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EVALUATION OF TIME-DEPENDENT INACTIVATION OF CYP3A IN CRYOPRESERVED HUMAN HEPATOCYTES

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Abbreviations:

CYP3A: cytochrome P450 3A; HLM: human liver microsomes; $IC_{50,\ Nominal}$: the nominal concentration of inactivator to cause 50% inactivation observed in hepatocytes; $IC_{50,}$

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$_{Corrected}$: IC_{50} corrected for non-specific binding and time-dependent loss due to metabolism in hepatocytes; $IC_{50,\ Predicted}$: predicted inactivator concentration in hepatocytes which produced 50% inactivation of CYP3A activity using microsomal parameters; I_u: free inactivator concentration in hepatocyte incubation medium; $\overline{[I]}$: time-averaged inactivator concentration in the hepatocyte incubation medium; K_I: apparent inactivation constant; k_{inact}: maximum inactivation rate constant; λ: apparent inactivation rate constant; TDI: time-dependent inactivation

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ABSTRACT

Irreversible CYP3A inhibition by drugs constitutes one of the major causes of inhibition-based drug interactions. We evaluated time-dependent inactivation of CYP3A in cryopreserved human hepatocytes for six structurally diverse compounds known to exhibit this property. Inactivation kinetic parameters were also determined using human liver microsomes. Except for diclofenac, which did not cause CYP3A inactivation either in microsomes or hepatocytes at concentrations up to 100 μM, time-dependent inactivation was observed in hepatocytes for amprenavir, diltiazem, erythromycin, raloxifene, and troleandomycin. The observed inactivation potency in hepatocytes (observed IC_{50}) was compared to the potency predicted using microsomal parameters (predicted IC_{50}). Despite satisfactory prediction for troleandomycin (1.35 and 2.14 μM for the predicted and observed IC_{50}, respectively), over-prediction of inactivation was observed for raloxifene, amprenavir and erythromycin (observed IC_{50} 6.2, 55, and 7.8-fold higher than the predicted $IC_{50,}$ respectively). By contrast, the observed IC_{50} for diltiazem in hepatocytes was approximately 4-fold lower than the IC_{50} predicted from microsomal data (under-prediction). After correcting for factors including non-specific binding and inactivator consumption, prediction was significantly improved for raloxifene (the observed IC_{50} then became 2-fold higher than the predicted IC_{50}) and for amprenavir to a lesser extent. A specific P-glycoprotein inhibitor CP-100356 modulated the observed CYP3A inactivation potency by erythromycin and troleandomycin. In summary, these studies reveal three important factors that must be considered when microsomal inactivation parameters are used to predict inhibition-based drug interactions in intact cell systems.

The cytochrome P450 3A (CYP3A) family of enzymes is responsible for the metabolism of more than 50% of marketed drugs and some important endogenous substances. Consequently, drug interactions arising through the inhibition of CYP3A are of significant clinical importance (Lin, 1998). Mechanisms of CYP3A inhibition include reversible inhibition and time-dependent inactivation (TDI, also known as mechanism-based inhibition), and the latter has been recognized for many drugs and new chemical entities (Lin, 1998, Ito et al, 1998, Mayhew et al, 2000). Mayhew and coworkers showed that the inhibition of CYP3A by N-desmethyl-diltiazem, clarithromycin, and fluoxetine in human was due to TDI instead of reversible inhibition (Mayhew et al, 2000). Traditionally, TDI is studied in human liver microsomes (HLM) or c-DNA expressed enzyme systems to obtain inactivation kinetic parameters including the maximum inactivation rate constant (k_{inact}) and the apparent inactivation constant (K_I). These parameters, along with the expected inactivator exposure ([I]) and the degradation rate constant of the enzyme, which is usually obtained from animal data (Kanamitsu et al, 2000 and Mayhew et al, 2000), are employed to predict the extent of enzyme inactivation *in vivo*.

Cryopreserved hepatocytes are widely used in drug discovery and development in pharmaceutical industries to predict ADME properties for new chemical entities. Hepatocytes have the advantage of maintaining a complete set of drug transporters and phase I/II metabolizing enzymes, not fully represented by HLM. It is anticipated that factors present in hepatocytes that are absent in microsomal systems may influence the

prediction of inhibition-based drug interactions *in vivo* by directly using microsomal kinetic parameters (k_{inact} and K_I).

In the present study, we have developed an approach to evaluate the TDI of CYP3A in cryopreserved human hepatocytes. Prediction of the extent of inactivation in hepatocytes was made using kinetic parameters obtained from HLM experiments. The predicted inactivation potency was compared to the values observed in hepatocytes. The reliability of utilizing HLM to predict TDI *in vivo* and the feasibility of using hepatocytes to assess TDI will be discussed.

Materials and Methods

Materials. Cryopreserved human hepatocytes and hepatocyte thawing medium were purchased from InVitro Technologies (Baltimore, MD). Pooled human liver microsomes from 60 individuals were obtained from a Pfizer Global R&D (PGRD, a division of Pfizer Inc) in-house supply. CP-100356 was synthesized at PGRD. Amprenavir was re-purified from the commercially available formulations at PGRD. HEPES was purchased from Invitrogen Life Technologies (Carlsbad, CA). All other reagents were from Sigma Co. (St Louis, MO) unless otherwise stated. The chemical structures of various compounds examined are shown in Figure 1.

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 in HLM was performed according to methods described elsewhere (Ito et al, 1998). Briefly, inactivators of varying concentrations were pre-incubated 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 concentrations of microsomal protein and NADPH were 0.2 mg/milliliter and 1 mM, respectively. The concentrations for the inactivators were: erythromycin, 5-80 μM; diltiazem, 0.25-4 μM; troleandomycin, 0.75-12 μM; diclofenac, 100 μM; amprenavir, 0.2-10 μM, and raloxifene, 1.25-10 μM. The experiments were performed in triplicate. At different times after the addition of NADPH, 10 microliters of the inactivator/microsome mixture (1^{st} incubation) were transferred to 90 microliters of potassium phosphate buffer containing 100 μM midazolam and 1 mM NADPH (10-fold dilution of inactivator in the incubation with midazolam), which had been pre-incubated

at 37°C for 5 minutes. The reaction lasted for 1 minute and was terminated by the addition of 100 microliters of a mixture of acetonitrile:methanol (3:1 v/v) containing alprazolam as internal standard (600 nM). In order to assess the inactivator consumption during 1st incubation, samples at time-zero and at the last time point after the addition of NADPH were quenched with acetonitrile:methanol (3:1, v/v) containing 600 nM alprazolam.

Microsomal protein binding of the inactivators 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 μL, n=3) of a mixture containing HLM (0.2 mg/milliliter in 0.1 M potassium phosphate buffer, initial incubation condition for TDI studies) and inactivators (nominal concentrations of 0.5 and 5 μM) 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) containing alprazolam as internal standard (20 nM). In general, equilibrium was reached between 4 and 6 hours for all inactivators (data not shown).

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

TDI of CYP3A in cryopreserved human hepatocytes. Hepatocyte suspensions were prepared according to the procedures provided by the manufacturer. A pool of five individuals (three male and two female) was used. Briefly, hepatocytes were thawed in

thawing medium (25 milliliters per 5 million hepatocytes) and centrifuged at 50 x g at room temperature for 5 minutes. The cell pellet was reconstituted in 30 milliliters of incubation medium (William's Medium E containing 10 mM HEPES) and centrifuged at 50 x g at room temperature for 5 minutes. The resulting cell pellet was reconstituted in fresh incubation medium and cell viability was determined by the trypan blue method (generally between 78 to 82% viability). Cell suspension was placed in 37°C incubator supplemented with 5% CO_2 before use.

TDI in hepatocytes

Hepatocytes were pre-incubated with and without time-dependent inactivators in a 48-well plate for 1 hour in a 37°C incubator under 5% CO_2. The final volume was 250 microliters and the final cell concentration was $0.5x10^6$ viable cells/milliliter. The inactivator concentrations were: amprenavir (0.1-5 μM), diclofenac (2.5-100 μM), diltiazem (0.5-4 μM), erythromycin (10-80 μM), raloxifene (2.5-20 μM), and troleandomycin (0.75-6 μM). The experiments were performed in triplicate. At the end of pre-incubation, 200 microliter aliquots of the suspension were transferred to 96-well plate micro-tubes (1.2 milliliter) and centrifuged at 50 x g for 5 minutes at room temperature. One hundred and fifty microliters of each supernatant were discarded and the pellets were re-suspended after the addition of 150 microliters of fresh medium for washing. The suspension was further centrifuged at 50 x g for 5 minutes at room temperature and 180 microliters of each supernatant were discarded. The post-wash pellet was re-suspended in 100 microliters of fresh medium containing midazolam. At this point, the hepatocyte concentration was approximately 1×10^6 cells/milliliters and midazolam concentration was 100 μM. The suspensions were immediately placed in the

incubator (37°C, 5% CO_2) and were incubated for 9 minutes before the addition of 2x volume of a mixture of acetonitrile:methanol (3:1 v/v) containing 300 nM alprazolam as internal standard. The use of 100 μM midazolam (to ensure saturation condition) and the choice of 9-minute incubation time were confirmed by preliminary kinetic studies.

To assess the effect of competitive inhibition of the inactivators, hepatocytes were pre-incubated in the absence of inactivator for 1 hour, and then co-incubated with midazolam (100 μM) and each inactivator. The final inactivator concentrations were: amprenavir (2.5 μM), diclofenac (50 μM), diltiazem (1 μM), erythromycin (10 μM), raloxifene (5 μM), troleandomycin (2.5 μM), and ketoconazole (2 μM, positive control). The experiments were performed in triplicate.

The effect of inactivators on cell viability was evaluated before and after 1-hour pre-incubation by determining glutathione S-transferase activities in the medium of the cell suspension (Habig et al, 1974).

Inactivator levels during 1-hour preincubation with hepatocytes

Free inactivator concentration (I_u) was equivalent to total inactivator concentration in hepatocyte medium since no protein was added in this study. Hepatocyte suspensions were centrifuged at 50 x g for 5 minutes at 0, 15, 40, and 60 minutes. The supernatant was quenched with an equal volume of a mixture of acetonitrile:methanol (3:1 v/v) containing alprazolam as internal standard (20 nM final concentration) for the analysis of I_u. In the cases where metabolic consumption and non-specific binding were significant, a mean time-averaged inhibitor concentration ($\overline{[I]}$) was obtained to represent average I_u during the 1 hour of pre-incubation. Total inactivator levels in the cell suspension before and after the 1-hour pre-incubation were also analyzed.

Effect of co-incubation with efflux transport inhibitor in hepatocytes

CP-100356, a P-glycoprotein inhibitor, was used to assess the possible involvement of P-glycoprotein in the modulation of CYP3A inactivation by the compounds tested in hepatocytes. CP-100356 (0.5 μM) was co-incubated with the time-dependent inactivator during the 1-hour pre-incubation of hepatocytes. The cells were washed and the activity of CYP3A was measured as described above.

LC-MS-MS methods. Samples quenched with organic solvent containing internal standard were vortexed and centrifuged at 1900 x g for 20 minutes. The supernatant was analyzed using a Micromass Quattro LC mass spectrometer (Quattro II) equipped with Waters 2790 HPLC system (Waters Corp., Milford, MA). Chromatographic separation was performed by a Zorbax C18 column (2x50 mm 5 micron, Agilent Technologies, Palo Alto, CA) coupled 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 milliliters/minute using a linear gradient elution consisting 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 described in this study. Alprazolam is the internal standard for all the analytes. The m/z transition for alprazolam is 309 → 205 . The retention time of alprazolam varied from 4.9 to 6.4 minutes due to different HPLC gradient condition of each analyte (Table 1).

Data analysis. The percent of inactivator unbound (% Free) in HLM was calculated according Equation 1:

$$\%\,Free = \frac{[I]_{buffer}}{[I]_{HLM}} \times 100\% \qquad \text{Equation 1}$$

Where $[I]_{buffer}$ and $[I]_{HLM}$ were the inactivator concentrations in the buffer side and the microsomal side at equilibrium, respectively.

Kinetic parameters (k_{inact} and K_I) of CYP3A inactivation 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 against the pre-incubation time. The apparent inactivation rate constant (λ) was determined from the slope of the initial linear phase on the semi logarithmic plots at each inactivator concentration. k_{inact} and K_I were obtained from the double-reciprocal plots of inactivator concentration [I] versus λ according to Equation 2 (Kitz et al, 1962).

$$\lambda = \frac{k_{inact} \bullet [I]}{K_I + [I]} \qquad \text{Equation 2}$$

TDI of CYP3A in hepatocytes was predicted using kinetic parameters obtained from microsomal incubations according to Equation 3, in which E_i and E_0 were CYP3A activities after 1-hour pre-incubation (t=60 min) with and without inactivator, respectively.

$$E_i = E_0 \times e^{-\lambda \times t} \qquad \text{Equation 3}$$

The $IC_{50,\,Predicted}$ was defined as the inactivator concentration that is required to cause $E_i = 0.5E_0$ in hepatocytes after 1 hour pre-incubation according to Equation 3. Theoretically, the value of λ that results in this effect is the same for all inhibitors (0.0115 min^{-1}, at t=60 min) according to Equations 2 and 3. Assumptions were made to aid the

prediction of hepatocyte inactivation potency using kinetic parameters generated from microsomal experiments. These assumptions are as follows: (a) I_u is represented by inactivator concentration in the hepatocyte medium; (b) equilibrium was reached instantaneously for the inactivators throughout the pre-incubation period in hepatocytes; and (c) the entry of the inactivator into the cell is mediated mainly by diffusion.

For inactivators that showed incomplete recovery or significant depletion in hepatocyte incubation, a time-averaged inactivator concentration in the medium ($\overline{[I]}$) was used to represent the I_u. $\overline{[I]}$ was calculated by trapezoidal integration using monoexponential Equation 4 by WinNonlin software (Version 3.01, Pharsight Inc, Mountain View, CA).

$$\overline{[I]} = \frac{\int_{t=0}^{t=60\ min} [I]_t \bullet dt}{60\ min} \qquad \text{Equation 4}$$

The observed CYP3A inactivation in hepatocytes was fitted to an inhibitory effect sigmoid E_{max} model (Equation 5)[1] using WinNonlin software, in which E_{max}, IC_{50}, and γ are the maximum enzyme activity, inactivator concentration at which 50% of activity was inhibited, and Hill's coefficient, respectively.

$$E = E_{max} \times \left(1 - \frac{[I]^{\gamma}}{[I]^{\gamma} + IC_{50}^{\gamma}}\right) \qquad \text{Equation 5}$$

[1] We have chosen the inhibitory E_{max} model to estimate the observed inactivation IC_{50} based on limited number of observations (4 for each inactivator). However, one should note that this is not an appropriate model to quantitatively assess the time-dependent event in hepatoctye incubations.

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Using Equation 5, the remaining CYP3A activity was fitted against both nominal inactivator concentrations and the concentrations corrected for nonspecific binding and metabolic consumption (I_u or $\overline{[I]}$) to obtain $IC_{50,\ Nominal}$ and $IC_{50,\ Corrected}$.

Simulations of the inactivation of CYP3A were performed using Microsoft Excel (Microsoft Co., Redmond, WA). HLM predicted inactivation was simulated according to Equation 3 and the observed inactivation was simulated according to Equation 5 with either $IC_{50,\ Nominal}$ or $IC_{50,\ Corrected}$.

Results

TDI in HLM. Figure 2 shows the time-dependent loss of CYP3A activity in HLM by six compounds reported to exhibit this property. The inactivation rate constants were obtained from the linear portion of the logarithmic ratio E_i/E_0 versus time at each inactivator concentration, as described under *Materials and Methods*. No apparent CYP3A inactivation was observed for diclofenac at concentrations up to 100 μM. The parameters from this study and literature reports are summarized in Table 2. In general, our findings are in agreement with those reported previously. Amprenavir and raloxifene rapidly inactivated CYP3A under the experimental conditions of this study, exhibiting k_{inact} values approximately one magnitude higher than those of diltiazem, erythromycin, and troleandomycin (Table 2).

Inactivator levels during the inactivation incubation (1st incubation) were analyzed at time zero and at the last time point after the addition of NADPH. Table 3 shows that inactivator consumption was not significant for diltiazem, erythromycin and troleandomycin. In contrast, loss of inactivator was observed for amprenavir and diclofenac at lower concentrations, and for raloxifene at all concentrations. Table 4 shows the inactivator microsomal free fraction at nominal 0.5 and 5 μM concentrations. The unbound fraction of raloxifene was 15 and 23% at 0.5 and 5 μM, respectively. Diclofenac also showed 47% microsomal binding at 0.5 μM. No significant microsomal protein binding was observed for the other compounds. Therefore, the apparent K_I for raloxifene and amprenavir (Table 2) are overestimates due to potential microsomal binding and/or inactivator consumption (Tables 3 and 4).

TDI of CYP3A in cryopreserved human hepatocytes. The use of cryopreserved human hepatocytes for the study of TDI was validated as described in the experimental section. Cell viabilities were not different before and after 1-hour pre-incubation in the absence or presence of the highest concentrations of inactivators (data not shown). CYP3A activities before and after 1-hour pre-incubation were 56.3±3.4 and 56.6±3.0 pmol 1-hydroxymidazolam per min per 10^6 cells, respectively (n=3 separate incubations). As shown in Figure 3 (open diamonds), amprenavir, diltiazem, erythromycin, raloxifene and troleandomycin showed concentration-dependent CYP3A inactivation in hepatocytes after 1-hour pre-incubation. Diclofenac had minimal effect on CYP3A activity at concentrations up to 100 μM (data not shown).

In order to discern the effect of reversible inhibition, inactivators and probe substrate midazolam were co-incubated with hepatocytes and the effect on CYP3A inhibition was compared with TDI experiments (Table 5). For amprenavir, diltiazem, erythromycin, raloxifene, and troleandomycin, co-incubation had minimal effect on CYP3A activity, as opposed to the inactivation observed in the TDI experiments where equal or lower inactivator concentrations were used in the pre-incubation. Co-incubation with diclofenac (50 μM) did not inhibit CYP3A activity. Ketoconazole is a potent non-competitive inhibitor of CYP3A (Gibbs, et al, 1999a) that served as a positive control in this study. CYP3A activity was inhibited by 81% when hepatocytes were co-incubated with 2 μM ketoconazole.

Calculation of I_u in hepatocyte experiments. Table 6 summarizes the inactivator levels measured in the hepatocyte incubations. Inactivator levels in the medium are equal to I_u

since no protein was added in the medium. Active uptake and/or non-specific binding can be evaluated by assessing the recovery of the inactivators (I_u as a percentage of total concentration) at time zero. The recovery of the inactivator in the medium at time zero was complete (near unity) for erythromycin and troleandomycin. However, the recovery ratios for raloxifene, amprenavir, diltiazem, and diclofenac were approximately 0.1, 0.4, 0.75, and 0.8, respectively. Inactivator consumption can be evaluated by measuring the percent remaining of both I_u and total inactivator concentration after 1-hour pre-incubation. Table 6 shows significant inactivator consumption for amprenavir (at lower concentrations), raloxifene and diclofenac. The time courses of I_u change for amprenavir and raloxifene are shown in Figure 4. Taken together, the data demonstrated that it was not appropriate to use I_u at any single time-point to represent the effective inactivator concentration for amprenavir and raloxifene. Instead, a time-averaged free inactivator concentration ($\overline{[I]}$) (Equation 4) was used. Therefore, for inactivators displaying low recovery in hepatocyte incubation medium, the nominal concentrations were replaced by I_u (for diltiazem) and $\overline{[I]}$ for amprenavir and raloxifene (Figure 4) for further evaluation (Table 7, Figure 3).

Prediction of TDI of CYP3A in cryopreserved hepatocytes using microsomal parameters. Comparison between the observed inactivation and HLM-predicted inactivation in hepatocytes was conducted. The practice was only to provide a descriptive relationship for the limited number of inactivators used in this study and was not meant to generate a quantitative correlation between the two systems. Diclofenac was excluded because of the absence of TDI under the conditions used in this study.

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Table 7 summarizes the observed inactivation ($IC_{50,Nominal}$ and $IC_{50,Corrected}$) and HLM-predicted inactivation ($IC_{50,\ Predicted}$). Under-prediction was defined when $IC_{50,Predicted}$ is higher than $IC_{50,Nominal}$ in hepatocytes whereas over-prediction was defined when $IC_{50,\ Predicted}$ is lower than $IC_{50,Nominal}$. Microsomal parameters of diltiazem apparently under-predicted the inactivation by approximately 4-fold ($IC_{50,\ Nominal}$ and $IC_{50,\ Predicted}$ of 3.22 and 12.32 μM, respectively). Correction of the observed inactivation using I_u in hepatocytes tends to magnify the under-prediction ($IC_{50,\ Corrected}$ became 2.41 μM). On the other hand, over-prediction was observed for the remaining four inactivators. The $IC_{50,\ Nominal}$ was 55, 6.2, and 7.8-fold higher than $IC_{50,\ Predicted}$ for amprenavir, erythromycin and raloxifene, respectively. Predicted inactivation by troleandomycin appears to be the most accurate ($IC_{50,\ Nominal}$ and $IC_{50,\ Predicted}$ of 2.14 and 1.35 μM, respectively). Correction of the observed inactivation using $\overline{[I]}$ markedly narrowed the difference between the observed and predicted IC_{50} values for raloxifene and amprenavir. The observed $IC_{50,\ Corrected}$ for raloxifene was 1.01 μM as compared with the $IC_{50,\ Predicted}$ of 0.62 μM. The $IC_{50,\ Corrected}$ for amprenavir became 0.36 μM (versus $IC_{50,\ Nominal}$ = 1.09 μM) as compared with the $IC_{50,\ Predicted}$ of 0.02 μM. I_u or $\overline{[I]}$ correction was not made for troleandomycin and erythromycin due to their relative complete recoveries and the absence of metabolic consumption (Table 7). The differences between the observed inactivation and the predicted inactivation can also be visualized by the simulated inactivation curves in Figure 3. Observed inactivation was simulated using parameters generated according to Equation 5 using both $IC_{50,\ Nominal}$ and $IC_{50,\ Corrected}$, whereas the predicted inactivation was simulated using HLM parameters (Equation 3, t=60 min) for each compound tested.

Effect of efflux transporter on TDI of CYP3A in hepatocytes. In order to assess the effect of P-glycoprotein on the inactivation potency of the compounds tested, hepatocytes were co-incubated with 0.5 μM CP-100356 and each inactivator during the 1-hour pre-incubation. The concentrations chosen for each inactivator (0.5, 2, 20, 5 and 1.5 μM for amprenavir, diltiazem, erythromycin, raloxifene and troleandomycin, respectively) are close to the observed respective $IC_{50,Nominal}$ values in this study (Figure 3 and Table 7). The concentration of CP-100356 (0.5 μM) was approximately 20-fold lower than its IC_{50} for CYP3A and about 5-fold higher than the IC_{50} for P-glycoprotein as reported by Wandel and co-workers (Wandel et al, 1999). CYP3A activity was not affected when 0.5 μM CP-100356 was present in the 1-hour pre-incubation (data not shown). Figure 5 shows the effect of co-incubation of CP-100356 on the inactivation potency for each compound tested. CP-100356 significantly enhanced CYP3A inactivation potency of the macrolides, troleandomycin and erythromycin ($p<0.01$ and $p<0.05$, respectively). CP-100356 did not significantly alter the inactivation potencies of raloxifene, diltiazem and amprenavir.

Discussions

The major findings of this study include: (1) observation of TDI of CYP3A in cryopreserved hepatocytes by inhibitors known to exhibit this property in HLM; (2) discovery that 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. These factors include, but are not limited to, non-specific binding, metabolic consumption, and active transport. To date, studies directed towards the prediction of TDI of CYP3A in hepatocytes using microsomal parameters have not been performed. The information from this work should therefore improve our understanding of the discrepancies associated with the prediction of drug interaction *in vivo* using HLM.

Time-dependent P450 inactivation results from the formation of reactive metabolites or intermediates, which bind tightly or irreversibly to the heme or apoprotein, leading to a catalytically inactive protein. Consequently, the concentration of active enzyme is controlled by the rate of enzyme inactivation as well as endogenous rates of *de novo* synthesis and degradation (Lin, 1998). The generally accepted approach for the prediction of the magnitude of TDI *in vivo* has been to apply microsomal kinetic parameters and the projected [I] (Equation 2), together with estimates of the endogenous degradation rate constant of the "victim" enzyme (Mayhew et al, 2000). Although successful for some compounds (Mayhew et al, 2000; Kanamitsu et al, 2000; Ito et al,

2003), direct use of microsomal parameters may fail to predict *in vivo* drug-interactions for marketed drugs known to have TDI properties.[2]

In this manuscript, we evaluated the TDI of CYP3A in two widely used systems, HLM and cryopreserved hepatocytes, for six compounds from different therapeutic classes. Pooled liver preparations were used to take into account the inter-individual variability associated with the CYP3A family. Raloxifene, a selective estrogen receptor modulator, was shown to inactivate CYP3A4 via a reactive intermediate from the active site, which can be attenuated by glutathione (Chen et el, 2002). Macrolide antibiotics, erythromycin and troleandomycin, are believed to form complexes with heme through a *nitroso* intermediate initiated by CYP3A through N-dealkylation (Periti et al, 1992). Similarly, TDI caused by the vasodilator diltiazem appears to be due to further metabolism of its N-desmethyl diltiazem metabolite (Jones et al, 1999; Mayhew et al, 2000). The HIV protease inhibitor amprenavir was recently shown to inhibit CYP3A by binding to the heme (Ernest et al, 2003). The non-steroidal anti-inflammatory agent diclofenac was reported to be a time-dependent inactivator of CYP3A with a rather high K_I of 1.6 mM (Masubuchi et al, 2002).

We did not observe TDI by diclofenac in both HLM and hepatocytes at concentrations up to 100 μM. Further investigation at higher concentrations was not conducted because 100 μM is already much higher than free diclofenac exposure observed in man. Indeed, diclofenac was not likely to cause clinically relevant CYP3A inhibition given its high microsomal K_I and high plasma protein binding (Masubuchi,

[2] Liras J (2003) Evaluation of an in vitro tool to predict clinical drug interactions from inactivation of cytochrome P450 3A4. Poster presentation. *6th international conference on Drug-Drug Interactions*, San Diego, CA. June, 2003.

2002). Thus, diclofenac was excluded from the HLM-hepatocyte prediction exercise. The remaining five inactivators demonstrated a concentration-dependent and time-dependent CYP3A inactivation in hepatocytes after 1-hour pre-incubation (Table 5).

In general, HLM-based prediction overestimates the inactivation potency observed in hepatocytes for troleandomycin, erythromycin, raloxifene and amprenavir by approximately 2 to 60-fold ($IC_{50,Predicted}$ lower than $IC_{50,Nominal}$, Table 7). The discrepancy may be due to metabolic stability, non-specific binding, active transport, or the combination of these factors. The depletion of the unbound inhibitor concentration available to the enzyme may introduce serious error into a quantitative prediction of an *in vivo* drug-drug interaction (Gibbs et al, 1999b; Xu et al, 2003). Among the inactivators tested, amprenavir and raloxifene had significantly low recoveries in the hepatocyte medium after the compounds were spiked into the cell suspension (approximately 10 to 40% recoveries, Table 6), suggesting concentrative uptake of these two compounds into the hepatocytes, mediated either by uptake transport or high lipophilicity. Indeed, the calculated $logD_{7.4}$ values for amprenavir and raloxifene were 4.2 and 5.5, respectively (ACD Labs, Toronto, Ontario, Canada), which are the highest among the compounds tested in this study. The significant microsomal binding for raloxifene may imply strong non-specific binding of this compound to cellular constituents in hepatocyte incubations (Tables 4 and 6). Metabolic consumption during the 1-hour pre-incubation in hepatocytes further decreased the availability of inactivator to the enzyme. The major biotransformation pathway for raloxifene is glucuronidation (Kemp et al, 2002). Effects of phase II enzyme are often not represented in HLM experiments. The discrepancy between I_u and nominal concentration for amprenavir may be partly attributed to the

metabolic consumption by CYP3A (Treluyer, 2003). Correction using $\overline{[I]}$ improved the prediction markedly for raloxifene and to a lesser extent for amprenavir. One should also note that the apparent K_I values are likely overestimates for raloxifene and amprenavir given that microsomal binding and/or HLM metabolic consumption (Tables 3 and 4) would theoretically widen the gap between the predicted IC_{50} and the observed IC_{50}.

The major assumption of these predictions is that entry of inactivator into the cell is not rate limiting and is mediated by diffusion only (free drug hypothesis). However, if the inactivator is a substrate of active transporter(s), I_u in the medium will no longer represent the intracellular inactivator concentration. Lam and Benet (2004) have demonstrated that the metabolism of digoxin (a substrate of both uptake and efflux transporters) in freshly isolated rat hepatocyte suspension can be modulated by inhibitors of both uptake and efflux transporters.

In this study, we chose CP-100356, a P-glycoprotein inhibitor (Wandel et al, 1999), to address the potential influencing factors associated with active transporters. If P-glycoprotein were functionally active in cryopreserved hepatocytes by reducing intracellular inactivator levels, or by efficiently removing obligatory metabolites of the pathway, one would observe decreased inactivation. To date, little is known about the expression, function, and orientation of P-glycoprotein after cryopreservation. In intact liver, hepatocytes are polarized, with transporters preferentially expressed on either the basolateral membrane or the bile canalicular membrane. The disruption of polarity upon collagenase isolation of hepatocytes may lead to the loss of P-glycoprotein. Roelofsen and coworkers (Roelofsen et al, 1995) have demonstrated the endocytosis of canalicular

multispecific organic anion transporter after isolation of rat hepatocytes, which was confirmed by the disappearance of transport activity from the cell surface and the appearance of intracellular vesicles that accumulate the substrate. Assuming P-glycoprotein undergoes similar changes, one would expect the decreased availability of a P-glycoprotein substrate to cytoplasmic components (such as CYP3A) due to efflux by the remaining cell membrane P-glycoprotein activity and/or accumulation into the intracellular vesicles by P-glycoprotein. We demonstrated that co-incubation of 0.5 μM CP-100356 (a concentration that did not inhibit CYP3A) significantly increased the CYP3A inactivation potency by troleandomycin and erythromycin (Figure 5). Interestingly, amprenavir has been shown to be a P-glycoprotein substrate (Polli, 1999; Huang et al, 2001; Choo et al, 2000); however, CP-100356 did not alter its inactivation potency (Figure 5). The strong concentrative effect observed for amprenavir may have masked the P-glycoprotein mediated efflux. Like many P-glycoprotein inhibitors, the selectivity of CP-100356 towards other transporters is not known, and the modulation of the CYP3A inactivation potency for macrolide antibiotics may have arisen from the inhibition of basolateral efflux transporter(s), which does not recognize amprenavir as a substrate. Our findings of effects of CP-100356 on macrolide induced inactivation suggested that P-glycoprotein (or other efflux proteins that can be inhibited by CP-100356) may have survived and maintained its efflux functionality during the hepatocyte cryopreservation and re-suspension. It has to be noted that cryopreserved hepatocytes are widely used to predict drug interaction and *in vivo* metabolic clearance of a new chemical entity. Thus, for compounds that are substrates for active transporters, care should be taken when interpreting data collected from cryopreserved hepatocytes to predict *in vivo*

outcome. It is also recognized that other transporters (both uptake and efflux) that survived cryopreservation and re-suspension can also contribute to the discrepancy between HLM and hepatocytes observed in this study. Further investigation of the mechanism through which efflux transporters alter the inactivation potency of macrolide antibiotics will be conducted.

The apparent discrepancy of the 4-fold difference between $IC_{50,\ Nominal}$ and $IC_{50,\ Predicted}$ for diltiazem (Table 7) is difficult to address. The low microsomal k_{inact} (0.012 min^{-1}) value of diltiazem is very close to the predicted λ value that would cause 50% inhibition (0.0115 min^{-1} at 60 min, Equation 3). Mathematically, predicting inactivator concentration that causes more than 50% inactivation in hepatocytes results in a negative value (Equation 3). It is also noted that the $IC_{50,\ Predicted}$ is greater than microsomal K_I (Table 7), implying that the prediction of 50% inactivation in hepatocytes is operated at $\lambda=k_{inact}$ (Equation 2). Together, one would expect a large degree of uncertainty of $IC_{50,\ Predicted}$ under the conditions set up in this manuscript. For compounds with low k_{inact} such as diltiazem, it appears to be very difficult to detect the existence of TDI from microsomal experiments (Figure 3), whereas individuals on diltiazem (60 mg, three times a day for three days) had a 2-fold increase of triazolam exposure (Kosuge et al, 1997). Our observation of CYP3A inactivation in hepatocytes for compounds with low k_{inact} such as diltiazem shows the potential advantage of using hepatocytes as a means to evaluate the projected *in vivo* potency of a time-dependent inactivator obtained from microsomal experiments.

In conclusion, when potential factors such as metabolic consumption, non-specific binding, and active transport are considered, cryopreserved hepatocytes can augment the use of HLM as a tool to evaluate a compound's potential to cause TDI *in vivo*.

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Footnotes:

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1. We have chosen the inhibitory E_{max} model to estimate the observed inactivation IC_{50} based on limited number of observations (4 for each inactivator). However, one should note that this is not an appropriate model to quantitatively assess the time-dependent event in hepatoctye incubations

2. Liras J (2003) Evaluation of an in vitro tool to predict clinical drug interactions from inactivation of cytochrome P450 3A4. Poster presentation. *6th international conference on Drug-Drug Interactions*, San Diego, CA. June, 2003.

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Legends for Figures

Figure 1. Chemical structures of CYP3A inactivators and CP-100356.

Figure 2. TDI of CYP3A in human liver microsomes. Legend shows the inhibitor concentrations in μM.

Figure 3. Effect of 1-hour pre-incubation of inactivators on CYP3A activities in cryopreserved human hepatocytes. Open square: HLM-predicted inactivator concentration that caused the same inactivation observed in hepatocytes. Open diamond: observed CYP3A activity at nominal concentration of the inactivator (n=3, C.V.=2-18%). Closed diamond: observed CYP3A activity at corrected concentration using I_u (diltiazem) or $\overline{[I]}$ (amprenavir and raloxifene). Solid lines: simulated inactivation using Equation 4, E_{max}, IC_{50} and γ. Broken lines: predicted inactivation using HLM parameters according to Equation 2 (Table 2).

Figure 4. Inactivator concentrations of raloxifene (left panel) and amprenavir (right panel) in hepatocyte incubation medium during 1-hour pre-incubation. Legends show the nominal concentrations of each inactivator.

Figure 5. Effect of P-glycoprotein inhibitor CP-100356 (0.5 μM) on the CYP3A inactivation potency by time-dependent inactivators in cryopreserved hepatocytes (Mean ± S.D., n=4 incubations). Inactivator concentrations: amprenavir, 0.5 μM; diltiazem, 2 μM; erythromycin, 20 μM; raloxifene, 5 μM; troleandomycin, 1.5 μM. *: $p<0.05$; **: $p<0.01$.

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Table 1. LC-MS-MS analytical parameters of 1-hydroxymidazolam and time-dependent inactivators.

Analyte	1-hydroxymidazolam	amprenavir	diclofenac	diltiazem	erythromycin	raloxifene	troleandomycin
Mobile phase gradient program: %B (min)	2(0)→25 (0.6)→55 (3.3)→55 (3.5)→98 (4.2)→2 (4.25)	2(0)→40 (0.6)→57 (3.1)→98 (4.2)→98 (5)→2 (5.2)	2(0)→25 (0.6)→55 (3.3)→55 (3.5)→98 (4.2)→2 (4.25)	2(0)→25 (0.6)→55 (3.3)→55 (3.5)→98 (4.2)→2 (4.25)	2(0)→30 (0.6)→55 (3.3)→55 (3.4)→98 (4.2)→2 (4.25)	2(0)→2 (0.2)→55 (0.8)→57 (3.1)→98 (4.2)→98 (5)→2 (5.2)	2(0)→25 (0.6)→55 (3.1)→55 (3.5)→98 (4.2)→2 (4.25)
Analyte m/z transition	342 →324	506→ 245	296→ 214	415→ 370	716→ 558	474→ 112	772 → 586
Mode	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Capillary voltage (kV)	2.50	2.52	2.52	2.52	2.52	2.52	2.52
Cone Voltage (V)	40	32	50	36	35	30	17
Desolvation temperature (°C)	300	300	300	300	300	300	300
Collision energy (eV)	20	17	40	18	15	27	25
Retention time (min)	6.2	5.6	5.7	6.7	5.4	5.3	6.6

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Table 2. Kinetic parameters of TDI of CYP3A in HLM.

Inactivator	**Kinetic parameters**		**Ref.**
	k_{inact} (min^{-1})	K_I (μM)	
amprenavir	0.45	0.64	
	0.60	0.37	Ernest, 2003
diclofenac	nd	nd	
	0.26	1600	Masubuchi, 2002
diltiazem	0.012	0.48	
	0.015	1	Dai, 2003
Erythromycin	0.072	15.2	
	0.081	9.9	Dai, 2003
raloxifene	0.36	18.7	
	0.16	9.9	Chen, 2002
troleandomycin	0.032	2.4	

Nd: not determined

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Table 3. Inactivator depletion in HLM during 1st incubation.

	Inactivator concentration (μM)	Percent parent drug remaining[a]	Reaction time (min)
amprenavir	0.1	56 (10)	2
	0.5	72 (15)	2
	2	76 (4)	2
	5	87 (4)	2
	10	96 (5)	2
diclofenac	1	54 (9)	8
	3	72 (1)	8
	10	86 (3)	8
	30	100 (14)	8
	100	97 (12)	8
diltiazem	0.25 to 4	79 (1) to 81 (8)	8
erythromycin	5 to 80	89 (2) to 99 (4)	8
raloxifene	1.25	72 (12)	3
	2.5	64 (5)	3
	5	79 (14)	3
	10	79 (15)	3
troleandomycin	0.75 to 12	102 (1) to 101 (8)	8

[a] Calculated as the percent remaining at the last time-point tested in the inactivation experiments. Mean (S.D.), n=3 incubations

Table 4. Microsomal free fractions (% Free) of inactivators at 0.2 mg/milliliter microsomal protein at 37°C. Mean (S.D.), n=3 incubations

Inactivator	Nominal concentration (μM)	% Free
amprenavir	0.5	95 (3)
	5	105 (2)
diclofenac	0.5	53 (6)
	5	110 (16)
diltiazem	0.5	112 (8)
	5	112 (6)
erythromycin	0.5	103 (4)
	5	85 (5)
raloxifene	0.5	15 (4)
	5	23 (2)
troleandomycin	0.5	103 (10)
	5	93 (6)

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Table 5. The effect of co-incubation with inhibitor and 1-hour pre-incubation with inactivator on CYP3A activities in cryopreserved human hepatocytes. Mean (S.D.), n=3 incubations.

Name	Co-incubation [a] (μM)	% of control without inhibitor	Pre-incubation [b] (μM)	% of control without inhibitor
amprenavir	2.5	100 (3)	1	46 (2)
diclofenac	50	97 (4)	100	102 (4)
diltiazem	1	95 (10)	1	75 (4)
erythromycin	10	97 (10)	10	65 (11)
raloxifene	5	105 (4)	5	48 (2)
troleandomycin	2.5	99 (4)	1.5	57 (7)
ketoconazole	2	19 (1)	-	-

[a]: Inhibitors were co-incubated with midazolam
[b]: Hepatocyte was pre-incubated with inactivators for 1 hour before the incubation with midazolam

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Table 6. Inactivator depletion and recovery from hepatocyte incubation media. Mean (S.D.), n=3 incubations.

	Concentration range (μM)	Recovery from medium [a]	% remaining in incubation [b]	% remaining in the medium [c]
amprenavir	0.1	0.34 (0.03)	63 (7)	18 (4)
	0.5	0.36 (0.04)	69 (9)	26 (4)
	1.0	0.36 (0.02)	80 (11)	80 (3)
	5.0	0.41 (0.04)	89 (9)	97 (4)
diclofenac	2.5 to 100	0.80 (0.01)	29 (3) to 92 (6)	27 (8) to 67 (8)
diltiazem	0.5 to 4	0.75 (0.01)	95 (4) to 98 (5)	85 (3) to 83 (4)
erythromycin	10 to 80	1.00 (0.04)	93 (1) to 103 (8)	105 (2) to 99 (2)
raloxifene	2.5	0.11 (0.02)	27 (1)	54 (3)
	5.0	0.09 (0.01)	23 (1)	51 (19)
	10.0	0.11 (0.06)	34 (4)	61 (15)
	20.0	0.11 (0.00)	69 (5)	70 (2)
troleandomycin	0.75 to 6	0.99 (0.01)	88 (5) to 94 (1)	86 (3) to 91 (2)

[a] Calculated as the ratio of concentration in the medium to the concentration in hepatocyte suspension immediately after hepatocytes were spiked with inactivators. (I_u/total inactivator concentration)

[b] Percent inactivator concentration after 1-hour pre-incubation (total concentration)

[c] Percent inactivator concentration after 1-hour pre-incubation (I_u)

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Table 7. Comparison of CYP3A inactivation between HLM-predicted potency and the potency observed in cryopreserved human hepatocytes

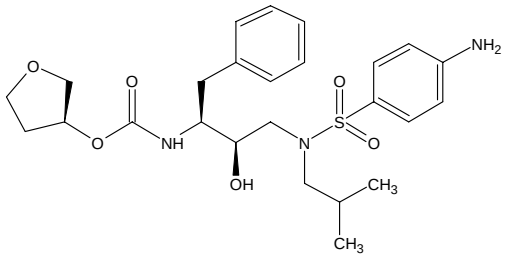
	amprenavir	diltiazem	erythromycin	raloxifene	troleandomycin
k_{inact} (min^{-1})	0.45	0.012	0.072	0.36	0.032
K_I (μM)	0.64	0.48	15.2	18.7	2.39
$IC_{50,\ Predicted}$ [a] (μM)	0.02	12.32	2.90	0.62	1.35
$IC_{50,\ Corrected}$ [b] (μM)	0.36 [c]	2.41	NC [d]	1.01 [c]	NC [d]
$IC_{50,\ Nominal}$ (μM)	1.09	3.22	18.02	4.85	2.14

[a]: Obtained from equation 2 and 3 using HLM parameters

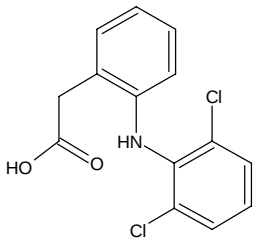
[b]: I_u that is required to produce 50% inactivation observed in hepatocytes

[c]: $\overline{[I]}$ was used for the determination of $IC_{50,Corrected}$

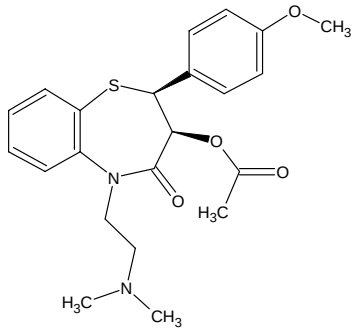
[d]: NC: Not calculated



Amprenavir



Diclofenac



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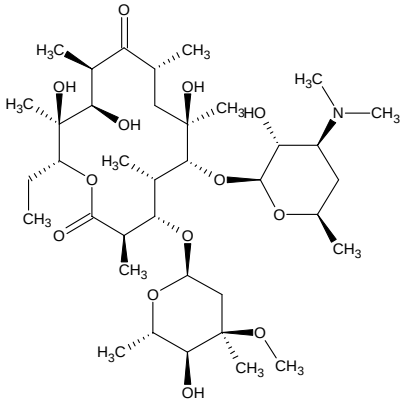

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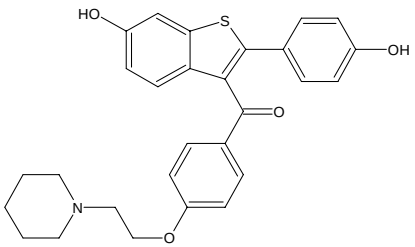

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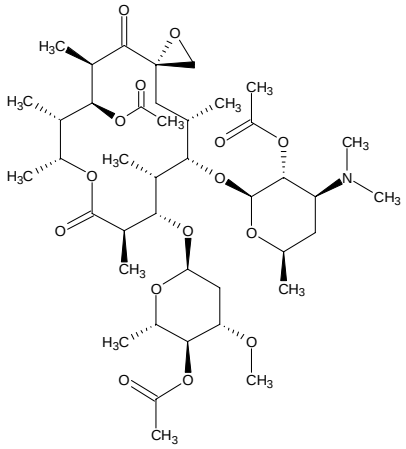
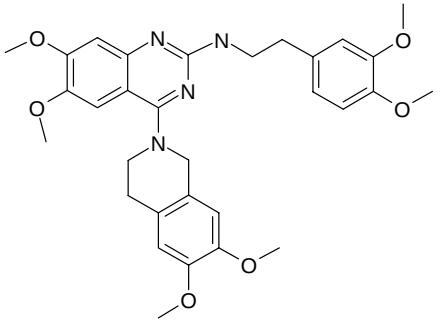


Troleandomycin



CP-100356

Figure 1

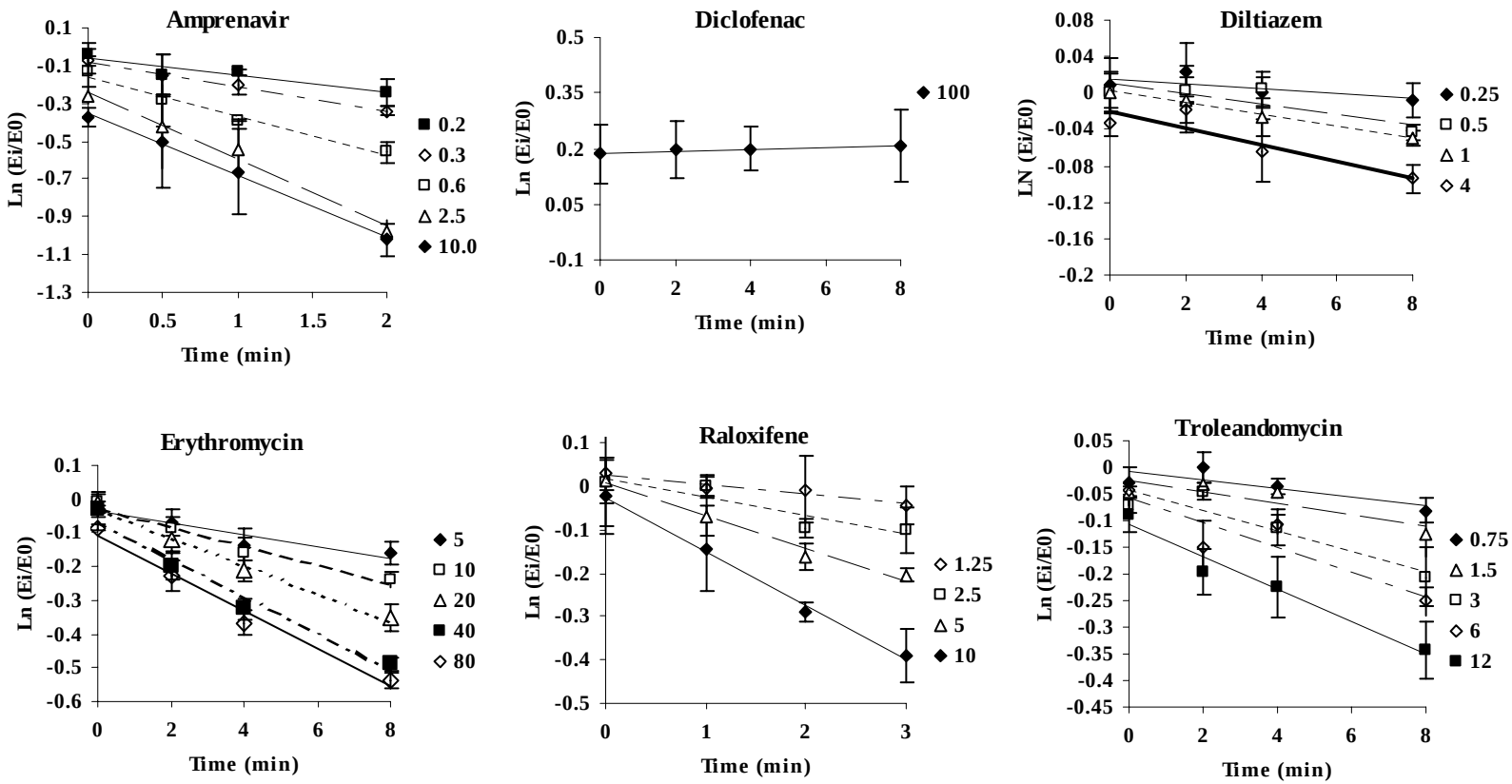


Figure 2

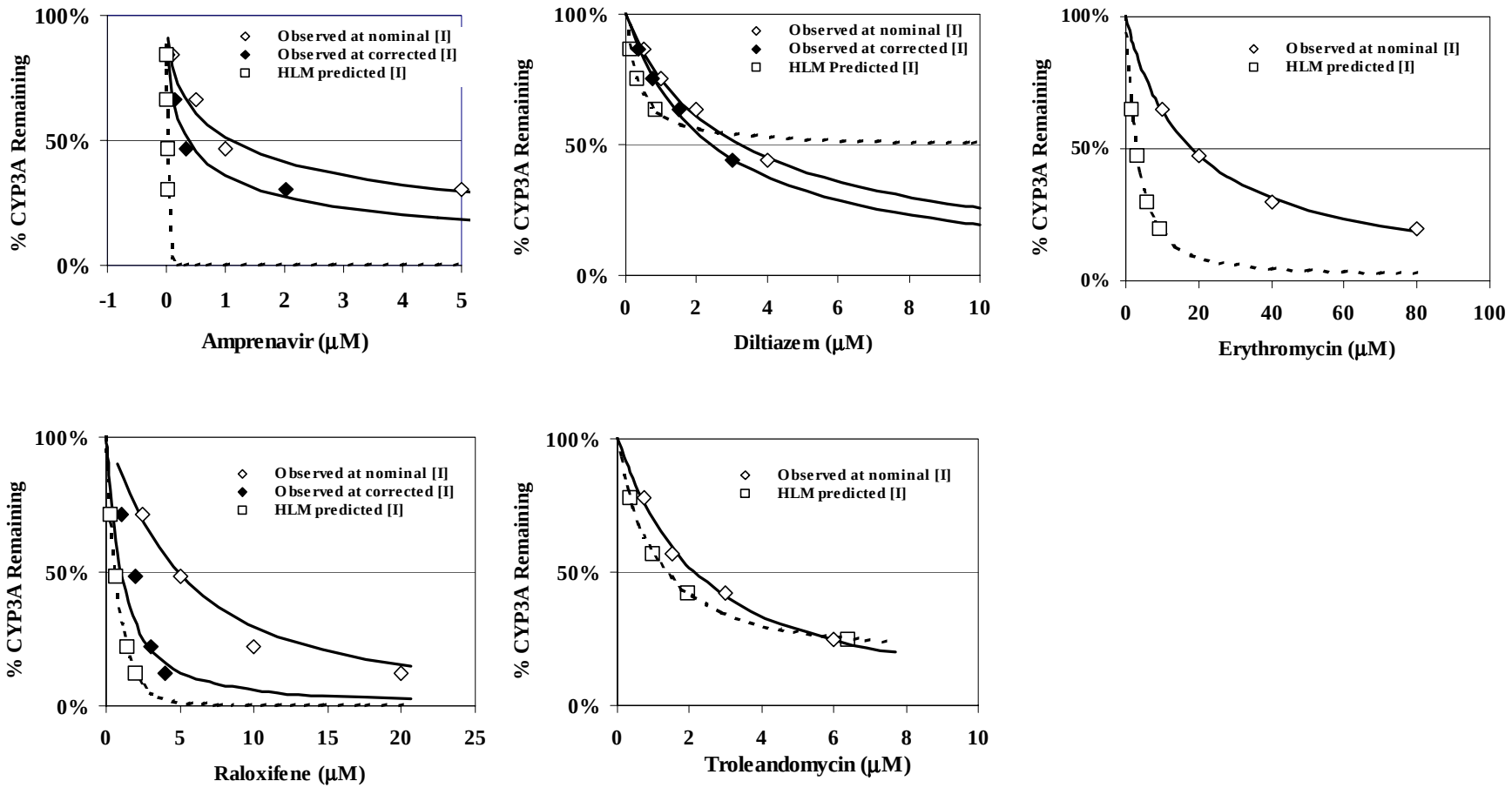


Figure 3

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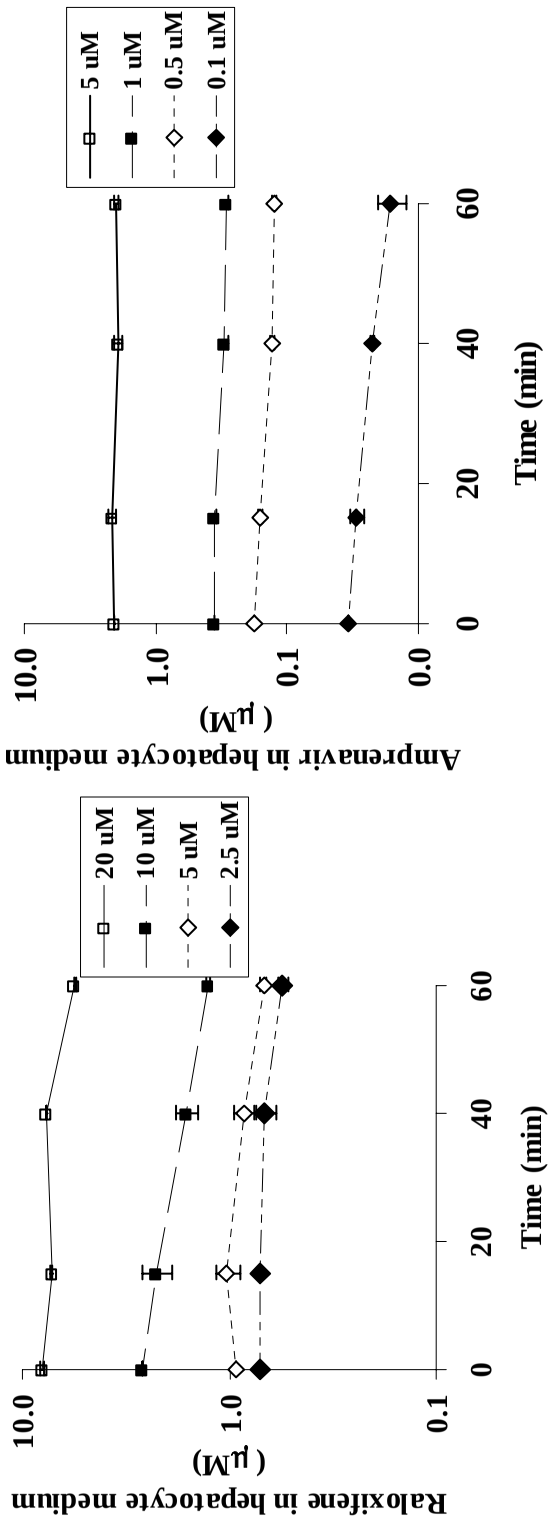

Figure 4

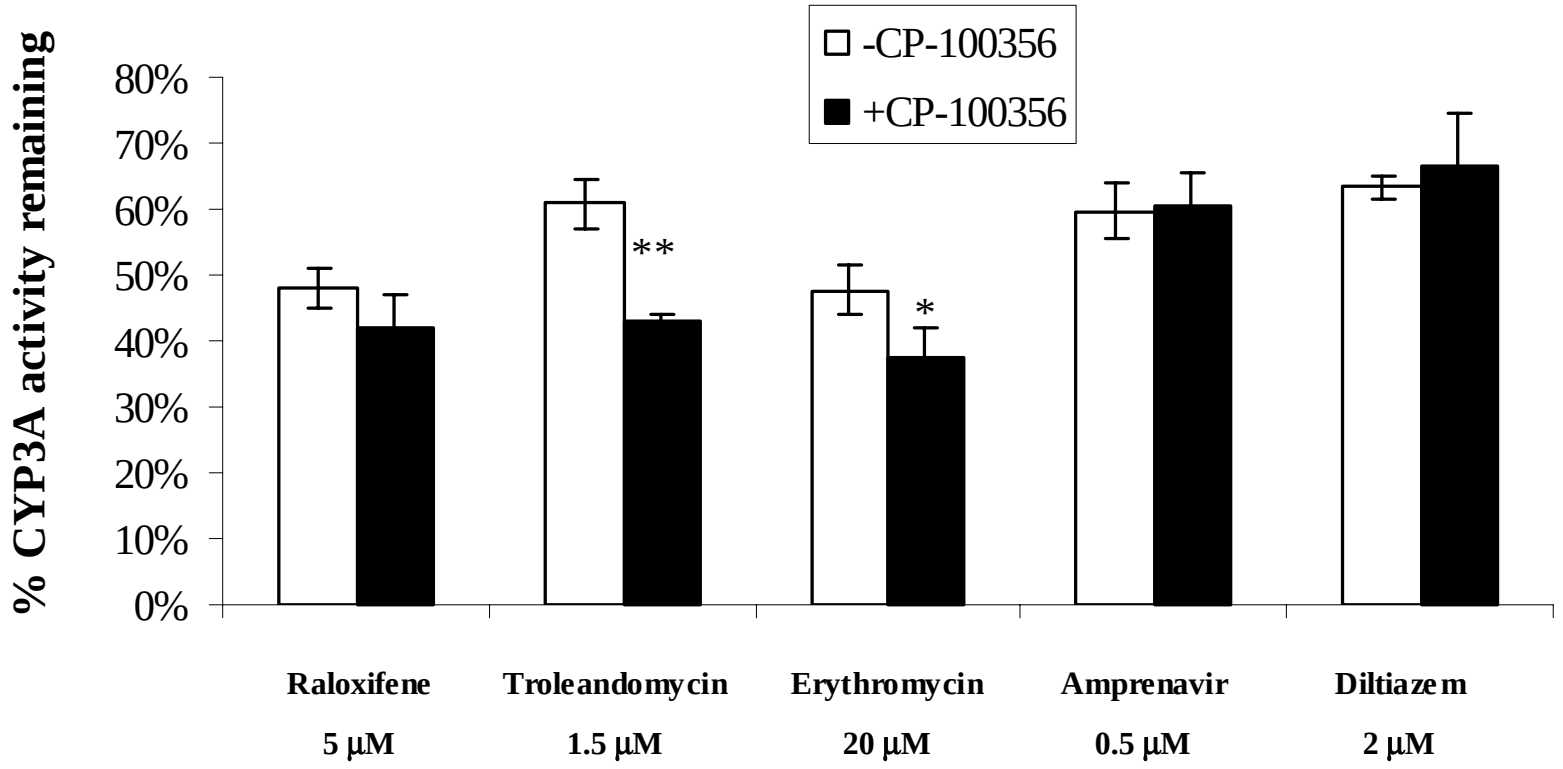

Figure 5