Prediction of Human Drug-Drug Interactions from Time-Dependent Inactivation of CYP3A4 in Primary Hepatocytes Using a Population-Based Simulator

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Time-dependent inactivation (TDI) of human cytochromes P450 3A4 (CYP3A4) is a major cause of clinical drug-drug interactions (DDIs). Human liver microsomes (HLM) are commonly used as an enzyme source for evaluating the inhibition of CYP3A4 by new chemical entities. The inhibition data can then be extrapolated to assess the risk of human DDIs. Using this approach, under- and overpredictions of in vivo DDIs have been observed. In the present study, human hepatocytes were used as an alternative to HLM. Hepatocytes incorporate the effects of other mechanisms of drug metabolism and disposition (i.e., phase II enzymes and transporters) that may modulate the effects of TDI on clinical DDIs. The in vitro potency ($K_I$ and $K_{\text{inact}}$) of five known CYP3A4 TDI drugs (clarithromycin, diltiazem, erythromycin, verapamil, and treleandomycin) was determined in HLM (pooled, $n = 20$) and hepatocytes from two donors (D1 and D2), and the results were extrapolated to predict in vivo DDIs using a Simcyp population trial-based simulator. Compared with observed DDIs, the predictions derived from HLM appeared to be overestimated. The predictions based on TDI measured in hepatocytes were better correlated with the DDIs ($R^2 = 0.601$ for D1 and 0.740 for D2) than those from HLM ($R^2 = 0.451$). In addition, with the use of hepatocytes a greater proportion of the predictions were within a 2-fold range of the clinical DDIs compared with using HLM. These results suggest that DDI predictions from CYP3A4 TDI kinetics in hepatocytes could provide an alternative approach to balance HLM-based predictions that can sometimes substantially overestimate DDIs and possibly lead to erroneous conclusions about clinical risks.

Clinical drug-drug interactions (DDIs) are a major source of adverse drug reactions and are becoming a growing problem with multiple-drug therapy. The most common DDIs are caused by inhibition of enzymes responsible for drug clearance and result in increased bioavailability, systemic exposure, and half-life of a victim drug (Gomez et al., 1995; Greenblatt et al., 1998b). The resulting increased exposure to the affected drug can lead to enhancement of the pharmacodynamic effects or serious side effects in humans. DDIs not only affect patient safety but also add to the cost of drug development because of the high costs of failure in clinical development. These issues have prompted the search for approaches to predict the potential for DDIs at the earliest possible stages of drug development (Ito et al., 1998; von Moltke et al., 1998; Mayhew et al., 2000; Obach et al., 2005, 2007). Information about the inhibition of drug-metabolizing enzymes obtained using in vitro systems can provide a basis for predicting DDIs in humans and thereby facilitate decisions about advancement of drug candidates and aid in planning of clinical DDI studies.

Human 3A4 is the most abundant cytochrome P450 (P450) isozyme and is responsible for the metabolism of approximately 50% of all drugs (Guengerich, 2003). The substrates of CYP3A4 are chemically diverse, and this broad substrate specificity renders CYP3A4 susceptible to reversible or irreversible inhibition by a variety of drugs. Irreversible, time-dependent inactivation (TDI) or mechanism-based inhibition of CYP3A4 refers to inactivation of the enzyme via formation of metabolic intermediates that bind tightly and irreversibly to the CYP3A4 enzyme in a time- and concentration-dependent manner. Two kinetic parameters, the maximum inactivation rate constant ($k_{\text{max}}$) and the inhibitor concentration leading to 50% of $k_{\text{inact}}$ ($K_I$), that describe the potency of the TDI are commonly used in the prediction of potential clinical DDIs. These TDI parameters are most commonly determined using human liver microsomes (HLM). However, it has been our observation that for some drugs the DDIs predicted by this method are far greater than any DDI that occurs clinically, even for highly potent inhibitors (M. Shou, unpublished observation). This result suggests that in some instances, microsomal incubations are inadequate to properly account for all of the factors that ultimately determine the extent of clinical DDIs.

Human hepatocytes have been widely used in drug discovery and development to measure intrinsic metabolic clearance and to measure excretion; RMRSS, root mean residual sum of square; MRS, mean residual sum; AUC, area under the curve.
the potential for a drug to cause DDIs due to either inhibition or induction of P450 enzymes. Because hepatocytes are an intact cellular system, not only are their P450 activity preserved, but also other microsomal enzymes that require cofactors and nonmicrosomal drug-metabolizing enzymes are active. In addition, the effects of passive and active uptake and efflux on intracellular concentrations of a drug are maintained in hepatocytes. This effect is important because inhibitory responses of a drug will depend on the intracellular concentration in hepatocytes, which for some drugs could be substantially different from the concentrations that occur extracellularly or in microsomes. In this study, in vitro TDI kinetic parameters of five known CYP3A4 inhibitors were measured in HLM and hepatocytes. The kinetic parameters derived from these studies were used for in vitro-in vivo extrapolations (IVIVEs) of clinical DDIs using Simcyp. Simcyp is a population-based clinical trial simulator that is increasingly being used for pharmacokinetic (PK) and DDI predictions. Simcyp incorporates information on properties of absorption, distribution, metabolism, elimination, and pharmacokinetics in a population and provides mean (or median) values with extremes of both PK and DDIs that may occur in the population (Rostami-Hodjegan and Tucker, 2007). In the present study, Simcyp was used to predict clinical DDIs from the in vitro TDI kinetic parameters of five CYP3A4 inhibitors measured in HLM and human hepatocytes (two donors), and the predictions were then compared with clinically observed DDIs. The study was conducted to better understand whether in vitro TDI data generated in human hepatocytes can be used to more reliably assess the risk of DDIs for compounds in preclinical development.

Materials and Methods

Materials. Cryopreserved human hepatocytes were obtained from two hepatic donors. Donor 1 (D1) was obtained from a female Caucasian donor aged 52 years (lot HH230; In Vitro Technologies, Baltimore, MA). Donor 2 (D2) was a pool of hepatocytes from three individuals, one female aged 20 years and two males at ages 42 and 81 years, respectively (Invitrogen-CellzDirect, Durham, NC). Enzymatic activities of the individual P450s in the hepatocytes were well characterized by the vendors. Pooled HLM (n = 20 donors, 10 from each gender) were purchased from XenoTech, LLC (Lenexa, KS). Clarithromycin (CLA), diltiazem (DIL), erythromycin (ERY), verapamil (VER), troleandomycin (TAO), 1-aminobenzotriazole (ABT), and Krebs-Henseleit buffer (KHB) were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam (MDZ) and 1-hydroxy midazolam (1-OH-MDZ) were purchased from BD Gentest (Woburn, MA).

Time-Dependent Inactivation of CYP3A4. Each inhibitor was preincubated at varying concentrations (five concentrations) at 37°C in an incubation mixture (0.2 ml) containing 1 mg/ml HLM, 1 mM NADPH, and 0.1 M phosphate buffer (pH = 7.4). The preincubation times at each inhibitor concentration were 0, 10, 20, 30, and 45 min. After the preincubation, an aliquot (10 µl) was transferred to a reaction mixture (190 µl, 20-fold dilution) containing 1 mM NADPH and 20 µM MDZ in 67 mM phosphate buffer. The reaction was incubated for an additional 5 min and terminated by the addition of 200 µl of acetonitrile containing 0.05% formic acid and internal standard (terfenadine; 0.5 µM final concentration). The sample was then centrifuged, and the supernatant was analyzed by LC-MS/MS (described below) to measure the disappearance of MDZ and formation of the metabolite 1-OH-MDZ (Fig. 1).

Measurement of Free Drug Concentrations in in Vitro Incubations and Plasma. Unbound fractions of the inhibitors in human hepatocytes were measured by two methods (treatment with alamethicin and ABT). Alamethicin is known to cause cells to become permeable to low molecular weight molecules, such as NADPH, resulting in ablation of metabolic capability (Matic et al., 2005). Human hepatocytes were incubated with 25 to 50 µM alamethicin in KHB in a 37°C incubator for 30 min. Under these conditions, the cells (10^6 cells/ml) remained intact, but the cell viability was <10% as monitored by dye exclusion analysis (trypan blue), and their ability to metabolize the inhibitors was minimized. The suspended cells were incubated for 15 min at 37°C in a water bath with the inhibitors at concentrations approaching their Ki and then centrifuged at 500g for 10 min. A 100-µl aliquot of the supernatants (unbound concentration) was analyzed by LC-MS. The total concentration was measured by quenching the cell suspensions (100-µl aliquot each) containing the inhibitor with acetonitrile (2 volumes) before centrifugation. Likewise, the unbound fractions in hepatocytes in the absence and presence of ABT (a mechanism-based inhibitor of multiple P450s) were also measured (Austin et al., 2005). Human hepatocytes were treated with 1 mM ABT dissolved in DMSO and preincubated in the presence of 1 mM NADPH in a water bath at 37°C for 60 min and then centrifuged (500g, 10 min) to remove the remaining ABT. The cell pellets were resuspended with buffer and incubated for an additional 15 min after the individual inhibitors were added. A 100-µl aliquot of the supernatants (unbound concentration) from the centrifuge was analyzed by LC-MS. Cells incubated in the absence of alamethicin or ABT served as a control. In each case the unbound fraction (fu, hep) was calculated as the ratio of unbound to total concentration. For measurement of unbound fractions in human plasma and HLM, frozen human plasma or HLM were thawed and preheated at 37°C. The plasma (2 ml) or HLM (1 mg/ml, 2 ml) in polypropylene centrifuge tubes (Corning Inc., Corning, NY) were incubated for 15 min with the individual inhibitors at the same concentrations tested in hepatocytes. After the incubation period, aliquots (800 µl, n = 2) were transferred to polyallomer tubes (Beckman Coulter, Fullerton, CA) for ultracentrifugation. The ultracentrifugation was performed for 3 h at 37°C and 627,000g using an MLA-130 rotor and an Optima Max Ultracentrifuge (Beckman Coulter). Supernatant (50 µl) from each sample was mixed with 200 µl of acetonitrile containing an internal standard (proprietary Amgen compound, 0.125 µg/ml). The samples were then centrifuged for 10 min at 405g, and 200 µl of supernatant was transferred into a 96-well plate and
dried under a nitrogen stream. The samples were reconstituted with 50% MeOH-H2O (100 μl) and analyzed by LC-MS/MS. Standard curves were generated by a sequential dilution of the analyte and quantified by the peak area ratio of analyte to internal standard added. Concentrations of the analytes were determined by the standard curves generated in the same matrix. The unbound fraction in plasma ($f_{u,p}$) was calculated as the ratio of the concentration measured in the ultracentrifugation supernatant to the concentration measured without the ultracentrifugation. Drug recovery values were also calculated by a ratio of the drug concentration in the plasma, HLM, or hepatocyte sample measured to the total concentration added. All of the LC-MS/MS analyses were performed in triplicate. The unbound fractions of the five inhibitors measured in plasma, HLM, and hepatocytes are listed in Table 1.

**Table 1. Measurement of Metabolite by LC-MS/MS.** The reaction samples (20 μl) obtained from the TDI experiments in HLM and hepatocytes were injected into a LC-MS/MS system equipped with a reverse-phase column (Spritz Armor C18 2.0 × 30 mm (10 μm) column), Shimadzu SCL-10AVP pumps (Shimadzu, Kyoto, Japan), a Leap HGPC PAL autosampler (LEAP Technologies, Carrboro, NC), and a Scien API 3000 mass spectrometer (Applied Biosystems, Foster City, CA). Analytes were eluted with a mobile phase (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) at a flow rate of 0.3 μl/min. A gradient elution was started at 95% A (0.3 min), then ramped to 5% A (0.4 min), and held at 5% A for 0.5 min before return to the initial condition. The metabolite, 1-OH-MDZ (transition 342,1/324,3), was detected in the positive ion mode by selective reaction monitoring. The peak area of the analyte was expressed as a ratio of the internal standard (terfenadine, mass transition 731/624). The concentrations of metabolite formed during the incubations were interpolated from the calibration curve.

**Data Analysis.** Fractions of CYP3A4 activity remaining ($E/E_{0}$) in the presence of an inhibitor ([I]) after a preincubation time (t) are described by eq. 1, where the rate of inactivation ($k_{inact}$) is the slope of the natural log of the percent enzyme activity ($\ln [E/E_{0}]$) versus preincubation time (t):

$$\ln \left(\frac{E}{E_0}\right) = -k_{inact} \cdot t + \ln \left(\frac{k_{max}}{K_i} + 1\right)$$

$K_i$ and $k_{max}$ were determined by nonlinear fitting of eq. 2 to the observed $k_{inact}$ in HLM and hepatocytes (Fig. 1) using SigmaPlot 10.0 (Systat Software, Inc., Chicago, IL). $k_{max}$ and $K_i$ were the maximal velocity of inactivation ($k_{max}$) and the inhibitor concentration that yields half of $k_{max}$, respectively.

The average (n = 3) remaining enzyme activity (percent) measured as MDZ 1-hydroxylation formation was expressed as a percentage of the time-matched control samples without inhibitor. The initial slope ($k_{obs}$) of each inactivation curve and its variance were determined by regression analysis using SigmaPlot. The kinetic parameters $k_{max}$ and $K_i$ were obtained from a nonlinear fitting of $k_{obs}$ against the inhibitor concentration ([I]) using eq. 2. The correlation analyses between predicted and clinical DDIs were performed by linear regression analysis between modeled values, were also determined. The DDIs were determined as the -fold increase in area under the curve and its variance were determined by regression analysis using SigmaPlot. The kinetic parameters $k_{max}$ and $K_i$ were obtained from a nonlinear fitting of $k_{obs}$ against the inhibitor concentration ([I]) using eq. 2. The correlation analyses between predicted and clinical DDIs were performed by linear regression analysis between modeled values, were also determined.

**Simcyp Simulator for Human DDI Prediction.** DDI simulations were performed using the Simcyp population-based ADME simulator (version 7.11). The physiologically based model and the differential equations used by the simulator have been described previously (Yang et al., 2006). In vitro $k_{max}$ and $K_i$ are incorporated into the model for simulating the level of CYP3A4 inhibition (Ito et al., 2003; Yang et al., 2006). In addition, experimentally determined or published $V_{max}$ and $K_m$ values (or intrinsic clearance) and unbound fractions ($f_u$) of the test substrates being modeled are used in the simulation. The simulator uses these data for all P450s that collectively contribute to the clearance of a particular substrate; consequently, the individual contribution of CYP3A4 ($f_u$,$\text{CYP3A4}$) to the total metabolic clearance of each substrate can be determined as shown in Table 2. This allows DDI simulations with substrates that are known to be poorly metabolized by CYP3A4, such as those tested here (theophylline, metoprolol, and imipramine). The model assumes first-order oral absorption, single-compartment distribution, and elimination by renal excretion and hepatic metabolism. The simulator also integrates a gut model because substantial levels of CYP3A4 are also expressed in that tissue and can contribute to the overall metabolism of a drug (Yang et al., 2007, 2008).

**Table 2.** The time-based simulations are performed according to the trial design (Einolf, 2007; Rostami-Hodjegan and Tucker, 2007). Virtual populations were generated within Simcyp using reported information on genetic (i.e., P450 genetic polymorphism and abundance in liver and gut), physiological, and demographic variability relevant to the appropriate clinical DDI studies. PK parameters (CL, $V_{max}$, $K_m$) gut absorption and hepatic metabolism, and others) for the inhibitors and victim drugs as well as other associated ADME properties such as in vitro turnover ($K_i$ and $V_{max}$ for the substrates) are incorporated in the Simcyp library. The mean CYP3A4 abundance of the population in liver (137 pmol/mg liver protein) and gut (66.2 nmol/total gut, predicted DDIs associated with gut CYP3A4 inhibition is also included) were used. The PK parameters of the five inhibitors for simulation of plasma concentration profiles are listed in Table 3. In vitro data measured, such as fractions unbound (plasma, HLM, and hepatocytes), kinetic constants ($K_i$ and $k_{inact}$) and PK parameters for the inhibitors were entered into Simcyp for the predictions. Dose, dose interval, and the duration of administration of the inhibitors and victim drugs were set according to the regimens of the clinical DDI trials described in the literature reports. The trials were designed and performed using a virtual population of healthy volunteers in 10 trials of 10 subjects, each aged 18 to 65 years with a ratio of 0.34, female/male). A half-life of 48 h for CYP3A4 degradation (kdeg = 0.0144 h⁻¹) was used for the CYP3A4 clearance for inactivation. The DDIs were determined as the -fold increase in area under the

<table>
<thead>
<tr>
<th>Drug</th>
<th>$f_{u,p}$ (Plasma)</th>
<th>$f_{u,max}$ (HLM)</th>
<th>$f_{u, hepat}$ (Hepatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA</td>
<td>0.175</td>
<td>0.323</td>
<td>0.806</td>
</tr>
<tr>
<td>DIL</td>
<td>0.212</td>
<td>0.820</td>
<td>0.709</td>
</tr>
<tr>
<td>ERY</td>
<td>0.326</td>
<td>0.870</td>
<td>0.465</td>
</tr>
<tr>
<td>VER</td>
<td>0.069</td>
<td>0.587</td>
<td>0.883</td>
</tr>
<tr>
<td>TAO</td>
<td>0.015</td>
<td>0.154</td>
<td>0.567</td>
</tr>
</tbody>
</table>

**Table 2.** $f_{u, \text{CYP3A4}}$ of the substrates (n = 100 individuals) used in Simcyp for simulation of DDIs

<table>
<thead>
<tr>
<th>Drug</th>
<th>$f_{u, \text{CYP3A4}}$</th>
<th>Mean (S.D.)</th>
<th>Median (5th-95th%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
<td>91.3 (14.0)</td>
<td>100 (63.9–100)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>90.8 (21.0)</td>
<td>100 (35.0–100)</td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td>2.0 (1.7)</td>
<td>1.5 (0.1–3.8)</td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td>14.7 (24.7)</td>
<td>6.7 (1.2–100)</td>
<td></td>
</tr>
<tr>
<td>Midazolam</td>
<td>83.7 (29.8)</td>
<td>100 (20.0–100)</td>
<td></td>
</tr>
<tr>
<td>Omeprazole</td>
<td>24.3 (20.6)</td>
<td>17.6 (4.2–60.7)</td>
<td></td>
</tr>
<tr>
<td>Sildenafil</td>
<td>82.3 (13.0)</td>
<td>87.0 (67.7–96.5)</td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>83.4 (11.7)</td>
<td>86.9 (58.0–97.1)</td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td>1.8 (1.3)</td>
<td>2.1 (0–5.0)</td>
<td></td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>2.1 (1.2)</td>
<td>1.6 (0–4.8)</td>
<td></td>
</tr>
<tr>
<td>Triazolam</td>
<td>85.7 (26.4)</td>
<td>100 (27.2–100)</td>
<td></td>
</tr>
</tbody>
</table>
hepatocytes are determined, metabolism of the inhibitors can con-
vert from 0.154 (TAO) to 0.870 (ERY) in HLM, and from 0.715
(ERY, VER, and TAO have been previously reported to be time-
dependent metabolism. We have demonstrated that under these experi-
mental conditions, alamethicin inhibited testosterone 6β-hydroxy-
lation in primary hepatocytes by 78% (M. Shou, unpublished data).
However, this approach does not correct for the effects of active
uptake or efflux transport. It may affect or negate active transport of
drugs. ABT is a mechanism-based inhibitor of multiple P450s. The
free concentrations of DIL, ERY, and TAO measured in the untreated
cells were lower than those measured when cells were pretreated with
either alamethicin or ABT (Table 1). The unbound fractions deter-
mined with ABT were slightly lower than that with alamethicin, but
they were still quite similar; therefore, the values measured from the
cells treated with alamethicin were used for all DDI predictions.

Results

Unbound Fractions in HLM and Hepatocytes. The unbound
fractions of the inhibitors in plasma (f_u,p, HLM (f_u,HLM), and hepa-
tocytes (f_u,hep)) are shown in Table 1. The unbound fractions among
the inhibitors varied widely, from 0.015 (TAO) to 0.326 (ERY) in
plasma, from 0.154 (TAO) to 0.870 (ERY) in HLM, and from 0.715
(TAO) to 0.980 (CLA) in hepatocytes. When the unbound fractions in
hepatocytes are determined, metabolism of the inhibitors can con-
found the measurement. Accordingly, hepatocytes were preincubated
with alamethicin (50 μM) or ABT (1 mM) to minimize metabolism of
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Time-Dependent Inactivation of CYP3A4 Activity. CLA, DIL,
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enzyme activity (log scale) versus preincubation time at various
concentrations of a given inhibitor were generated, and maximal rates
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(k_obs, versus [I]) (Fig. 2; Table 4). K_i values were also corrected by
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![Fig. 2](image-url)

**Fig. 2.** Fitting curves of observed k_obs versus inhibitor concentration by nonlinear regression analyses with eq. 2. Observed k_obs values are the slopes obtained from the linear regression of percent enzyme activity remaining (in scale) versus preincubation time of each inhibitor at varying concentrations. The fitting curves of the five inhibitors are obtained from HLM (A), hepatocyte donor 1 (B), and hepatocyte donor 2 (C), respectively.
### TABLE 4

**Kinetic parameters of the five inhibitors for the time-dependent CYP3A4 inhibition from human pooled liver microsomes and hepatocytes from two donors**

<table>
<thead>
<tr>
<th>Inhibitor and Conc. Range</th>
<th>Hepatocytes</th>
<th>HLM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 1</td>
<td>Donor 2</td>
</tr>
<tr>
<td></td>
<td>$K_{inact}$</td>
<td>$k_{inact}$</td>
</tr>
<tr>
<td></td>
<td>$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>CLA, 12.8–80 $\mu M$</td>
<td>7.88 (3.09)</td>
<td>7.64</td>
</tr>
<tr>
<td>DIL, 0.25–16 $\mu M$</td>
<td>3.55 (1.33)</td>
<td>3.12</td>
</tr>
<tr>
<td>ERY, 0.5–500 $\mu M$</td>
<td>21.40 (4.60)</td>
<td>20.03</td>
</tr>
<tr>
<td>VER, 0.31–20 $\mu M$</td>
<td>2.99 (0.35)</td>
<td>2.80</td>
</tr>
<tr>
<td>TAO, 0.05–1.6 $\mu M$</td>
<td>0.56 (0.084)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* $K_{inact}$ and $k_{inact}$ of each inhibitor in hepatocytes and HLM (S.D. in parentheses) were obtained from nonlinear regression analyses of observed $k_{inact}$ values shown in Figure 2.

* $K_{inact}$ values were calculated by $K_{i}$ multiplied by $f_{j}$ shown in Table 2. In addition to the $K_{i}$ values measured in the study, they were also reported in literature: CLA: $K_{m}$ 5.49 $\mu M$, 0.072 min$^{-1}$ (Mayhew et al., 2000); DIL: $K_{m}$ 3.3 $\mu M$, 0.057 min$^{-1}$ (Yeo and Yeo, 2001); ERY: $K_{m}$ 10.9 $\mu M$, 0.046 min$^{-1}$ (McConn et al., 2004); VER: 2.9 $\mu M$, 0.15 min$^{-1}$ (Yeo and Yeo, 2001); DIL: 1.6 $\mu M$, 0.092 min$^{-1}$ (Polasek et al., 2004); and TAO: 0.26 $\mu M$, 0.15 min$^{-1}$ (Atkinson et al., 2005).

### TABLE 5

**Predictions of human DDIs from in vitro TDI of CYP3A4 using Simcyp simulator**

<table>
<thead>
<tr>
<th>Inhibitor and Dose Regimen</th>
<th>Predicted DDI*</th>
<th>Observed Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simulated $k_{inact}$</td>
<td>Substrate</td>
</tr>
<tr>
<td>ERY, 400 mg t.i.d., 10 days</td>
<td>0.96</td>
<td>Alprazolam 0.8 mg</td>
</tr>
<tr>
<td>500 mg q.i.d., 3 days</td>
<td>2.03</td>
<td>Cyclosporine 350 mg</td>
</tr>
<tr>
<td>500 mg t.i.d., 5 days</td>
<td>1.20</td>
<td>Midazolam 15 mg</td>
</tr>
<tr>
<td>500 mg q.d., 2 days</td>
<td>0.48</td>
<td>Imipramine 100 mg</td>
</tr>
<tr>
<td>400 mg t.i.d., 2 days</td>
<td>0.037</td>
<td>Triazolam 0.125 mg</td>
</tr>
<tr>
<td>500 mg q.d., 3 days</td>
<td>0.86</td>
<td>Omeprazole 20 mg</td>
</tr>
</tbody>
</table>

* $K_{inact}$ values were calculated by $K_{i}$ multiplied by $f_{j}$ shown in Table 2. In addition to the $K_{i}$ values measured in the study, they were also reported in literature: CLA: $K_{m}$ 5.49 $\mu M$, 0.072 min$^{-1}$ (Mayhew et al., 2000); DIL: $K_{m}$ 3.3 $\mu M$, 0.057 min$^{-1}$ (Yeo and Yeo, 2001); ERY: $K_{m}$ 10.9 $\mu M$, 0.046 min$^{-1}$ (McConn et al., 2004); VER: 2.9 $\mu M$, 0.15 min$^{-1}$ (Yeo and Yeo, 2001); DIL: 1.6 $\mu M$, 0.092 min$^{-1}$ (Polasek et al., 2004); and TAO: 0.26 $\mu M$, 0.15 min$^{-1}$ (Atkinson et al., 2005).

* $K_{inact}$ and $k_{inact}$ of each inhibitor in hepatocytes and HLM (S.D. in parentheses) were obtained from nonlinear regression analyses of observed $k_{inact}$ values shown in Figure 2.

* $K_{inact}$ values were calculated by $K_{i}$ multiplied by $f_{j}$ shown in Table 2. In addition to the $K_{i}$ values measured in the study, they were also reported in literature: CLA: $K_{m}$ 5.49 $\mu M$, 0.072 min$^{-1}$ (Mayhew et al., 2000); DIL: $K_{m}$ 3.3 $\mu M$, 0.057 min$^{-1}$ (Yeo and Yeo, 2001); ERY: $K_{m}$ 10.9 $\mu M$, 0.046 min$^{-1}$ (McConn et al., 2004); VER: 2.9 $\mu M$, 0.15 min$^{-1}$ (Yeo and Yeo, 2001); DIL: 1.6 $\mu M$, 0.092 min$^{-1}$ (Polasek et al., 2004); and TAO: 0.26 $\mu M$, 0.15 min$^{-1}$ (Atkinson et al., 2005).
(Table 4). In most circumstances, values for unbound $K_I$ in HLM were smaller than those in hepatocytes from both donors.

The overall potency of a time-dependent inhibitor can be described as the intrinsic clearance of enzyme via inactivation [$C_{\text{int,F}} = k_{\text{max}}/(K_I/f_a)$]. Using this method the inhibitor potencies were ranked as TAO (2.22) $\gg$ DIL (0.035) $>$ VER (0.026) $>$ CLA (0.012) $=$ ERY (0.012) in HLM and as TAO (0.13, 0.05) $>$ VER (0.011, 0.008) $>$ DIL (0.009, 0.004) $>$ CLA (0.005, 0.002) $>$ ERY (0.001, 0.001) in hepatocytes from the two donors, respectively. The inhibitors were shown to be 2 (VER)- to 44 (TAO)-fold more potent in HLM than in hepatocytes. Of all the inhibitors, TAO was the most capable of inactivating CYP3A4-mediated MDZ 1-hydroxylation activity with a low $K_I$ (0.19–1.05 μM) and high $k_{\text{max}}$ (0.038–0.065 min$^{-1}$) in both HLM and hepatocytes, respectively.

**In Vivo Drug-Drug Interactions.** To assess the reliability of in vivo-in vitro correlations, clinically observed DDIs reported in the literature were collected. A total of 37 pairs of inhibitor-substrate interactions were used in the correlation analysis (Table 5). In these cited clinical studies the doses, dose regimens, and durations of inhibitor and substrate administration were provided. These study designs were used in an identical manner for the Simcyp predictions. In addition, the predicted $I_{\text{max,ss}}$ of the inhibitors at the given dose regimens (maximum plasma concentrations of the individual inhibitors at steady state) were simulated (Table 5). A Simcyp simulated plasma concentration-time profile of CLA at a given dose (500 mg b.i.d. for 9 days) is shown in Fig. 3A as an example. The $C_{\text{max,ss}}$ values of all inhibitors were generally in agreement with the in vivo concentrations reported in literature, which fall within 2-fold or less (Table 3).

**Simulation of DDIs from in Vitro Data.** The in vitro TDI kinetic parameters ($K_I$ and $k_{\text{max}}$) and protein binding data of the inhibitors in plasma, HLM, and hepatocytes were used to predict in vivo DDIs in Simcyp simulations. The degradation rate of CYP3A4 used for the simulation was selected ($k_{\text{deg}}$ for CYP3A4 = 0.0144 h$^{-1}$, equivalent to a half-life of 48 h). As seen in Table 5, the majority of the victim drugs used in this investigation are the known substrates of CYP3A4 (i.e., alprazolam, simvastatin, triazolam, sildenafil, cyclosporine, and MDZ). In addition, some of the drugs are also metabolized by other P450s (i.e., imipramine, metoprolol, omeprazole, and theophylline). $K_m$ and $V_{\text{max}}$ of the victim drugs for individual P450s were entered in Simcyp to identify contribution of CYP3A4 to total clearance of the drugs ($f_{\text{m,CYP}3A4}$) (Table 2). It is clear that the $f_{\text{m,CYP}3A4}$ values of the poor substrates for CYP3A4 are low. Input concentrations in the gut and hepatic portal vein were also estimated using Simcyp (data not shown).

The simulated plasma concentration-time profile of a drug (simvastatin) in the presence and absence of a given inhibitor (CLA) is shown in Fig. 3B. Exposure of simvastatin is markedly increased because of the inhibition of CYP3A4 by CLA, resulting in the profound decrease in intrinsic clearance in both liver and gut as a function of time depicted in Fig. 3, C and D. The DDIs predicted from the in vitro data in HLM and hepatocytes (D1 and D2) are listed in Table 5. The median values and the 5th and 95th percentiles in the population (10 trials) are shown. The predicted in vivo DDIs ($n = 37$)
from TDI of the five inhibitors between the two hepatocyte donors (D1 and D2) were reasonably well correlated ($R^2 = 0.747$) (Fig. 4C). The TDI values predicted from D1 and D2 were slightly different as should be expected from heterogeneity among human donors. The correlations of predicted DDIs between HLM and hepatocytes from each donor were 0.813 (D1) and 0.549 (D2), respectively (Fig. 4, A and B).

Figure 5 shows the correlations of 37 pairs of substrate-inhibitor interactions between predicted and observed DDIs. Three correlation analyses of the predictions (HLM, D1, and D2) with the in vivo observations were performed (Fig. 5, A, C, and E). Predictions based on the two hepatocyte donors ($R^2 = 0.601$ for D1 and $R^2 = 0.740$ for D2) (Fig. 5, C and E) were better correlated than those based on HLM ($R^2 = 0.451$) (Fig. 5A). In addition, the majority of the DDIs (35 and 30 of 37 DDI pairs for D1 and D2, respectively) predicted from hepatocyte data were within 2-fold of the actual clinical DDIs. In comparison, 27 of 37 DDI pairs from HLM were within a 2-fold range. Most of the HLM outliers were ERY-based DDIs (Fig. 5B). In addition, 24 of 37 of the HLM-based predictions overestimated the DDIs actually observed. The DDIs based on hepatocytes from D1 were uniformly distributed about unity (19:18) (Fig. 5D). In contrast, 35 of 37 DDIs predictions based on hepatocytes from D2 underpredicted DDIs observed in the clinic. In the ANOVA analyses, the RMRSS values for the predictions from D1 and D2 were smaller (1.25 and 1.63) than those from HLM (3.95), indicating a small degree of variability between the predicted and observed values. In addition, the positive mean residual sum (MRS) in the predictions from HLM (1.87) are consistent with an overprediction of the results. Conversely, the negative MRS value from D2 ($-1.17$) indicates a bias for underprediction of the data. The predictions from D1 had no significant bias with a MRS of 0.25.

**Discussion**

In vitro approaches are being increasingly used to predict clinical DDI risks associated with new chemical entities (Ito et al., 1998; Mayhew et al., 2000; Obach et al., 2005, 2007). Successful predictions can result in decreased compound attrition during drug development and can reduce the costs and time associated with failed clinical trials. The TDI of CYP3A4 has been reported to be a major cause of clinical DDIs. Because of the primary role of CYP3A4 in drug disposition, there is a need for better tools to effectively predict the potential for clinical DDI from in vitro data when the inhibition is mechanism-based. The present study provides an initial demonstration of the potential utility of hepatocytes to empirically account for the various factors that affect a reliable in vitro assessment of DDIs and to assess potential clinical DDIs using a Simcyp-directed IVIVE.

The capability of CLA, DIL, ERY, VER, and TAO, to inactivate CYP3A4 activity in HLM and hepatocytes was confirmed. The kinetic parameters of CYP3A4 inactivation ($K_i$ and $k_{max}$) by the five inhibitors in HLM have been characterized previously (Mayhew et al., 2000; Yeo and Yeo, 2001; Polasek et al., 2004; Atkinson et al., 2005). The findings in the current study were consistent with those previously reported parameters (see the values in footnote b of Table 4). Hepatocytes were also used to determine estimates of the TDI kinetic parameters for comparison with HLM. Although cryopreserved hepatocytes were used, activities of the major P450s in these hepatocytes were well characterized by the vendor. The inactivation of CYP3A4 in hepatocytes was observed to exhibit the typical characteristics of concentration and preincubation time dependence. The TDI caused by the five inhibitors and measured as enzyme inactivation ($CL_{int, E}$) was...
found to be 2- to 44-fold greater in HLM than in hepatocytes. Such differences can, of course, affect the accuracy of any in vivo DDI predictions. CLA, DIL, ERY, and TAO have been shown to be selective mechanism-based inhibitors of CYP3A4 so the predicted in vivo DDIs are largely due to the TDI of CYP3A4. However, VER was shown to inhibit not only CYP3A4 but also competitively inhibit CYP2C9, CYP2C19, and CYP2D6. Its IC$_{50}$ or $K_i$ values in pooled HLM were reported to be 118 μM for the CYP2C9-catalyzed formation of 4-hydroxytolbutamide (Wester et al., 2000), 43.8 μM for the CYP2C19-catalyzed formation of 4-hydroxymephenytoin (Foti and Wahlstrom, 2008), and 30.4 μM for the CYP2D6-catalyzed formation of O-desmethylmetoprolol (Kim et al., 1993). As shown in Table 5, predictions of VER-dependent DDIs with the test substrates metoprolol, imipramine, and theophylline that are metabolized by P450s other than CYP3A4 were apparently underestimated.

As shown by the correlation analyses in Fig. 5, DDIs predicted from TDI parameters measured in HLM were generally greater than those predicted from hepatocytes, due largely to lower $K_i$ values measured in HLM. These apparent differences in the TDI parameters measured between the two preparations can probably be attributed to differences in the actual concentrations of the inhibitors available to the target enzyme; that is, intracellular concentrations of the inhibitors were different from the nominal or extracellular concentrations used for calculation of the kinetic parameters. Factors that may cause this difference include rapid metabolism and active uptake or efflux of the inhibitors by transporters. For example, VER (Crivellato et al., 2002), DIL (Saeki et al., 1993), and ERY (Takano et al., 1998) are known to...
be either substrates for or inhibitors of multidrug resistance 1 and/or multidrug resistance-associated proteins in humans. In addition, it has been reported that VER (Tracy et al., 1999), DIL (Sutton et al., 1997), ERY (Wang et al., 1997b), and CLA (Rodrigues et al., 1997) were metabolized by CYP3A4 and other P450s. Uptake, efflux, or rapid metabolism of the inhibitors can alter the equilibrium between the intra- and extracellular concentrations, which affects the apparent $K_i$ and leads to differences in the predictions.

The unbound fractions of the inhibitors in plasma were used in the predictions in adherence to the principle that under conditions of rapid equilibrium only free drug is available for metabolism or enzyme inhibition. Corrections in Simcyp were also made for the substantial differences in drug concentration in systemic circulation, in the hepatic portal vein, and in the gut. These corrections help prevent underestimations of DDIs that can result from using only systemic blood concentrations for the predictions (Ito et al., 1998). The effect of the inhibitors on first-pass metabolism of the victim drugs in the gut was estimated using Simcyp and incorporated into the predictions. Because CYP3A4 is the major P450 enzyme in the gut wall, knowledge of the intrinsic gut clearance of a drug by this enzyme allows the prediction of net clearance by the gut (Thummel et al., 1996). Exposure of a drug to enzymes during its transit through the gut wall also depends on uptake and efflux transporters, passive membrane permeability and enterocyte blood flow. All of these parameters are incorporated into the Simcyp simulation.

The in vivo degradation of CYP3A4 ($k_{deg}$) is a key input parameter for IVIVE of TDI-mediated DDIs. Because this is a physiological rather than drug-dependent parameter, it is often associated with uncertainty, with only indirect approaches available to estimate its value in humans. There are two approaches used to determine $k_{deg}$ in vitro: (1) in vitro liver models (liver slices and primary hepatocytes) and pharmacokinetically based estimations (Ghanbari et al., 2006). Additional uncertainty in the estimates of $k_{deg}$ results from the assumptions that underlie the experimental models and the approaches used to analyze the results. Together, these uncertainties result in a relatively wide range of reported values. In the present study, a median of the reported values (0.0144 h$^{-1}$ or $1/2$ days $= 48$ h) was chosen for the prediction by Simcyp (Yang et al., 2008).

In the case of drugs whose metabolism is mediated by multiple isozymes of P450 (e.g., diazepam) the prediction of DDIs may be further complicated by possible dose-dependent changes in the contribution of each enzyme to overall metabolism (Rodrigues, 1999). The contribution of a given metabolic pathway to the total clearance of a drug is an important determinant of the accurate prediction of drug interactions. If the contribution of a pathway is small, its inhibition would not be predicted to have a significant effect on the AUC of the drug. To accurately measure the contribution of CYP3A4 to the metabolism of the drugs used in this study, Simcyp was used to incorporate kinetic capacities ($K_m$ and $V_{max}$) of each individual P450 responsible for the metabolism of the drugs to ensure that the predicted DDIs were due solely to the inhibition of CYP3A4.

In conclusion, predictions of in vivo DDIs from in vitro TDI data ($K_i$ and $k_{max}$) measured in both HLM and hepatocytes were made using Simcyp, a software simulator that incorporates a library of ADME information about the inhibitors and substrates, as well as information about genetic and physiological variability in the human population. The predicted DDIs from HLM and hepatocytes were correlated with clinically observed DDIs ($n = 37$). Based on the kinetic measurements, the magnitude of TDI in hepatocytes appeared to be generally weaker than that in HLM, suggesting that the intra- and extracellular concentrations of the inhibitors in hepatocytes were lower than the nominal concentrations in the incubations. As a consequence, the magnitudes of clinical DDIs predicted from HLM were generally greater than those from hepatocytes, and some predictions were substantially higher than the clinically observed values. Indeed, correlation analyses indicated a general trend of overestimations in the DDI predictions from HLM. In contrast, one set of hepatocyte-based predictions (D1) showed close concordance between predicted and observed clinical DDIs, whereas the other (D2) underestimated the clinical DDIs. Taken together, these results show that hepatocytes could provide an alternative way to balance HLM-based predictions that can sometimes substantially overestimate DDIs and possibly lead to erroneous conclusions about clinical risks.

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**References**


