations of microsomal protein and NADPH were 0.2, 0.4 or 0.8 mg/mL and 1 mM, respectively. The nominal concentrations of MA in these incubations were 0.25, 0.5, 1, 2, and 4 μ M. The experiments were performed in triplicate. At different times after the addition of NADPH (0, 2, 5, and 10 min), 10 μ L of the inactivator/microsome mixture (primary incubation) were transferred to 90 μ L of potassium phosphate buffer containing 100 μ M midazolam and 1 mM NADPH, which had been pre-incubated at 37°C for 5 minutes. This resulted in a 10-fold dilution of inactivator in the incubation with midazolam. The mixture was incubated for 1 minute and was terminated by the addition of 100 μ L of a mixture of acetonitrile:methanol (3:1

v/v) containing alprazolam as internal standard. In parallel, the primary incubation samples at time-zero and at different incubation time points after the addition of NADPH were quenched with acetonitrile:methanol (3:1, v/v) containing alprazolam to evaluate the metabolic consumption of MA over time during the primary incubation.

The effect of microsomal protein concentration on TDI of CYP3A by diltiazem was evaluated by incubating 10 μ M of diltiazem with 0.2, 0.4, or 0.8 mg/mL microsomal protein. These protein concentrations were the same as those used in the primary incubations with MA. At different times after the addition of NADPH (0, 2, 6, 12, and 20 min), 10 μ L of the primary incubation was transferred to 90 μ L of potassium phosphate buffer containing 100 μ M midazolam and 1 mM NADPH, which had been pre-incubated at 37°C for 5 min, for the analysis of CYP3A activity. In order to assess the metabolism of diltiazem during the primary incubation, a separate set of samples was quenched with acetonitrile: methanol (3:1, v/v) containing alprazolam as internal standard for the analyses of diltiazem, MA and MD.

Since MD is the end product of the N-demethylation processes of diltiazem, dependence of microsomal protein concentration of MD-induced TDI of CYP3A was not evaluated. TDI of CYP3A by MD was measured by incubation of 1, 2, and 4 μ M of MD with 0.2 mg/mL HLM, the lowest concentration used in the TDI studies with MA and diltiazem. Using the dilution method, as described above, aliquots (10 μ L) were taken from the primary incubation at 0 and 12 min.

Reversible inhibition of CYP3A by diltiazem, MA and MD. Reversible inhibition experiment was conducted under conditions similar to the midazolam incubations mentioned above for TDI experiments. Diltiazem, MA, or MD was co-incubated with

midazolam and HLM in potassium phosphate buffer (0.1 M, PH 7.4) at 37°C for 5 min prior to the addition of NADPH. The final concentrations were: 0.02 mg/mL HLM protein, 1 mM NADPH, varying concentrations of inhibitors (Diltiazem: 0.5, 1, 2, and 4 μ M; MA: 0.5, 1, 2, and 4 μ M; and MD: 0.25, 0.5, 1, and 2 μ M) and substrate (midazolam: 1.25, 2.5, 5, and 10 μ M). The 0.02 mg/mL HLM matched the protein concentration used in the secondary incubation of the TDI studies (with 0.2 mg/mL HLM in primary incubation). This concentration was chosen to minimize nonspecific binding and substrate/inhibitor depletion. The experiments were performed in triplicate. Each reaction lasted for 1 min and was terminated by the addition of 100 μ L of a mixture of acetonitrile:methanol (3:1 v/v) containing alprazolam as internal standard.

Kinetics of diltiazem and MA metabolism and the effect of ketoconazole. Formation kinetics of MA and MD was determined in HLM using diltiazem and MA as substrates, respectively. The incubation mixture contained 0.2 mg/mL HLM protein, 1 mM NADPH, and varying concentrations of substrates: 6.25, 12.5, 25, 50, 100, and 200 μ M diltiazem or 0.8, 1.6, 3.1, 6.3, and 12.5 μ M MA. The microsomal concentration of 0.2 mg/mL matched the lowest concentration used in primary incubation of the TDI studies to ensure minimal substrate depletion and nonspecific binding. Substrate and HLM protein were pre-incubated in potassium phosphate buffer (0.1 M, PH 7.4) at 37°C for 5 min prior to the addition of NADPH. The effect of CYP3A inhibitor ketoconazole on diltiazem metabolite formation was assessed by co-incubating ketoconazole (1 μ M) and substrate (10 μ M diltiazem or 5 μ M MA) for 5 min at 37°C prior to the addition of NADPH. The experiments were performed in duplicate. The reactions were stopped at 0, 0.5, 1, and 2 min by the addition of equal volume of a mixture of acetonitrile:methanol

(3:1 v/v) containing alprazolam as internal standard. The linear portion of the time course of metabolite formation was used to obtain reaction velocity.

Microsomal binding of MA and MD. Microsomal protein binding was determined using equilibrium dialysis as described previously (Obach, 1997; Banker et al, 2003). A multi-well Teflon dialysis apparatus was manufactured at PGRD and the dialysis membrane (molecular weight cutoff 12-14 kDa) was purchased from Spectrum Medical Industries (Los Angeles, CA). An aliquot (150 μ L, n=3) of a mixture containing HLM (0.2, 0.4, and 0.8 mg/mL in 0.1 M potassium phosphate buffer, primary incubation condition for TDI studies) and inactivator (nominal concentrations of 0.25, 0.5, 1, 2, and 4 μ M for MA or 0.4 and 4 μ M for MD) was placed on one side of the membrane and blank phosphate buffer was added to the other side. The dialysis chamber was maintained at 37°C for 6 hours. Samples were quenched with an equal volume of a mixture of acetonitrile:methanol (3:1 v/v) containing alprazolam as internal standard.

Sample extraction and analytical methods for the measurement of inactivators and 1-hydroxymidazolam are described below.

Hepatocyte studies

Loss of CYP3A activity in cryopreserved human hepatocytes incubated with diltiazem.

Hepatocyte study was performed using the same pool of donors (3 males and 2 females) and the same condition as described previously (Zhao et al, 2005). Briefly, hepatocytes were thawed in thawing medium (25 mL per 5 million hepatocytes) and centrifuged at 50 x g at room temperature for 5 min. The cell pellet was reconstituted in 30 mL of incubation medium (William's Medium E containing 10 mM HEPES) and centrifuged at 50 x g at room temperature for 5 min. The resulting cell pellet was reconstituted in 30

mL of fresh incubation medium and cell viability was determined by the trypan blue method (generally between 78 to 82% viability). Cell suspension was placed in a 37°C incubator supplemented with 5% CO₂ before use.

Hepatocytes were pre-incubated in the presence of different concentrations of diltiazem (0.5, 1, 2, 4, 10, and 20 µM) in a 48-well plate for 1 hour in a 37°C incubator under 5% CO₂. The final volume was 250 µL and the final cell concentration was 0.5x10⁶ viable cells/mL. The experiments were performed in triplicate. At the end of pre-incubation, 200 µL aliquots of the suspension were transferred to 96-well plate micro-tubes (1.2 mL) and centrifuged at 50 x g for 5 min at room temperature. One hundred and fifty µL of each supernatant were discarded and the pellets were re-suspended after the addition of 150 µL of fresh incubation medium for washing. The suspension was further centrifuged at 50 x g for 5 min at room temperature and 180 µL of each supernatant were discarded. The post-wash pellet was re-suspended in 100 µL of fresh incubation medium containing midazolam. At this point, the concentrations were approximately 1 x 10⁶ cells/mL for hepatocytes and 100 µM for midazolam. The suspensions were immediately placed in the incubator (37°C, 5% CO₂) and were incubated for 9 min before the addition of 2x volume of a mixture of acetonitrile:methanol (3:1 v/v) containing alprazolam as internal standard. The use of 100 µM midazolam (to ensure saturation condition) and the choice of 9-min incubation time were confirmed by preliminary kinetic studies.

MA and MD formation in hepatocytes incubated with diltiazem. Free drug concentrations of diltiazem, MA, and MD in hepatocyte suspension (I_u) were believed to be equivalent to concentrations in hepatocyte medium since no protein was added to the medium.

Hepatocyte suspensions (incubated at 37°C for 0, 15, 40, and 60 min) were centrifuged at 50 x g for 5 min to obtain supernatant for the quantitation of I_u . Samples were quenched with equal volume of acetonitrile:methanol (3:1 v/v) containing alprazolam as internal standard. In parallel, aliquot of hepatocyte suspension was quenched with equal volume of acetonitrile:methanol (3:1 v/v) containing alprazolam for the quantitation of total drug concentrations.

LC-MS-MS methods. The supernatant from samples extracted by organic solvent was analyzed for diltiazem and its metabolites using Micromass Quattro Ultima mass spectrometer (Waters Corp., Milford, MA) equipped with Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) and LEAP autosampler (LEAP technologies, Carrboro, NC). Chromatographic separation was performed on a Zorbax C18 column (2x50 mm 5 micron, Agilent Technologies, Palo Alto, CA), in line with a Keystone Javelin C18 guard column (2x20 mm, Western Analytical Products, Inc. Murrieta, CA), at a flow rate of 0.2 to 0.3 mL/min using a linear gradient elution of A: 50 mM ammonium acetate in water and B: acetonitrile:methanol (89:11 v/v). Table 1 shows the analytical parameters for each analyte. The analysis of 1-hydroxymidazolam was performed using a Micromass Quattro LC mass spectrometer (Quattro II) equipped with a Waters 2790 HPLC system (Waters Corp., Milford, MA) as described previously (Zhao et al, 2005).

Data Analysis.

Microsomal protein binding and correction of metabolic consumption: The percent inactivator/inhibitor unbound (% Free) in HLM was calculated as the ratio of free

concentration to total concentration at equilibrium in the dialysis experiment. Mean percent MA remaining at each time point (from three experiments) of the primary incubation was plotted versus time and the area under the percent remaining-time curve from time zero to the last time point was calculated using a linear trapezoidal method by Microsoft Excel software (Microsoft Corp., Redmond, WA). A time-averaged factor was then calculated by dividing the area by time (zero to last time of primary incubation) to account for the metabolic consumption. Free MA concentrations in microsomal TDI studies were corrected for both %Free and metabolic consumption ($[I]_{u,ave}$).

TDI of CYP3A in HLM: Inactivation parameters in HLM were obtained by plotting the logarithm of the ratio of the remaining enzyme activity in the presence of inactivator to the activity in the absence of inactivator versus the pre-incubation time (Figures 2A to 2C). The apparent inactivation rate constant (λ) was determined from the slope of the initial linear phase on the semi logarithmic plots at each nominal inactivator concentration. Maximum inactivation rate constant (k_{inact}) and unbound inactivation constant ($K_{I,u,ave}$) were obtained by nonlinear regression of the $[I]_{u,ave}$ versus λ data using Equation 1. Data fitting was performed by the numerical module of SAAM II software (Version 1.2.1, SAAM Institute, Seattle, WA). A fractional standard deviation of 10% was assigned to each datum.

$$\lambda = \frac{k_{inact} \bullet [I]_{u,ave}}{K_{I,u,ave} + [I]_{u,ave}} \quad \text{Equation 1}$$

The predicted remaining CYP3A activity in hepatocytes or HLM was then calculated as a fraction of control without inactivator by Equation 2:

$$E_i/E_0 = e^{-\lambda t} \quad \text{Equation 2}$$

Where E_i and E_0 are CYP3A activities at specific primary incubation time point and time zero, respectively.

Reversible inhibition by MA and MD in HLM: Competitive inhibition was assumed to be the mechanism of reversible inhibition of CYP3A by MA and MD (Sutton, 1997), which is further confirmed by Dixon plots of the inhibition data from this study (data not shown). Inhibition constant values (K_i) and the Michaelis-Menten kinetic parameters for 1-hydroxymidazolam formation (K_m and V_{max}) were obtained simultaneously by fitting the 1'-hydroxymidazolam formation velocity (v) data according to Equation 3. Because the microsomal concentration (0.02 mg/mL) used in the reversible inhibition experiment was low and the reaction time was kept short, no correction of $[I]$ regarding nonspecific binding and depletion was performed. Data fitting was performed by nonlinear regression analysis using the numerical module of SAAM II software. A fractional standard deviation of 10% was assigned to each datum.

$$v = \frac{V_{max} \cdot [S]}{(K_m + [I] / K_i) + [S]} \quad \text{Equation 3}$$

Michaelis-Menten kinetics of diltiazem metabolite formation in HLM: Metabolite formation velocity was plotted against $v/[S]$ (Eadie-Hofstee plot) for each reaction. In general, each reaction was well described by a simple one-enzyme Michaelis-Menten model (data not shown). Parameters (K_m and V_{max}) of MA or MD formation were determined by nonlinear regression analysis using the numerical module of SAAM II software Equation 4. A fractional standard deviation of 10% was assigned to each datum.

$$v = \frac{V_{\max} \bullet [S]}{K_m + [S]} \quad \text{Equation 4}$$

Results

TDI of CYP3A by MA and MD in HLM. MA caused concentration- and time-dependent inactivation of CYP3A (Figures 2A to 2C). To ensure a fair comparison of parameters across varying microsomal protein concentrations, inactivator concentrations were corrected for microsomal binding and metabolic consumption during the primary incubation (Table 2) to obtain $[I]_{u,ave}$. Thus, the reported K_I values for MA are unbound K_I ($K_{I,u,ave}$, Figure 2D). Inactivation kinetic parameters for MA measured at different microsomal protein concentrations (0.2 to 0.8 mg/mL) are summarized in Table 3. Microsomal protein concentration seems to have little effect on the TDI parameters. The values for $K_{I,u,ave}$ ranged from 1.0 to 1.5 μ M and k_{inact} values from 0.045 to 0.061 min^{-1} . Table 3 also includes parameters obtained from diltiazem at 0.2 mg/mL HLM for comparison. Maximum rate of inactivation by MA is approximately four times faster than that of the parent drug (k_{inact} of 0.045 to 0.061 min^{-1} for MA vs. 0.012 min^{-1} for diltiazem). MD did not exhibit measurable time-dependent CYP3A inactivation (Table 4). Microsomal binding and metabolic consumption of MD were minimal.

Reversible inhibition of CYP3A in HLM. Reversible inhibition by diltiazem, MA, and MD was evaluated in HLM under conditions where TDI was evaluated. MA and MD caused concentration dependent inhibition of CYP3A. The estimated competitive K_i values are summarized in Table 5, which agree with the literature in general (Sutton, 1997). MD is approximately 10 times more potent than MA. The %free at 0.2 mg/mL HLM protein concentration was near unity for both MA and MD (Tables 2 and 4). Therefore, the competitive K_i values of MA and MD do not require further correction for metabolic consumption and microsomal binding at a 10-fold lower protein

concentration (0.02 mg/mL) used in reversible inhibition experiment. The Michaelis-Menten kinetic parameters for 1'-hydroxymidazolam formation (K_m and V_{max}) were simultaneously fitted with K_i according to Eq. 3 for the experiments using MA or MD as inhibitor. The values of parameter estimate (standard error) for K_m are 2.4 (0.2) and 2.2 (0.2) μ M, and for V_{max} 5.0 (0.1) and 4.9 (0.2) nmol 1'-hydroxymidazolam/min/mg protein from the experiments with MA and MD, respectively. The K_m values are similar to that reported by Walsky and Obach (2004), which was approximately 2 μ M. As expected from literature report (Sutton, 1997), diltiazem caused minimal inhibition of CYP3A under our experimental conditions (data not shown).

Diltiazem-induced TDI of CYP3A and sequential metabolism in HLM – effect of microsomal protein concentration. Figure 3A shows the time-dependent loss of CYP3A activities in HLM incubated with 10 μ M of diltiazem at varying concentrations of microsomal protein (0.2, 0.4 and 0.8 mg/mL). The observed inactivation of CYP3A appears to be dependent on microsomal protein concentrations. Figures 3B to 3D show the corresponding profiles of parent drug loss (percent remaining vs. time) and metabolite formation (concentration vs. time) during the 1st incubation at varying microsomal protein concentrations. At the end of 20 min, the percent remaining of diltiazem was protein concentration dependent ($86 \pm 5\%$, $73 \pm 12\%$, and $65 \pm 4\%$ at 0.2, 0.4, and 0.8 mg/mL protein, respectively), which corresponds to the formation of sequential metabolites: the higher the protein concentration, the more the metabolites were formed.

CYP3A dependent N-demethylation of diltiazem and MA. Michaelis-Menten parameters for MA and MD formation from diltiazem and MA are shown in Table 6.

The apparent K_m and V_{max} are 54 μ M and 1881 pmol/min/mg for MA formation, and 19

μM and 385 pmol/min/mg for MD formation from MA, respectively. Sutton and colleagues (1997) have reported a K_m of 23 μM and V_{\max} ranging from 1.0 to 5.4 nmol MA/min/mg in HLM from 3 individuals. The apparent intrinsic clearance (V_{\max}/K_m) for the formation of MA from diltiazem appears to be greater than that for MD formation from MA (Table 6). A classic CYP3A inhibitor ketoconazole (1 μM) caused more than 90% inhibition of each N-demethylation process.

CYP3A inactivation by diltiazem in hepatocytes. Figure 4A shows the loss of CYP3A activity in cryopreserved human hepatocytes incubated with varying concentrations of diltiazem for 60 min. The activity study was performed in hepatocytes washed with fresh medium (at least 20 times dilution, see Materials and Methods). Figures 4B and 4C show the MA and MD formation in hepatocytes incubated with diltiazem. MD formation at diltiazem concentrations of 0.5, 1, and 2 μM is not reported due to nothing observed at given detection limits. At 10 and 20 μM diltiazem, MA concentration increased to 0.88 and 1.44 μM after 60 min, which approach its microsomal $K_{I,u,ave}$ and K_i values (Tables 3 and 5). At 20 μM diltiazem, MD level reached 74 nM after 60 min, which is approximately 30% of its competitive K_i value of 0.2 μM (Table 5). Washing with medium will result in at least 20 times dilution, making MA and MD levels in the medium of activity experiments significantly below their competitive K_i values (2.7 and 0.2 μM for MA and MD, respectively, Table 5).

Table 7 summarizes the free fraction of inhibitor in hepatocyte incubation (f_c), which is calculated by dividing free concentration in incubation medium (I_u) by total concentration. The f_c values for diltiazem are near unity, whereas low recovery was

apparent for MA and MD (f_c from 0.50 to 0.66), suggesting the possible trapping of both metabolites inside the cells upon formation. Therefore, intracellular concentrations of diltiazem are expected to be higher than the observed total concentrations in Figures 4B and 4C.

Discussions

Identification of the mechanism responsible for diltiazem-induced loss of CYP3A.

The results of the present study implicate TDI by MA as the major contributor responsible for diltiazem-induced loss of CYP3A. This is supported by the following findings: i) the primary metabolite of diltiazem, MA, is a more potent time-dependent CYP3A inactivator than its parent drug; ii) the dependence of enzyme inactivation on microsomal protein concentrations for diltiazem, but not for MA, suggests a significant contribution of this sequentially formed primary metabolite to *in vivo* drug interaction formerly attributed to the parent compound ; and iii) among the different mechanisms induced by diltiazem and its sequential metabolites MA and MD, time-dependent CYP3A inactivation by MA seems to be the major contributor responsible for diltiazem induced loss of CYP3A in HLM and human hepatocytes.

Inhibitory metabolites have been shown to play an additional role of inhibiting enzymes other than the parent compound. Isoherranen and colleagues (2004) have demonstrated that itraconazole metabolites are equal or more potent CYP3A4 inhibitors compared to the parent drug in HLM. Consequently, these inhibitory species may contribute to the inhibition of CYP3A4 observed *in vivo* after administration of itraconazole. Similarly, this report demonstrates that sequential metabolites of a time-dependent inactivator can significantly contribute to the observed drug interaction.

Inactivation of CYP3A by diltiazem and MA results from the formation of an MI complex between ferrous prosthetic heme iron and a highly oxidized nitroso metabolite (Jones et al, 1999, Mathew et al, 2000). Because MD is not a TDI of CYP3A (Table 4), we hypothesize that MA causes mechanism-based inactivation by forming reactive

intermediate that modifies the enzyme upon formation. Additionally, MA and MD have been reported to be competitive inhibitors of CYP3A (Sutton et al, 1997). It is generally believed that drug interaction arising from co-administration of diltiazem with CYP3A substrates *in vivo* is mainly due to enzyme inactivation rather than reversible inhibition by diltiazem and its metabolites. This conclusion is supported by the facts that the observed free plasma concentrations of MA are much lower than its K_i (Mayhew et al, 2000), and plasma levels of MD appears to be negligible (Molden et al, 2003). Additionally, a prolonged half-life of diltiazem was observed in hypertension patients taking multiple doses (Montamat and Abernethy, 1987), consistent with a time-dependent auto inactivation of diltiazem metabolism. In fact, CYP3A is the major isoform responsible for N-demethylation reactions of diltiazem (Sutton et al, 1997). The inhibiting effect of ketoconazole in this study further confirmed the role of CYP3A in N-demethylation pathways for diltiazem (Table 6). In this report, we comprehensively assessed the contribution of different mechanisms to the loss of CYP3A activity by conducting microsomal studies to obtain inhibition/inactivation parameters of the sequential N-desmethyl metabolites of diltiazem. Most of the parameters we have obtained agree with literature reports. In depth evaluation of each mechanism revealed the significance of each mechanism of inactivation/inhibition by both parent drug and metabolites on diltiazem-induced loss of CYP3A. Among different mechanisms, TDI by MA appears to be the major contributor, which is further supported by results from hepatocyte experiments.

Hepatocyte is a more integrated system than liver microsomes and has been used to predict metabolic clearance and drug interactions *in vivo* in pharmaceutical industries

(McGinnity, 2006, Lu, 2006, 2007). Given its unique position along the hierarchy between liver microsomes and *in vivo*, hepatocytes can be used to test the predictability of parameters obtained using liver microsomes. We have demonstrated that the discrepancies between the predicted inactivation using microsomal kinetic parameters and those observed in hepatocytes can be partially explained by factors governing the accessibility of inactivators to CYP3A in hepatocytes (Zhao, 2005). These factors include, but are not limited to, non-specific binding, metabolic consumption, and active transport. Results from this work demonstrate that sequentially formed metabolites are another important factor to consider when discrepancies are observed between HLM and hepatocyte.

In hepatocytes incubated with 10 and 20 μM diltiazem, MA concentration increased to 0.88 and 1.44 μM , respectively after 60 min (Figure 4B), concentrations approaching its microsomal $K_{I,u,ave}$ and K_i values (Tables 3 and 5). At 20 μM diltiazem, MD concentration was 74 nM at 60 min (Figure 4C), which is approximately 30% of its microsomal competitive K_i value (Table 5). The values of f_c are lower than unity for both MA and MD (Table 7), implicating possible entrapment of metabolites inside the cells upon formation. Preliminary calculation of hepatocyte volume in the initial incubation (primary incubation) with diltiazem suggests that the cells account for less than 5% of total incubation volume (data not shown). Assuming f_c of 0.6, the small cell volume results in approximately 10-fold higher cellular concentrations for MA and MD than those observed in Figures 4B and 4C. Under our experimental condition, the activity study (secondary incubation with midazolam) was carried out after the cell pellets were washed twice with fresh medium to achieve at least a 20-fold dilution of the primary

incubation. At this stage, the competitive inhibition is expected to become less important if the washing caused similar dilution of intracellular levels of metabolites, especially for hepatocytes incubated with lower concentrations of diltiazem ($<4 \mu\text{M}$). Thus, TDI by MA was likely the major cause of decreased CYP3A activity described in Figure 4A.

Theoretically, the time-dependent loss of CYP3A in hepatocytes can be modeled if the following information are available: microsomal parameters of metabolite formation (i.e. K_m , V_{max}), inhibition/inactivation (K_i , K_I and k_{inact}), and the contribution of each inhibitory mechanism for the parent and each of its sequential metabolites. For example, if MA induced TDI is the predominant mechanism, a model consisting of the loss of CYP3A depicted by Equations 1 and 2, and by a response relationship relating CYP3A activity loss as a function of MA levels, can be established. However, the selection of inhibitor concentration and the contribution of reversible inhibition will significantly complicate the model. Since MA is formed from diltiazem in the active site inside the cell, it likely causes inactivation before appearing in the extracellular space. Thus, a kinetic profile of MA based on its I_u is not appropriate. Similarly, cellular accumulation of MD may result in an appreciable concentration to cause reversible inhibition of CYP3A, at higher diltiazem concentrations. The trapping effect of MA and MD in hepatocytes incubated with diltiazem is not understood. This poor partitioning may be due to non-specific binding to cellular compartment or active transport process(es). Nonetheless, if the accumulation of MA and MD is true *in vivo*, predicting diltiazem induced clinical drug interaction would be more complicated.

In this study we focused on the inhibitory effect of diltiazem and its N-desmethyl metabolites MA and MD. Diltiazem also undergoes O-demethylation and deacetylation

(“other pathways” in Figure 1, Homsy, 1995, Murray and Butler, 1996, Sutton, 1997).

O-desmethyl diltiazem did not inhibit CYP3A in HLM (Sutton, 1997). Although no report on the inhibitory effects of deacetylated metabolites on human CYP3A is available, a study conducted in rats demonstrated that metabolites associated with deacetylation and O-demethylation were ineffective inhibitor of CYP3A2 mediated 6- β hydroxylation of testosterone, and MA and MD were more potent competitive inhibitors of CYP3A2 than diltiazem (Murray and Butler, 1996). These findings appear to support the hypothesis that metabolites other than MA and MD do not inhibit human CYP3A, although it needs to be confirmed when these metabolites are available.

Diltiazem showed different inhibition/inactivation kinetics toward CYP3A4 and CYP3A5 (McConn, 2004). Because both CYP3A isoforms catalyze 1'-hydroxylation reaction of midazolam (Williams, 2002, Huang, 2004), one can not disregard the impact that differential expression of these two CYP3A isoforms in HLM and hepatocytes may have on the interpretation of the results presented in this study. We have chosen gender pooled HLM (n=59) and hepatocytes (n=5) to minimize this impact as well as the influence by large inter-individual variability known to human CYP3A family (Khuehl, 2001). Although beyond the scope of this manuscript, studies of the inhibition/inactivation by diltiazem in CYP3A4 and CYP3A5 supersomes should be performed to further understand the mechanism. For example, if quantitative prediction were to be performed in hepatocytes using HLM data, caution should be made because of the differences in inhibition/inactivation kinetics by diltiazem and its metabolites and the kinetics of 1-hydroxy midazolam formation between CYP3A4 and CYP3A5.

Impact of sequential metabolism on the prediction of drug interaction using microsomal parameters of parent compound. The second objective of this study was to investigate the impact of sequential metabolism of inhibitor on the validity of using microsomal kinetic parameter of the parent compound to predict its inactivation potential *in vivo*. The results presented in this manuscript suggest that prediction of *in vivo* drug interaction by utilizing microsomal kinetic parameters (i.e. K_I and k_{inact}) obtained under one experimental condition may be inadequate.

Because TDI has been recognized as an important mechanism to cause drug interactions, pharmaceutical industries have implemented methodologies to evaluate the potential for new chemical entities' to cause time-dependent inhibition (Favreau, 1999, Lim, 2005). The advancement of automation in drug discovery and development allows companies to obtain more information when screening TDI. Compounds can be tested for TDI by monitoring the time-dependent change in inhibition potency, i.e., the value of inhibitor concentration causing 50% of enzyme inhibition ("IC₅₀ shift" method) at early discovery stage, or by definitive TDI studies at later development stage to obtain k_{inact} and K_I , as described by Ghanbari and colleagues (2006). These TDI parameters are then used to predict *in vivo* drug interactions. Our findings that diltiazem-induced TDI in HLM is protein-concentration dependent clearly demonstrated that kinetic parameters obtained from a microsomal study under one condition can be misleading. For diltiazem, increased TDI of CYP3A is associated with loss of parent drug and simultaneous accumulation of sequential metabolites MA and MD (Figure 3). Thus, if definitive TDI experiments were performed for diltiazem using different microsomal protein concentrations, one would expect to obtain different k_{inact} (depending on reaction

time and the extent of metabolite formation) and possibly different K_I (lower $I_{u,ave}$ of diltiazem at higher protein concentration). Whereas data collected from the condition with the lowest protein concentration may implicate diltiazem as a remote inactivator, resulting in false negative.

In conclusion, we have demonstrated that TDI by MA is the main contributor responsible for the loss of CYP3A activity in microsomes and hepatocytes incubated with diltiazem. Given the complexity of inhibition/inactivation mechanisms by diltiazem and its metabolites, caution should be made when predicting inhibition/inactivation using microsomal kinetic parameters derived from parent drug only and/or from one set of conditions.

Acknowledgements:

The authors would like to acknowledge David Neul for providing human cryopreserved hepatocytes and Tanabe Seiyaku Co. Ltd. for providing N-desmethyl metabolites of diltiazem for the studies reported in the manuscript. We also thank Dr. Ellen Wu for critical review of manuscript.

References:

Banker MJ, Clark TH, Williams JA (2003) Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. *J Pharm Sci* **92**:967-974.

Dai R, Wei X, Luo G, Sinz M, Marathe P (2003) Metabolism-dependent P450 3A4 inactivation with multiple substrates. Abstract from 12th North American ISSX Meeting. Providence, RI. *Drug Metab Rev* **35** (Suppl. 2): 341.

Ghanbari F, Rowland-Yeo K, Bloomer JC, Clarke SE, Lennard MS, Tucker GT, Rostami-Hodjegan A (2006) A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition, with implications for in vitro-in vivo extrapolation. *Curr Drug Metab.* **7**:315-334.

Homsy W, Lefebvre M, Caille G, du Souich P (1995) Metabolism of diltiazem in hepatic and extrahepatic tissues of rabbits: in vitro studies. *Pharm Res.* **12**:609-614.

Huang W, Lin YS, McConn DJ 2nd, Calamia JC, Totah RA, Isoherranen N, Glodowski M, Thummel KE (2004) Evidence of significant contribution from CYP3A5 to hepatic drug metabolism. *Drug Metab Dispos.* **32**: 1434-1445.

Isoherranen N, Kunze KL, Allen KE, Nelson WL, Thummel KE (2004) Role of itraconazole metabolites in CYP3A4 inhibition. *Drug Metab Dispos* **32**:1121-1131.

Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H, Sugiyama Y (1998) Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interaction in the liver. *Pharmacol Rev* **50**:387-412.

Jerling M, Huan BL, Leung K, Chu N, Abdallah H, Hussein Z (2005) Studies to investigate the pharmacokinetic interactions between ranolazine and ketoconazole, diltiazem, or simvastatin during combined administration in healthy subjects. *J Clin Pharmacol* **45**:422-33.

Jones DR, Gorski JC, Hamman MA, Mayhew BS, Rider S, Hall SD (1999) Diltiazem inhibition of cytochrome P-450 3A activity is due to metabolite intermediate complex formation. *J Pharmacol Exp Ther* **290**:1116-1125.

Jones TE and Morris RG (2002) Pharmacokinetic interaction between tacrolimus and diltiazem: dose-response relationship in kidney and liver transplant recipients. *Clin Pharmacokinet*. **41**:381-388.

Kitz R and Wilson IB (1962) Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol Chem* **237**: 3245-3249.

Kosuge K, Nishimoto M, Kimura M, Umemura K, Nakashima M, Ohashi K (1997) Enhanced effect of triazolam with diltiazem. *Br J Clin Pharmacol* **43**:367-372.

Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E (2001) Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet.* **27**:383-391.

Lu C, Li P, Gallegos R, Uttamsingh V, Xia CQ, Miwa GT, Balani SK, Gan LS (2006). Comparison of intrinsic clearance in liver microsomes and hepatocytes from rats and humans: evaluation of free fraction and uptake in hepatocytes. *Drug Metab Dispos.* **34**:1600-1605.

Lu C, Miwa GT, Prakash,SR, Gan L-S, Balani SK (2007) A novel model for the prediction of drug-drug interactions in humans based on in vitro cytochrome P450 phenotypic data. *Drug Metab Dispos* **35**: 79-85.

Mayhew BS, Jones DR, Hall SD (2000) An in vitro model for predicting in vivo inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation. *Drug Metab Dispos* **28**:1031–1037.

McConn DJ 2nd, Lin YS, Allen K, Kunze KL, Thummel KE (2004) Differences in the inhibition of cytochromes P450 3A4 and 3A5 by metabolite-inhibitor complex-forming drugs. *Drug Metab Dispos.* **32**:1083-1091.

McGinnity DF, Berry AJ, Kenny JR, Grime K, Riley RJ (2006). Evaluation of time-dependent cytochrome P450 inhibition using cultured human hepatocytes. *Drug Metab Dispos.* **34**:1291-1300.

Molden E, Helen Boe G, Christensen H, Reubsaet L (2003) High-performance liquid chromatography-mass spectrometry analysis of diltiazem and 11 of its phase I metabolites in human plasma. *J Pharm Biomed Anal.* **33**:275-285.

Montamat SC, Abernethy DR (1987) N-monodesmethyldiltiazem is the predominant metabolite of diltiazem in the plasma of young and elderly hypertensives. *Br J Clin Pharmacol* **24**: 185-189.

Murray M, Butler AM. (1996) Enhanced inhibition of microsomal cytochrome P450 3A2 in rat liver during diltiazem biotransformation. *J Pharmacol Exp Ther.* **279**:1447-1452.

Obach RS (1997) Nonspecific binding to microsomes: impact on scale-up of in vitro intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab Dispos* **25**:1359-1369.

Sutton D, Butler AM, Nadin L, Murray M (1997) Role of CYP3A4 in human hepatic diltiazem N-demethylation: inhibition of CYP3A4 activity by oxidized diltiazem metabolites. *J Pharmacol Exp Ther* **282**: 294-300.

Walsky RL, Obach RS (2004). Validated assays for human cytochrome P450 activities. *Drug Metab Dispos.* 32:647-660.

Williams JA, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, Hamman MA, Hall SD, Wrighton SA (2002) Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos.* **30**:883-891.

Zhao P, Kunze KL, Lee CA (2005) Evaluation of time-dependent inactivation of CYP3A in cryopreserved human hepatocytes. *Drug Metab Dispos* **33**:853-861.

Footnotes:

This work was partially supported by NIH GM32165.

Address correspondence to: Ping Zhao, PhD. Sonus Pharmaceuticals, Inc.

22026 20th Ave SE, Bothell, WA 98021, USA

* Current affiliation: Sonus Pharmaceuticals, Bothell, Washington

Legends for Figures

Figure 1. Metabolic scheme of diltiazem to MA and MD.

Figure 2. TDI of CYP3A in HLM by MA. Panels A to C, HLM protein concentration of 0.2, 0.4, and 0.8 mg/mL, respectively; legends show the nominal concentration of MA. Data are Mean \pm SD (n=3 individual experiments). Panel D, apparent inactivation rate constant (λ) versus $[MA]_{u,ave}$ plot. Lines represent simulations according to equation 1 and legends show microsomal protein concentrations.

Figure 3. (A) Loss of CYP3A activity (A), metabolic consumption of diltiazem (B) and metabolite formation (C and D) upon incubation of HLM with 10 μ M diltiazem.

Legends show microsomal protein concentration. Data are Mean \pm SD (n=4-5 individual experiments for A and B, n=3-5 individual experiments for C and D).

Figure 4. (A): Effect of diltiazem incubation on CYP3A activity in cryopreserved human hepatocytes at the end of 1-hour incubation. Open square: observed CYP3A activities (Mean \pm SD, n=3-4 individual experiments); closed square: remaining CYP3A activity predicted using microsomal TDI parameters of diltiazem according to Equation 2. (B and C): time courses of total MA formation (B) and MD formation (C) in hepatocytes incubated with diltiazem.

Table 1. LC-MS-MS analytical parameters of diltiazem and metabolites.

Analyte	diltiazem	MA	MD	alprazolam
Mobile phase gradient program: % B (min)	10(0)→10 (0.6)→66 (3.2)→66(3.6)→90 (4.2)→90 (4.3) →10 (4.4)			
Analyte m/z transition	415→ 178	401→ 178	387→ 178	309→ 205
Mode	Positive	Positive	Positive	Positive
Capillary voltage (kV)	3.1	3.1	3.1	3.1
Cone Voltage (V)	40	69	69	50
Desolvation temperature (°C)	350	350	350	350
Collision energy (eV)	36	20	20	80
Retention time (min)	5.9	5.4	5.3	5.6

Table 2. Time-averaged free concentration of MA in liver microsomes.

Microsomal protein (mg/mL)	Nominal concentration of MA (μ M)	%Free ^a	Time-averaged factor ^b	Time averaged free concentration of MA ^c (μ M)
0.2	0.25	0.81(0.10)	0.94	0.19
	0.5	0.77(0.08)	0.99	0.38
	1	0.77 (0.09)	0.94	0.73
	2	0.80 (0.08)	1.01	1.60
	4	0.79 (0.07)	1.02	3.20
0.4	0.25	0.64 (0.04)	0.96	0.15
	0.5	0.65 (0.04)	0.85	0.28
	1	0.68 (0.08)	0.92	0.63
	2	0.68 (0.08)	0.94	1.28
	4	0.65 (0.08)	0.92	2.38
0.8	0.25	0.63 (0.01)	0.80	0.13
	0.5	0.63 (0.04)	0.79	0.25
	1	0.63 (0.03)	0.87	0.55
	2	0.64 (0.02)	0.87	1.11
	4	0.59 (0.04)	0.92	2.16

^a: %Free Mean (SD), n=3 incubations

^b: Time-averaged factor (accounting for metabolic consumption) was calculated according to that described Materials and Methods

^c: Nominal concentration multiplied by %Free and Time-averaged factor

Table 3. Time-dependent CYP3A inactivation in HLM by MA.

	HLM protein concentration (mg/mL)	k_{inact} (min^{-1})	$K_{\text{I,u,ave}}$ (μM) ^a
MA	0.2	0.047 (0.005) ^b	1.1(0.2) ^b
	0.4	0.045 (0.006)	1.0 (0.2)
	0.8	0.061 (0.010)	1.5 (0.3)
diltiazem ^c	0.2	0.012	0.48

^a Value corrected for HLM protein binding and metabolic consumption according to table 2.

^b Estimate (standard error) by SAAM II software (Materials and methods).

^c Zhao et al, 2005

Table 4. Lack of Time-dependent CYP3A inactivation by MD.

MD (μ M)	HLM protein binding ^a (% Free)	% MD remaining ^b	% CYP3A activities ^b
0.4	88 (5)	ND	ND
1	ND	109 (7)	96 (5)
2	ND	101 (6)	103 (9)
4	83 (5)	111 (13)	106 (5)

^a Calculated according to equation 1. Mean (S.D.), n=4 incubations at 0.2 mg/mL HLM

^b Calculated as the percent remaining at 12-min tested in the inactivation experiments. Mean (S.D.), n=3 incubations

Table 5. Parameters of reversible inhibition of CYP3A in HLM by MA and MD.
Results from this study are estimates (standard error) obtained using SAAM II software.

	Findings from this study		Literature report	
	K_i (μ M) ^a	Type	K_i (μ M)	Type
MA	2.7 (0.2)	Competitive	2.2, 2.5 ^b	Competitive
MD	0.2 (0.0)	Competitive	0.07, 0.1 ^b	Competitive

^a: Results are estimate (standard error). Compounds were co-incubated with midazolam (1.25 to 10 mM) for 1-minute after NADPH was added

^b: Determined from two individuals (Sutton et al, 1997)

Table 6. Michaelis-Menten kinetic parameters of metabolite formation from diltiazem and MA in HLM. Results are parameter estimate (standard error) obtained by SAAM II software assuming one enzyme reaction.

	K_m (μ M)	V_{max} (pmol/mg/min)	Intrinsic clearance ^a (μ L/mg/min)	% inhibition by 1 μ M ketoconazole
Diltiazem to MA	54 (5)	1881 (115)	35	94% ^b
MA to MD	19 (8)	385 (1)	20	93% ^c

^a: Calculated as V_{max} / K_m

^b: n=4, diltiazem 10 μ M, 18-min reaction

^c: n=3, MA 5 μ M, 2-min reaction

Table 7. Free fraction (f_c) of diltiazem and its sequential metabolites in hepatocyte incubations over time. Calculated as the ratio of supernatant concentration to total concentration. Mean (SD), n=3 individual incubations.

	Time (min)			
	0	15	40	60
Diltiazem	0.89 (0.12)	1.04 (0.06)	0.94 (0.11)	0.92 (0.14)
MA	-	0.51 (0.01)	0.55 (0.07)	0.66 (0.01)
MD	-	0.61 (0.10)	0.55 (0.01)	0.63 (0.06)

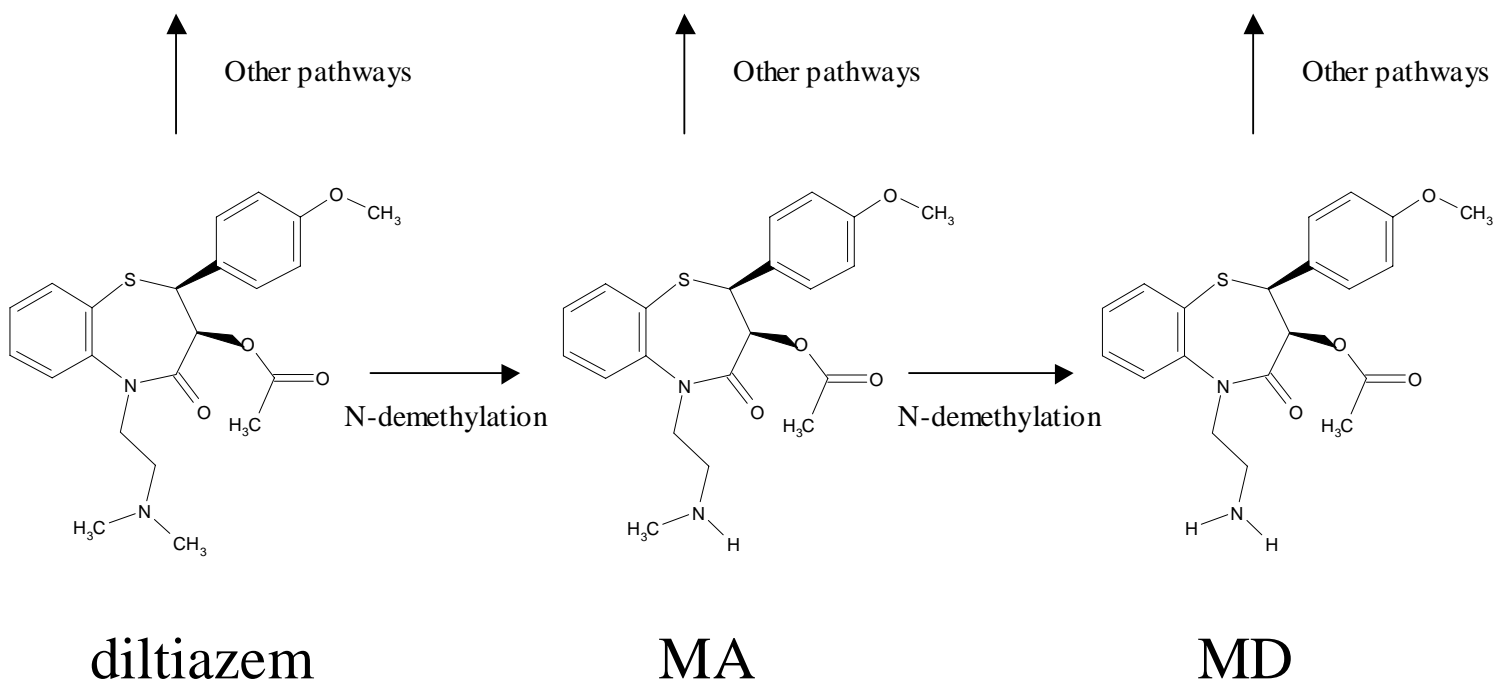


Figure 1

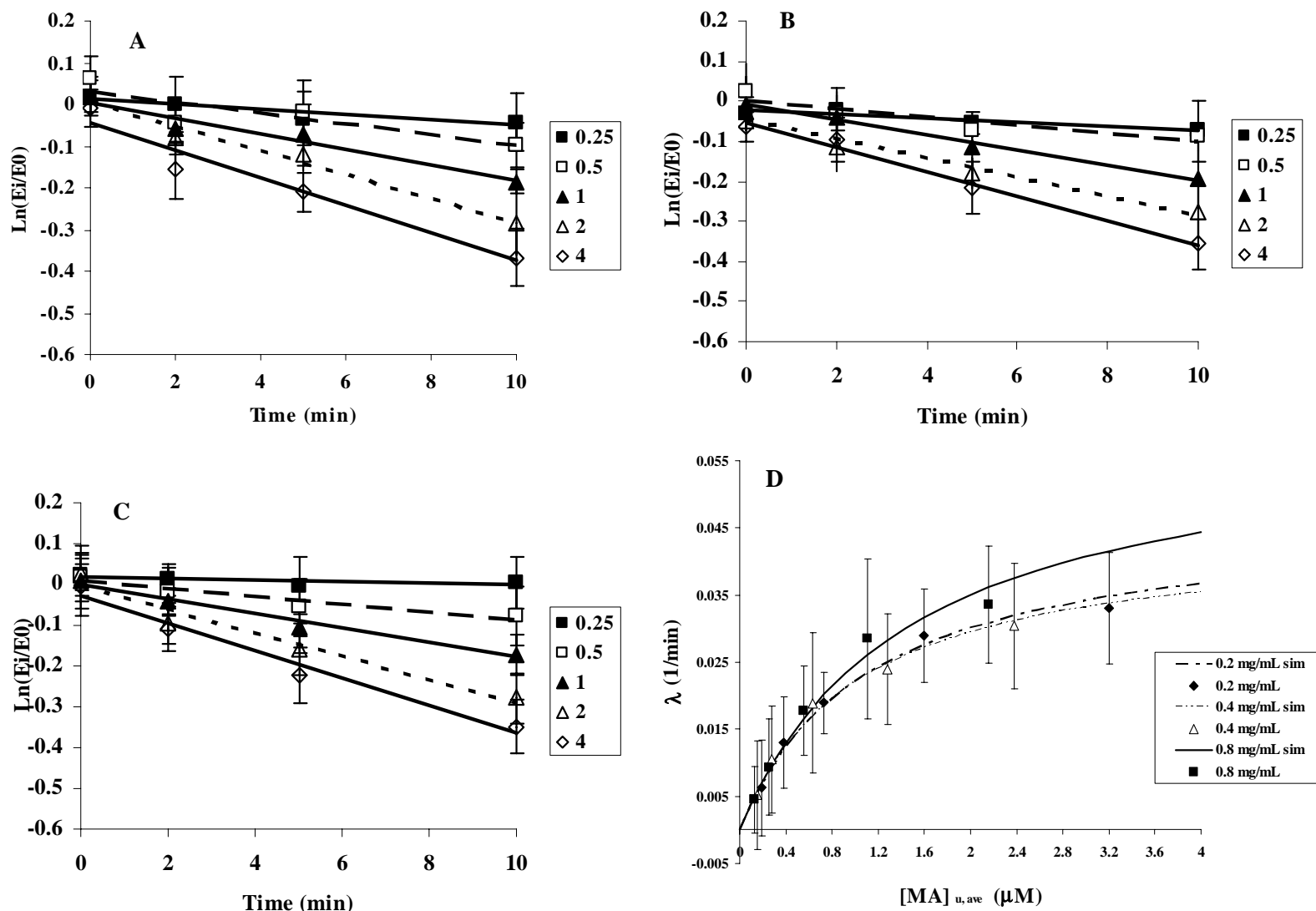


Figure 2

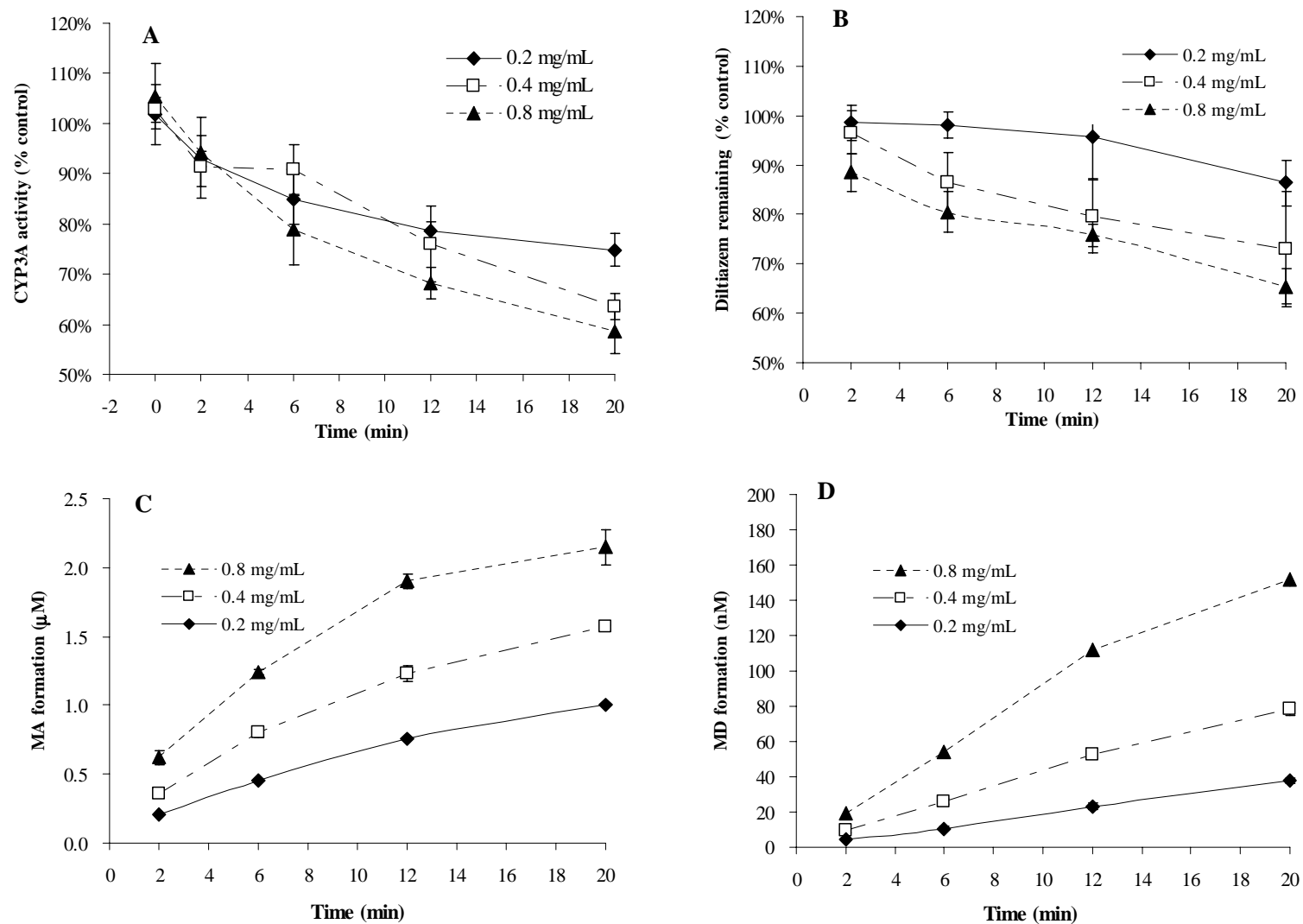


Figure 3

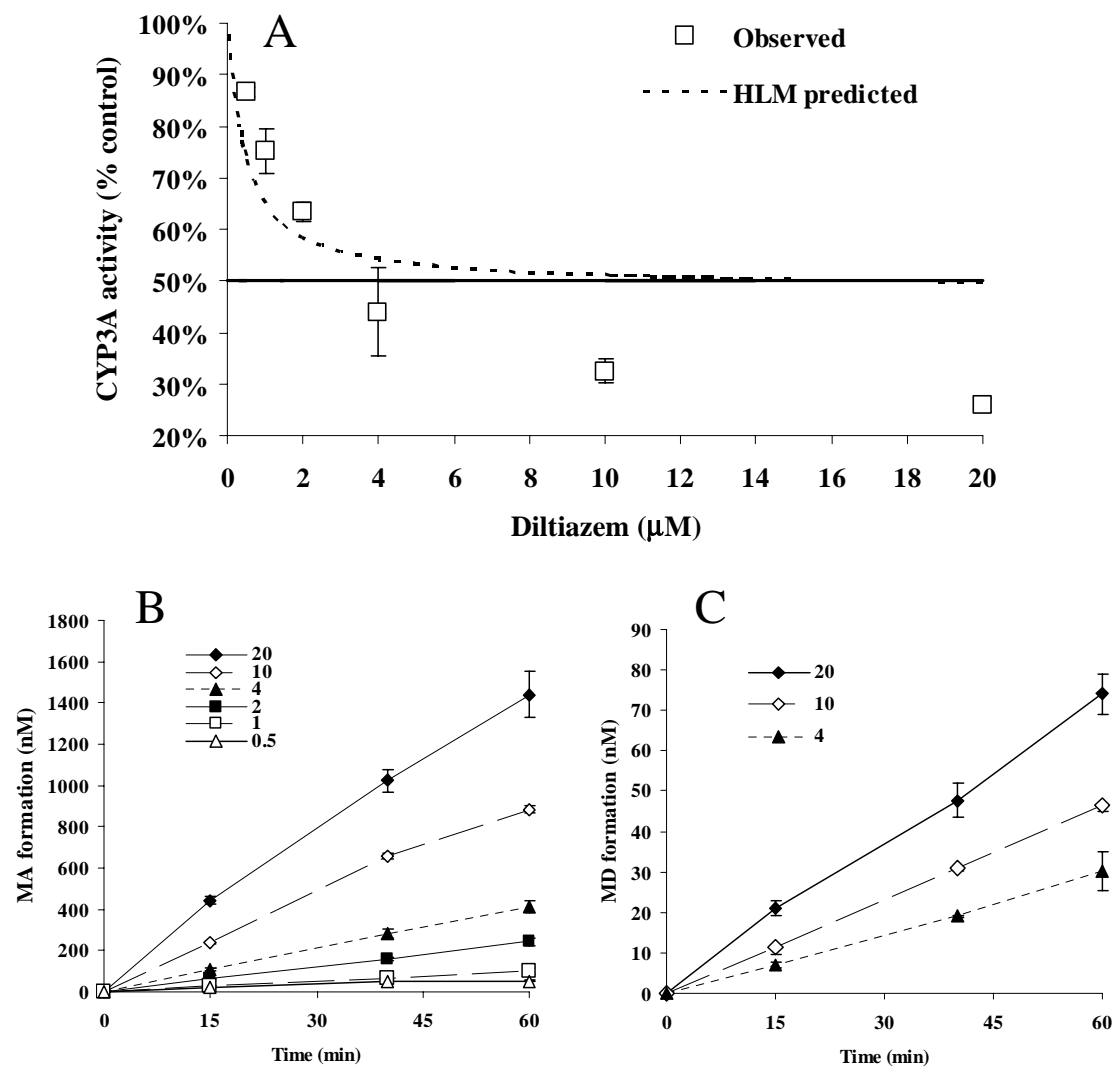


Figure 4