Predictions of Cytochrome P450-Mediated Drug-Drug Interactions Using Cryopreserved Human Hepatocytes: Comparison of Plasma and Protein-Free Media Incubation Conditions

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ABSTRACT:
Cryopreserved human hepatocytes suspended in human plasma (HHSHP) have previously provided accurate CYP3A drug-drug interaction (DDI) predictions from a single IC50 that captures both reversible and time-dependent inhibition. The goal of this study was to compare the accuracy of DDI predictions by a protein-free human hepatocyte system combined with the fraction unbound in plasma for inhibitor(s) with those obtained with protein-containing incubations. Seventeen CYP3A, CYP2C9, or CYP2D6 inhibitors were incubated with hepatocytes in human plasma or hepatocyte maintenance medium (HMM) for 20 min over a range of concentrations after which midazolam 1'-hydroxylation, diclofenac 4'-hydroxylation or [R]-bufuralol 1'-hydroxylation were used to quantify the corresponding cytochrome P450 (P450) catalytic activities. Two methods were used to predict the human exposure ratio of the victim drug in the presence and absence of inhibitor. The HMM K_i,app values were combined with the free average systemic plasma concentration (“free [I] with HMM K_i,app”) and the plasma K_i,app values were combined with the total average systemic plasma concentration (“total [I] with plasma K_i,app”). Of 63 clinical DDI studies, the total [I] with plasma K_i,app method predicted 89% of cases within 2-fold of the reported interaction whereas the free [I] with HMM K_i,app method predicted only 59%. There was a general underprediction by the free [I] with HMM K_i,app method, which is consistent with an underestimation of in vitro inhibition potency in this system. In conclusion, the HHSHP system proved to be a simple, accurate predictor of DDIs for three major P450s and superior to the protein-free approach.

Introduction
In studies of drug disposition, it has often been assumed that in vitro drug-metabolizing systems used for clearance and DDI predictions perform equally well in the presence or absence of protein. Therefore, the expectation is that after correction for drug protein binding, the intrinsic clearance and inhibition potencies obtained with or without protein should be equivalent, but exceptions to this expectation have been noted. Enhanced unbound clearance of rose bengal, oleic acid, sulfobromophthalein, and bilirubin in the presence of albumin, relative to the absence of albumin, led to postulates that an albumin receptor present on the hepatocellular membrane facilitated hepatic elimination or that a protein binding dissociation rate limitation existed (Svenson et al., 1974; Goresky and Rose, 1977; Reed, 1977; Weisger et al., 1981; Forker et al., 1982). Although the unexpected effects of albumin on hepatic clearance led to complex hypotheses, the reliability of the intrinsic clearance estimation in the presence and absence of protein was not investigated in these studies.

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Human hepatocytes have been used in several formats to predict drug clearance in vivo (Houston and Carlile, 1997; Li et al., 1999; Blanchard et al., 2006; Hallifax et al., 2008) and more recently to predict DDIs (McGinnity et al., 2005; Zhao et al., 2005; Lu et al., 2007; Xu et al., 2009). Hepatocytes with a functioning cell membrane barrier and a realistic complement of phase I and phase II enzymes and their necessary coenzymes and cofactors can be seen as better in vitro model for the quantitation of DDIs. However, a systematic comparison of the performance of hepatocyte DDI assays with and without human plasma has not been conducted (Lu et al., 2007, 2008a,b). A previous study from this laboratory demonstrated that a method using an IC50 measured from a single time point in human hepatocytes suspended in human plasma (HHSHP) provided a highly reliable CYP3A-mediated DDI prediction for both reversible inhibitors and TDIs (Mao et al., 2011). Therefore, the similar approach has been expanded to CYP2C9 and CYP2D6 inhibitors. A comprehensive evaluation was undertaken to determine whether HHSHP or protein-free hepatocyte maintenance medium (HMM) provides reliable and comparable DDI predictions using four reversible and two time-dependent CYP3A inhibitors [HHSHP data were obtained from our previous publication (Mao et al., 2011), six reversible CYP2C9 inhibitors, and six CYP2D6 inhibitors (five reversible inhibitors and one TDI)].
Materials and Methods

Materials. Cryopreserved human hepatocytes (pool of five individuals) and InVitroGRO HT medium were obtained from Celsis In Vitro Technologies, Inc. (Baltimore, MD). Midazolam, 1'-hydroxymidazolam, [13C5]-1'-hydroxymidazolam, 4'-hydroxydiclofenac, [13C5]-4'-hydroxydiclofenac, 1'-hydroxybufuralol, and [H2]-1'-hydroxybufuralol were obtained from BD Gentest (Woburn, MA). Conivaptan was obtained from an internal Lilly chemical library. Diclofenac, diphenhydramine, duloxetine, flucloxacilone, fluostavatin, fluoxetine, ibuprofen, ketoconazol, nefazodone, miconazol, paroxetine, quinidine, sertraline, sulfaphenazol, and tolbutamide were obtained from Sigma-Aldrich (St. Louis, MO). Aprepitant, (R)-bufuralol, and voriconazol were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). Human plasma (sodium heparin) was obtained from Lampire Biological Laboratories, Inc. (Pipersville, PA). HMM was obtained from Lonza Walkersville, Inc. (Walkersville, MD).

Hepatocyte Studies. Hepatocytes were thawed in InVitroGRO HT medium (25 ml/5 million hepatocytes) and centrifuged (50g) at room temperature for 5 min. The cell pellet was reconstituted in HMM, and cell viability was found to be at least 80% using a Vi-CELL XR cell viability analyzer (Beckman Coulter, Brea, CA). After the cell viability was determined, hepatocytes were centrifuged at 50g at room temperature for 5 min and resuspended in HMM or human plasma (1 × 10^6 cells/ml for kinetic studies and 2 × 10^6 cells/ml for inhibition studies). The cell suspension was incubated at 37°C with 5% CO2 and 95% O2 before use. HMM is protein-free hepatocyte maintenance medium and its content is similar to that of Williams’ E medium, containing water, inorganic salts, amino acids, and vitamins with no growth factors.

Kinetic studies. The stock hepatocyte suspension in HMM or human plasma (50 μl) was added to 50 μM of HMM or human plasma containing probe substrates such that the final concentration of hepatocytes was 0.5 × 10^6 cells/ml. Preliminary studies were conducted to determine the substrate range necessary to achieve maximal activity for each P450-substrate pair. For CYP3A, final midazolam concentrations were 1.25 to 160 μM (HMM) and 15 to 1920 μM (human plasma). For CYP2C9, final diclofenac concentrations were 1.5 to 192 μM (HMM) and 75 to 9600 μM (human plasma). For CYP2D6, the final concentration range of (R)-bufuralol in both HMM and human plasma was 2.25 to 288 μM. All incubations were conducted at 37°C and 5% CO2. Incubations were terminated after 35, 45, and 25 min for CYP3A, CYP2C9, and CYP2D6, respectively, by addition of 200 μM of acetonitrile-methanol (3:1, v/v) containing the respective internal standard (150 nM [13C5]-1'-hydroxymidazolam for CYP3A, 945 nM [13C5]-4'-hydroxydiclofenac for CYP2C9, and 80 nM [H2]-1'-hydroxybufuralol for CYP2D6). Samples were centrifuged at 4000 rpm for 20 min, and an aliquot of the supernatant was analyzed by liquid chromatography-tandem mass spectrometry.

Inhibition studies. The studies with HHSHP and CYP3A inhibitors have appeared elsewhere (Mao et al., 2011) and are included here to allow for direct correction of the studies with hepatocytes suspended in protein-free HMM. The same hepatocyte lot was used for conducting all studies presented here. CYP3A inhibitor concentrations in HMM and human plasma were 0.03 to 20 μM for ketoconazole and 0.13 to 100 μM for aprepitant, conivaptan (TDE), fluconazol, nefazodone (TDE), and voriconazol. CYP2C9 inhibitor concentrations in HMM and human plasma were 0.13 to 100 μM for fluconazol, miconazol, sulfaphenazol, and fluostavatin and 1.65 to 1200 μM for ibuprofen and tolbutamide. CYP2D6 inhibitor concentrations in HMM and human plasma were 0.13 to 100 μM for diphenhydramine, duloxetine, and sertraline, 0.0013 to 1 μM for quinidine, 0.013 to 10 μM for paroxetine (TDE), and 0.03 to 20 μM for fluoxetine. The final organic vehicle concentration was 0.5% methanol.

Data Analysis. Kinetic data. Kinetic parameters (Vmax and Km) for each substrate in HMM and human plasma (based on the nominal concentration, no correction of unbound fraction) were obtained by fitting the untransformed data to the Michaelis-Menten equation (eq. 1) using weighted nonlinear regression (WinNonlin 5.0; Pharsight, Mountain View, CA).

\[ Y = \frac{[S] \times V_{max}}{K_m + [S]} \]  

(1)

Inhibition data. The same approach, in which the relationship between the P450 activities in hepatocytes incubated in both systems at a given time and inhibitor concentration relative to the baseline P450 activity was used to determine an IC50, was described previously (Mao et al., 2011). IC50 values were calculated with the mean of triplicate determinations using eq. 2 by weighted nonlinear regression (WinNonlin 5.0; Pharsight).

\[ Y = \frac{a}{1 + \left(\frac{IC_{50}}{X}\right)^b} \]  

(2)

where X is the nominal concentration of an inhibitor [no correction of fraction unbound in human plasma (fu)] for the human plasma incubation. Y represents the percentage of baseline P450 activity remaining, a is the estimated μM in human plasma (1/[R]-bufuralol). After addition of the probe substrate, the incubations were continued for 35, 45, and 25 min for CYP3A, CYP2C9, and CYP2D6, respectively. Incubations were terminated similar to the method used for kinetic studies by the addition of 200 μl of acetonitrile-methanol (3:1, v/v) containing the respective internal standard. Samples were centrifuged at 4000 rpm for 20 min, and an aliquot of the supernatant was analyzed by liquid chromatography-tandem mass spectrometry.
response at zero concentration of inhibitor, and \( y \) is the slope factor, which describes the steepness of the curve.

The apparent \( K_{i, app} \) for each inhibitor in HMM or human plasma was calculated by eq. 3:

\[
K_{i, app} = \frac{IC_{50}}{1 + [S]/K_m}
\]

(3)

where \( K_m \) is the Michaelis-Menten constant for 1'-hydroxymidazolam, 4'-hydroxydiclofenac, and 1'-hydroxybufuralol formation obtained in either HMM or human plasma, and \([S] \) represents the concentration of midazolam, diclofenac, and (R)-bufuralol in the HMM and plasma inhibition assays.

**Predictions of Drug-Drug Interactions.** CYP3A. A general model (eq. 4) of enzyme inhibition was used to predict a potential increase in exposure to a drug as a result of the inhibition of hepatic and intestinal CYP3A (Ito et al., 1998; Wang et al., 2004; Obach et al., 2006). For competitive inhibitors, the \( K_{i, app} \) would be equivalent to the inhibition constant \( K_i \). In the case of TDIs, \( K_{i, app} \) was equivalent to \( K_i \times k_{deg} \times k_{max} \) when \( [I] \ll K_i \) where \( k_{deg} \) is the degradation rate constant of CYP3A, \( k_{max} \) is the inhibitor concentration required for half-maximal inactivation, and \( k_{max} \) is the maximum inactivation rate constant (Wang et al., 2004).

\[
\frac{AUC_{p.o, i, app}}{AUC_{p.o}} = \frac{C_{L,m, int,i, app}}{C_{L,m, int,i, app}} = \frac{1}{\left(\frac{f_m,CYP3A}{1 + [I]/K_{i, app}}\right) + (1 - f_m,CYP)} \times \left(\frac{1}{(1 - F_u)p/(1 + [I]/K_{i, app} \times F_u)}\right) + F_u
\]

(4)

\( AUC_{p.o, i, app} \) is the predicted ratio of in vivo exposure of a CYP3A-cleared drug with and without oral coadministration of the inhibitor, \( f_m,CYP \) is the fraction of total clearance CYP3A contributes for the affected drug, \( F_u \) is the fraction of the dose of the affected drug that passes through the intestine unchanged after oral administration in the control state. In four clinical studies (ketoconazole, aprepitant, conivaptan, and voriconazole), intravenous midazolam dosing was used and the AUC ratio was predicted using eqs. 4 and 5 with \( F_u = 1 \) under the reasonable assumption that midazolam has a low to moderate hepatic extraction ratio.

The fraction of midazolam metabolized by CYP3A was assumed to be 0.93 as observed previously (Obach et al., 2006). The fraction of drug metabolized by CYP3A in the intestine was assumed to be 1 (assuming no other P450s other than CYP3A metabolize midazolam in the intestine), and the \( F_u \) value for midazolam was assumed to be 0.57, as described previously (Ernest et al., 2005; Obach et al., 2006). Inhibitor concentrations were collected from three main sources. The average systemic plasma concentration of the inhibitor reported or calculated [i.e., plasma concentration area under the curve from 0 to the dosing interval (AUC_{int,i} divided by the dosing interval) from the primary literature was the preferred source. The second option used the inhibitor concentration at a specific time point reported in the primary literature. If the inhibitor concentration was not reported in the primary literature, values were obtained from secondary literature sources (Ito et al., 2003; Einolf, 2007; Fahmi et al., 2008, 2009). These values were previously derived from other literature sources in which similar dosing regimens were used for individual inhibitors.

The concentration of the inhibitor in the enterocyte during absorption (\([I]_g \)) (eq. 5) was estimated on the basis of the assumptions that 1) no significant protein binding occurred in the gut lumen and 2) inhibitors were not subject to any first-pass metabolism (Rostami-Hodjegan and Tucker, 2004; Galetin et al., 2008). \( D \) is the dose of the inhibitor (milligrams), \( k_g \) is the oral absorption rate constant of the inhibitor, \( F_s \) is the fraction of the inhibitor absorbed into the gut wall from the intestinal lumen after oral administration, \( Q_{ent} \) represents the blood flow to the enterocyte, and \( MW \) is the molecule weight for each inhibitor. For \( k_g \) and \( Q_{ent} \), values of 0.03 min⁻¹ and 248 m/minute, respectively, were used, as described previously (Obach et al., 2006). An \( F_u \) value of 1 was used for all drugs (Einolf, 2007).

\[
[I]_g = \frac{D \times k_g \times F_s}{Q_{ent} \times MW}
\]

(5)

For HMM \( K_{i, app} \), the free systemic plasma concentration ([I] × \( f_u \)) was used for DDI predictions because the HMM \( K_{i, app} \) was generated in a plasma protein-free environment (the "free [I] with HMM \( K_{i, app} \)" method).

For plasma \( K_{i, app} \), the total systemic plasma concentration was used for hepatic DDI prediction (the "total [I] with plasma \( K_{i, app} \)" method). The plasma \( K_{i, app} \) values were based on total inhibitor concentration in plasma, and, therefore, there was no need to use unbound plasma concentrations in the predictions. The prediction of intestinal inhibition, \( K_{i, app} \), values were converted to unbound values (\( K_{i, app} \times f_u \)) consistent with the assumption of no significant plasma protein binding in the gut lumen. The fraction unbound in human plasma (\( f_u \)) for each compound was collected according to Goodman & Gilman’s *The Pharmacological Basis of Therapeutics* (Hardman et al., 2001).

**CYP2C9 and CYP2D6.** A similar model (eq. 6) without considering the intestinal interaction was used for CYP2C9 and CYP2D6 inhibition:

\[
\frac{AUC_{p.o, i}}{AUC_{p.o}} = \frac{C_{L,m, int,i}}{C_{L,m, int,i}} = 1 + \frac{1}{(1 + [I]/K_{i, app}) + (1 - f_m,CYP)}
\]

(6)

The values for the fraction of object drug metabolized by CYP2C9 were assumed to be the same as those reported previously for tolbutamide, phenytoin, S-warfarin, lovastatin, fluvastatin, glimepiride, and diclofenac (0.80, 0.75, 0.87, 0.81, 0.60, 0.95, and 0.75, respectively) (Brown et al., 2005). Likewise, the values for the fraction of object drug metabolized by CYP2D6 were 0.83, 0.88, 0.46, 0.86, 0.49, and 0.76 for metoprolol, desipramine, imipramine, encainide, mexiletine, and propafenone, respectively (Brown et al., 2005). Values for the inhibitor concentration were adopted from the calculated average systemic plasma concentration after repeated oral administration (Ito et al., 2003).

**Data Sources.** Data from 63 clinical DDI studies were collected from the primary literature after having been identified by the University of Washington Metabolism & Transport Drug Interaction Database (http:// www.druginteractioninfo.org/). The database was accessed on June 24, 2009, and the data are reported in Table 3.

Seventeen inhibitors were chosen on the basis of the following considerations: 1) representative reversible inhibitors (i.e., ketoconazole, a strong CYP3A inhibitor; sulfaphenazole, fluvastatin, and miconazole, strong CYP2C9 inhibitors; duloxetine, fluoxetine, and quinidine, strong CYP2D6 inhibitors; fluconazole, a moderate CYP3A and CYP2C9 inhibitor; voriconazole and aprepitant, moderate CYP3A inhibitors; ibuprofen and tolbutamide, moderate CYP2D6 inhibitors; and diphenhydramine and sertraline, moderate CYP2D6 inhibitors) and TDIs (nefazodone and conivaptan, moderate CYP3A TDIs; and paroxetine, strong CYP2D6 TDI); and 2) compounds with various levels of plasma protein binding (conivaptan, ketoconazole, fluvastatin, miconazole, ibuprofen, tolbutamide, duloxetine, fluoxetine, paroxetine, quinidine, and sertraline, high plasma protein binding; voriconazole, sulfaphenazole, and diphenhydramine, moderate plasma protein binding; and fluconazole, low plasma protein binding).

Nineteen clinical DDI studies involved the inhibition of CYP3A with midazolam as the victim drug (3 studies were related to TDIs), 15 studies involved the inhibition of CYP2C9 with tolbutamide, phenytoin, S-warfarin, lovastatin, fluvastatin, glimepiride, and diclofenac as victim drugs, and 29 studies involved the inhibition of CYP2D6 with metoprolol, desipramine, imipramine, encainide, mexiletine, and propafenone as the victim drugs (eight studies were related to paroxetine).

**Data Analyses.** Among 63 clinical DDIs, there were 15 strong interactions (AUC ratio >5), 25 moderate interactions (2 ≤ AUC ratio ≤5), 17 weak interactions (1.25 ≤ AUC ratio <2), and 6 no interaction (AUC ratio <1.25, assuming no induction). Two methods were used to quantify the accuracy of predicted DDIs. One was to compare the fold error of the ratio of predicted to observed AUC values (2-fold cutoff). The second method was called "categorical prediction" on the basis of the definition of strong, moderate, weak, and no interactions (Draft Food and Drug Administration Guidance for Industry, 2006, Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling, http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm).

In addition, the bias and precision of both methods were evaluated by the geometric mean fold error (GMFE) and the root mean square error (RMSE) with eqs. 7 and 8, respectively.

\[
\text{Bias} = \frac{\text{Mean of GMFE}}{\text{Mean of RMSE}}
\]

(7)

\[
\text{Precision} = \frac{\text{stddev of GMFE}}{\text{stddev of RMSE}}
\]

(8)
TABLE 1  
Kinetic parameters for CYP3A, CYP2C9, and CYP2D6 substrates from human hepatocyte suspensions in HMM or human plasma

<table>
<thead>
<tr>
<th>Substrate Parameters</th>
<th>HMM</th>
<th>Plasma</th>
<th>Unbound Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midazolam $K_{i,u}$, $\mu$M</td>
<td>8.11 ± 0.65</td>
<td>45.83 ± 4.38</td>
<td>1.47 ± 0.14</td>
</tr>
<tr>
<td>Midazolam $V_{max}$, nmol · min$^{-1}$ · million cells$^{-1}$</td>
<td>0.021 ± 0.001</td>
<td>0.036 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Diclofenac $K_{i,u}$, $\mu$M</td>
<td>34.58 ± 2.14</td>
<td>1408 ± 304</td>
<td>4.22 ± 0.91</td>
</tr>
<tr>
<td>Diclofenac $V_{max}$, nmol · min$^{-1}$ · million cells$^{-1}$</td>
<td>0.41 ± 0.01</td>
<td>0.49 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>(R)-Bufuralol $K_{i,u}$, $\mu$M</td>
<td>12.22 ± 1.42</td>
<td>54.83 ± 7.38</td>
<td>10.42 ± 1.40</td>
</tr>
<tr>
<td>(R)-Bufuralol $V_{max}$, nmol · min$^{-1}$ · million cells$^{-1}$</td>
<td>0.059 ± 0.002</td>
<td>0.048 ± 0.002</td>
<td></td>
</tr>
</tbody>
</table>

* Two systems (nominal values) showed a significant difference ($P < 0.05$) by t test.

$K_{i,u}$ was generated using the total inhibitor concentration, whereas the HMM $K_{i,u}$ was generated using the free inhibitor concentration. Therefore, to compare the values for $K_{i,u}$ generated from two matrices, the plasma $K_{i,u}$ corrected by $f_{u,p}$ was plotted against HMM $K_{i,u}$ in Fig. 1. To prevent bias in the collection and use of $f_{u,p}$, the values for $f_{u,p}$ were obtained from a single source, Goodman & Gilman’s *The Pharmacological Basis of Therapeutics* (Hardman et al., 2001). For all five CYP3A inhibitors, the HMM $K_{i,u}$ values were higher than the plasma $K_{i,u}$ values corrected by $f_{u,p}$.

CYP2C9. Six inhibitors were incubated with human hepatocytes suspended in human plasma or HMM for 20 min over a range of concentrations in the absence of diclofenac and a further 45 min in the presence of diclofenac. The $IC_{50}$ value for each inhibitor was estimated using eq. 1, and the values are shown in Table 2. For fluconazole, the plasma $IC_{50}$ value was comparable to that reported previously (Lu et al., 2007, 2008a,b). These $IC_{50}$ values were converted to the corresponding $K_{i,u}$ in HMM and plasma (listed in Table 3), considering the difference in $K_{i,u}$ values measured in each system according to eq. 3. With the exception of sulfaphenazole, the HMM $K_{i,u}$ values were higher than the plasma $K_{i,u}$ values.

TABLE 2  
$IC_{50}$ values for CYP3A, CYP2C9, and CYP2D6 inhibitors from human hepatocyte suspensions in HMM and human plasma

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>HMM</th>
<th>Plasma</th>
<th>$f_{u,p}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.28 ± 0.02</td>
<td>1.26 ± 0.23$^a$</td>
<td>0.01</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>27.00 ± 1.50</td>
<td>3.71 ± 2.67$^a$</td>
<td>0.89</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>22.40 ± 4.90</td>
<td>3.01 ± 0.85$^a$</td>
<td>0.42</td>
</tr>
<tr>
<td>Conivaptan</td>
<td>1.90 ± 0.18</td>
<td>1.70 ± 0.56$^a$</td>
<td>0.01</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>0.49 ± 0.08</td>
<td>1.70 ± 0.31$^a$</td>
<td>0.01</td>
</tr>
<tr>
<td>Aprepitant</td>
<td>Poor solubility</td>
<td>24.10 ± 7.30$^a$</td>
<td>0.05</td>
</tr>
<tr>
<td>CYP2C9 inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micofenolate</td>
<td>2.12 ± 0.37</td>
<td>2.02 ± 0.37</td>
<td>0.1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>53.96 ± 2.95</td>
<td>14.34 ± 12.77</td>
<td>0.89</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>0.29 ± 0.06</td>
<td>9.49 ± 1.89</td>
<td>0.32</td>
</tr>
<tr>
<td>Fluvasatin</td>
<td>4.07 ± 1.84</td>
<td>6.78 ± 1.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Busprofen</td>
<td>151.30 ± 29.25</td>
<td>&gt;1200</td>
<td>0.01</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>101.08 ± 22.70</td>
<td>&gt;1200</td>
<td>0.04</td>
</tr>
<tr>
<td>CYP2D6 inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>1.71 ± 0.19</td>
<td>30.54 ± 4.34</td>
<td>0.22</td>
</tr>
<tr>
<td>Sertraline</td>
<td>3.10 ± 0.34</td>
<td>13.97 ± 3.49</td>
<td>0.01</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>0.03 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>0.04 ± 0.00</td>
<td>0.35 ± 0.09</td>
<td>0.06</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>0.22 ± 0.04</td>
<td>0.67 ± 0.37</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$^a$ Data are from Mao et al. (2011).
than the plasma $K_{i,app}$ values corrected by $f_{u,app}$ (Fig. 1), which was also observed with CYP3A inhibitors. Fluconazole had a higher $K_{i,app}$ value in HMM than in plasma for both CYP3A and CYP2C9.

CYP2D6. Six inhibitors were incubated with human hepatocytes suspended in human plasma or HMM for 20 min over a range of concentrations in the absence of the probe substrate and a further 25

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TABLE 3

Predictions of clinical DDIs from in vitro inhibition parameters of CYP3A, CYP2C9, and CYP2D6 inhibitors (63 clinical DDIs)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Victim Drug</th>
<th>$[I]_{av,total}$</th>
<th>$K_{i,app}$</th>
<th>Predicted Fold Increase in AUC by $K_{i,app}$</th>
<th>Observed Fold-Increase in AUC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HMM</td>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$f_{u,app}$ corrected by $K_{i,app}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Notes:**

- $K_{i,app}$ values corrected by $f_{u,app}$ (Fig. 1).
- $f_{u,app}$ values corrected by $K_{i,app}$ (Fig. 1).

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A New Drug Application (NDA) 021697 (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2005/021697s000_VaprisolTOC.cfm).
in the presence of (R)-bufuralol. (R)-Bufuralol was selected rather than racemic bufuralol because CYP2D6 displays substrate enantioselectivity for (R)-bufuralol over (S)-bufuralol (Dayer et al., 1987; Narimatsu et al., 2003; Masuda et al., 2005). CYP2D6 is responsible for 95% of racemic bufuralol 1'-hydroxylation clearance, whereas CYP2C19 is responsible for 5% and CYP1A2 has a small contribution (<1%) (Mankowski, 1999).

The IC_{50} value for each inhibitor was estimated using eq. 1, and the values are shown in Table 2. There were no major differences in IC_{50} values in HMM and plasma for quinidine and paroxetine. All IC_{50} values were converted to the corresponding K_{i, app} in HMM and plasma (listed in Table 3), considering the difference in K_{i, app} values measured in each system according to eq. 3. With the exception of diphenhydramine, the HMM K_{i, app} values were higher than the plasma K_{i, app} values corrected by f_{u,p} (Fig. 1), which was also observed with the CYP3A and CYP2C9 inhibitors.

**Prediction of DDIs.** Two methods were used for DDI predictions with six CYP3A inhibitors, six CYP2C9 inhibitors, and six CYP2D6 inhibitors in 63 clinical studies (Table 3). The assumptions behind these two methods were those for the “free [I] with HMM K_{i, app}” method: HMM K_{i, app} represents the “free” inhibition potency; therefore, the in vivo inhibitor concentration needs to be corrected by f_{u,p}. On the other hand, for the “total [I] with plasma K_{i, app}” method, plasma K_{i, app} represents the “total” inhibition potency; therefore, there is no need to correct the in vivo inhibitor concentration by f_{u,p}. Two criteria (2-fold cutoff and categorical prediction) were used to evaluate the prediction outcomes (refer to Materials and Methods for details). The predictive performance from both methods is summarized for individual P450s and for all P450s combined in Table 4.

**CYP3A: Free [I] with HMM K_{i, app}**. Five of 19 studies were predicted within 2-fold (26% accuracy). This method failed to predict 4 of 19 studies (Fig. 2; Tables 3 and 4). The HMM system could not be used to predict four aprepitant clinical DDIs as no measurement of K_{i, app} could be obtained because of poor aprepitant solubility in HMM. In addition, this method underpredicted the other 15 clinical studies by up to 8.24-fold for the ketoconazole study reported by Olkkola et al. (1994). With respect to categorical prediction, neither of the two clinical studies was correctly predicted as a weak interaction (0% accuracy), two of nine clinical studies were correctly predicted as moderate interactions (22% accuracy), and none of the eight clinical studies were correctly predicted as strong interactions (0% accuracy).

**CYP3A: Total [I] with plasma K_{i, app}**. Sixteen of 19 studies were predicted within 2-fold (84% accuracy) including four aprepitant clinical DDIs (Fig. 3; Tables 3 and 4). Two of two clinical studies were correctly predicted as weak interactions (100% accuracy), six of nine clinical studies were correctly predicted as moderate interactions (67% accuracy), and five of eight clinical studies were correctly predicted as strong interactions (63% accuracy).

**CYP2C9: Free [I] with HMM K_{i, app}**. Fourteen of 15 studies were predicted within 2-fold (93% accuracy) (Fig. 2; Tables 3 and 4). For fluvastatin, ibuprofen, and tolbutamide, this method predicted all three clinical studies within 2-fold and correctly predicted each as no interaction (100% accuracy). Although the majority of clinical studies related to fluconazole and miconazole were predicted within 2-fold, there was a bias toward underprediction (Fig. 2). Four of five clinical studies were correctly predicted as weak interactions (80% accuracy), two of six clinical studies were correctly predicted as moderate interactions (33% accuracy), and the only clinical study with a strong interaction was not correctly predicted (0% accuracy).

**CYP2C9: Total [I] with plasma K_{i, app}**. Fourteen of 15 studies were predicted within 2-fold (93% accuracy) (Fig. 3; Tables 3 and 4). For fluvastatin, ibuprofen, and tolbutamide, this method predicted all three clinical studies within 2-fold and correctly predicted each as no interaction (100% accuracy). With this method the predictions for eight studies with fluconazole did not demonstrate a bias. An accurate DDI prediction for sulfaphenazole was also observed. Because of the high average plasma concentration of sulfaphenazole (160–640 μM), the maximum inhibition was reached even though the plasma K_{i, app} was high. Four of five clinical studies were correctly predicted as weak interactions (80% accuracy), four of six clinical studies were correctly predicted as moderate interactions (66% accuracy), and the
only clinical study with a strong interaction was not correctly predicted (0% accuracy).

**CYP2D6: Free [I] with HMM $K_{i,\text{app}}$.** Eighteen of 29 studies were predicted within 2-fold (62% accuracy) (Fig. 2; Tables 3 and 4). Similar to the observations with CYP3A and CYP2C9, this method underpredicted the majority of clinical DDI studies with a maximum 6.55-fold underprediction for the paroxetine study reported by He-meryck et al. (2000). Three of three clinical studies were correctly predicted as no interactions (100% accuracy), 2 of 10 clinical studies were correctly predicted as weak interactions (20% accuracy; the rest were underpredicted as no interactions), 1 of 10 clinical studies was correctly predicted as a moderate interaction (10% accuracy), and none of six clinical studies were correctly predicted as a strong interaction (0% accuracy).

**CYP2D6: Total [I] with plasma $K_{i,\text{app}}$.** Twenty-six of 29 studies were predicted within 2-fold (90% accuracy) (Fig. 3; Tables 3 and 4). Three of three clinical studies were correctly predicted as no interactions (100% accuracy) and 4 of 10 clinical studies were correctly predicted as weak interactions (40% accuracy; the rest were underpredicted as no interaction). Six of 10 clinical studies were correctly predicted as moderate interactions (60% accuracy), and two of six clinical studies were correctly predicted as strong interactions (33% accuracy).

**All P450s: Free [I] with HMM $K_{i,\text{app}}$.** Thirty-seven of 63 clinical DDIs (59% accuracy) were predicted within 2-fold, and the accuracy in predicting no, weak, moderate, and strong interactions was 100, 35, 20, and 0%, respectively (Table 4). The values for GMFE and RMSE were 0.51 and 3.94, respectively.

**All P450s: Total [I] with plasma $K_{i,\text{app}}$.** Fifty-six of 63 clinical DDIs (89% accuracy) were predicted within 2-fold, and the accuracy in predicting no, weak, moderate, and strong interactions was 100, 59, 64, and 47%, respectively (Table 4). The values for GMFE and RMSE were 0.86 and 2.61, respectively.

### Discussion
To evaluate the relative utility of the total [I] with plasma $K_{i,\text{app}}$, and free [I] with HMM $K_{i,\text{app}}$ methods for predicting in vivo DDIs with cryopreserved human hepatocytes, the apparent inhibition constants were determined from protein-containing and protein-free systems, respectively. After correction for protein binding, the $K_{i,\text{app}}$ values were generally higher in the protein-free hepatocyte system, with cases showing approximately 10- to 100-fold variation (Fig. 1). These system differences result in most inhibitors appearing to be more potent in the protein-containing system. One explanation for this observation is that the system is modified by the presence of protein such that an enhancement of unbound intracellular inhibitor concent-

### Table 4

<table>
<thead>
<tr>
<th>Performance</th>
<th>CYP3A</th>
<th>CYP2C9</th>
<th>CYP2D6</th>
<th>All P450s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free [I] with HMM $K_{i,\text{app}}$</td>
<td>Total [I] with Plasma $K_{i,\text{app}}$</td>
<td>Free [I] with HMM $K_{i,\text{app}}$</td>
<td>Total [I] with Plasma $K_{i,\text{app}}$</td>
</tr>
<tr>
<td>2-Fold of observed</td>
<td>26% (5/19)$^b$</td>
<td>84% (16/19)</td>
<td>93% (14/15)</td>
<td>93% (14/15)</td>
</tr>
<tr>
<td>No interaction</td>
<td>N.A.</td>
<td>100% (3/3)</td>
<td>100% (3/3)</td>
<td>100% (3/3)</td>
</tr>
<tr>
<td>Weak interaction</td>
<td>0% (0/2)</td>
<td>100% (2/2)</td>
<td>80% (4/5)</td>
<td>80% (4/5)</td>
</tr>
<tr>
<td>Moderate interaction</td>
<td>22% (2/9)$^c$</td>
<td>67% (6/9)</td>
<td>33% (2/6)</td>
<td>66% (4/6)</td>
</tr>
<tr>
<td>Strong interaction</td>
<td>0% (0/8)</td>
<td>63% (5/8)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
</tr>
<tr>
<td>GMFE</td>
<td>0.51</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMSE</td>
<td>3.94</td>
<td>2.61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.A., there is “no interaction” in 19 CYP3A clinical studies.

$^b$ 26% (5/19) means that there are total 19 clinical studies related to CYP3A inhibitors. This method was able to predict 5 of 19 within 2-fold of observed data.

$^c$ 59% (37/63) means that there are total 63 clinical studies related to CYP3A, CYP2C9, and CYP2D6 inhibitors. This method was able to predict 37 of 63 within 2-fold of observed data.

$^d$ 100% (6/6) means that there are 6 (of total 63) CYP3A clinical studies as “no interaction.” Among these 6 studies, this method was able to predict all clinical studies as no interactions.

$^e$ 22% (2/9) means that there are 9 (of total 19) CYP3A clinical studies as “moderate interaction.” Among these 9 studies, this method was able to predict 2 clinical studies as moderate interactions.
tation occurs. This proposition would be analogous to previous discussions that question the free drug hypothesis by postulating the existence of hepatocyte membrane “albumin receptors” or an albumin-inhibitor complex “dissociation rate limited” uptake proposed for the hepatic clearance of iopanoic acid, rose bengal, sulfobromophthalein, oleate, bilirubin, and palmitate (Wilkinson, 1987). The main assumption behind the “free drug” theory in the context of clearance is that the hepatic uptake of drug is solely dependent on the unbound concentration available at the surface of the liver cell and the binding equilibrium existing within the sinusoid is maintained by the spontaneous dissociation of the protein-drug complex. However, apparent saturation kinetics were observed when the albumin-oleate complex concentration was increased at a constant fraction unbound (molar ratio of oleate to albumin constant at 1:1) in isolated perfused rat livers (Weisiger et al., 1981). This finding suggests that the uptake of highly protein-bound compounds, such as oleate, may be attributable to an albumin receptor on the liver cell surface, but this theory remains highly controversial despite many attempts to identify the molecular basis for such a phenomenon (Burczynski et al., 1989; Tang et al., 2002; Cui and Walter, 2003). An alternative explanation to the albumin receptor suggested that uptake removes the unbound moiety more rapidly than it can be replenished by spontaneous dissociation from albumin (for review, see Wilkinson, 1987). In this hypothesis, uptake is rate-limited by dissociation from albumin and becomes proportional to the concentration of bound drug for a rapidly cleared compound (Ockner et al., 1983). Later, Iwatsubo et al. (1996) reported that the rate of hepatic uptake of compounds highly bound to albumin did not necessarily depend on the extracellular free concentration.

However, the greater unbound inhibitor potency and unbound substrate affinity observed here in the protein-containing system can be explained without dismissing the free drug hypothesis but rather by hypothesizing that there is an artifically low intracellular inhibitor concentration in the protein-free system because of nonspecific loss of inhibitor from the extracellular environment. Although extracellular inhibitor concentrations were not measured in the current report, others have shown that substantial binding to system components can occur in nonprotein media, such as the adsorption of compounds to plastic plates (DeWitte, 2006; Palmgrén et al., 2006). This phenomenon can also explain why the HMM $K_{i.app}$ values for fluconazole and voriconazole were higher than the corresponding plasma $K_{i.app}$ values before correction by $f_u$. This explanation is attractive for the observations reported here not only because it is simple and has a precedent but also because the in vivo DDIs examined were best predicted from the protein-containing system, which can be reasonably expected to be free from the nonspecific loss of inhibitor.

The higher HMM $K_{i.app}$ values versus plasma $K_{i.app} \times f_u$ values and the less accurate prediction from HMM $K_{i.app}$ suggested that the free inhibition potency represented by HMM $K_{i.app}$ has underpredicted the in vivo inhibition potency. However, this does not mean that it could not serve as an in vitro system to measure P450 inhibition assays. Either measuring the unbound fraction of inhibitor with human hepatocytes in the protein-free incubation (equivalent medium to HMM) to correct for the nonspecific binding (McGinnity et al., 2005; Zhao et al., 2005) or incorporating the inhibitor loss to correct the inhibition potency (Zhao et al., 2005) has been used when cryopreserved hepatocytes were utilized to generate the inhibition potency. However, both studies suggested that the improvement in DDI prediction after additional correction steps was limited to one or two compounds, and no general recommendation for the protein-free medium was given despite the rational basis for such corrections. It is also possible that the hepatocytes maintain key metabolic capabilities in the presence of plasma and consequently provide better DDI predictions.

McGinnity et al. (2005) compared the IC$_{50}$ values of 14 drugs obtained from cDNA-expressed CYP2C9 (rCYP2C9) and with those from cryopreserved human hepatocytes in hepatocyte suspension buffer (similar to HMM). The CYP2C9 IC$_{50}$ in vitro values generated in human hepatocytes were systematically higher than those determined with the rCYP2C9, and there was a correlation between IC$_{50}$ unbound values generated in the different milieu after correction for nonspecific binding. It is interesting to note that the unbound fractions of 14 drugs in hepatocytes suspension buffer are lower than those of recombinant enzyme, which suggests that there is more nonspecific binding in hepatocyte suspension buffer for CYP2C9 inhibitors. How-
ever, the method used by these investigators significantly underpredicted the majority of the DDIs examined.

Zhao et al. (2005) compared the IC$_{50}$ values of six CYP3A inhibitors measured in cryopreserved human hepatocytes (suspended in William’s E medium) with those predicted with human liver microsomes (HLM), although there was no attempt to predict DDIs from the in vitro parameters. Hepatocyte IC$_{50}$ values were 2- to 60-fold higher than those measured in HLM after correction for factors such as nonspecific binding and inhibitor consumption in hepatocytes. In addition, the reported hepatocyte IC$_{50}$ values for diltiazem, erythromycin, and troleandomycin from the investigation of Zhao et al. (3.22, 18.02, and 2.14 μM, respectively) were higher than plasma IC$_{50\text{app}}$ values (2.28, 2.58, and 0.23 μM, respectively) from incubations with hepatocytes in plasma as reported in a previous investigation from this laboratory (Mao et al., 2011). As a result, it is reasonable to speculate that if the hepatocyte IC$_{50}$ values from the study of Zhao et al. were combined with the free inhibitor concentration used in the current studies, an underprediction would be expected. This example suggests that the correction for nonspecific binding and inhibitor depletion in the HMM equivalent system may not be sufficient to result in robust DDI predictions. In addition, the hepatocellular synthesis and excretion of CYPs was assessed with cryopreserved human hepatocytes in the presence and absence of human serum, and more accurate predictions of in vivo clearance were observed in the presence of the human serum (Blanchard et al., 2006).

For the purpose of predicting in vivo DDIs with human cryopreserved hepatocytes, the total [I] with plasma K$_{i\text{app}}$ method is superior to the free [I] with HMM K$_{i\text{app}}$ method under the conditions used. For 19 clinical CYP3A-mediated DDIs, the total [I] with plasma K$_{i\text{app}}$ method demonstrated a greater accuracy in predicting the observed in vivo DDIs (Tables 3 and 4). More clinical DDIs were predicted within 2-fold of the observed DDI magnitude by the total [I] with plasma K$_{i\text{app}}$ method, and more accurate categorical predictions of weak, moderate, and strong interactions were observed. Because of the limited solubility of the CYP3A inhibitor aperitant in HMM, no IC$_{50}$ value was obtained. In the plasma system, however, an IC$_{50}$ value was obtained for this poorly soluble compound and an accurate prediction was made for two weak and moderate interactions precipitated by aperitant. The total [I] with plasma K$_{i\text{app}}$ method was also the most predictive method relative to the free [I] with HMM K$_{i\text{app}}$ method for the 29 clinical CYP2D6 DDIs (Tables 3 and 4). More clinical DDIs were predicted within 2-fold of the observed DDI magnitude by the total [I] with plasma K$_{i\text{app}}$ method (89% accuracy), and more accurate categorical predictions of no, weak, moderate, and strong interactions were observed. However, the majority of weak interactions related to diphenhydramine and sertraline were underpredicted as “no interactions” by both methods. These observations may be explained by the “substrate-dependent inhibition” phenomenon (Vandenbrink et al., 2012) whereby the in vitro victim-inhibitor pairs (R)-bufuralol-diphenhydramine and (R)-bufuralol-sertraline may not fully reflect the in vivo interaction of the clinical victim-inhibitor pairs metoprolol-diphenhydramine and desipramine/imipramine-sertraline.

The total [I] with plasma K$_{i\text{app}}$ method was clearly superior to the free [I] with HMM K$_{i\text{app}}$ method for predicting DDIs with CYP3A and CYP2D6 inhibitors. However, the difference in methods was much less apparent for CYP2C9 inhibitors because of the high inhibitor concentrations achieved in the sulfaphenazole clinical studies (insensitive to the different K$_{i}$ estimates) and the weak inhibition by fluconazole that is captured by both hepatocyte systems. Previous studies with HLM failed to predict CYP2C9-mediated DDIs (Andersson et al., 2004). In contrast, the total [I] with plasma K$_{i\text{app}}$ method accurately predicted no inhibition by fluvastatin, ibuprofen, and tolbutamide (the observed and predicted AUC changes in diclofenac were 1.07 and 1.12, 0.99 and 1.08, and 0.93 and 1.11, respectively). Although use of the HHSHP did not yield an accurate measurement of K$_{i\text{app}}$ for ibuprofen and tolbutamide (plasma IC$_{50\text{app}}$ > 1200 μM), the maximum prediction (assuming IC$_{50\text{app}}$ = 1200 μM) predicted the interaction as 1.08 and 1.11. Therefore, the true interactions should be weaker than predicted, and this speculation was confirmed with the observed data (0.99 and 0.93, respectively).

A possible concern with this method is that there is no correction for the possible depletion of the inhibitor concentration in HHSHP. However, on the basis of the kinetic data for midazolam, an efficiently metabolized CYP3A substrate, it is unlikely that inhibitor depletion would be a significant concern. In particular, the media concentration of 1’-hydroxymidazolam was at most 0.7% of midazolam after the 35-min incubation with various concentrations of the parent drug in HHSHP. By assuming that 90% of midazolam is eliminated by 1’-hydroxylation and half of the 1’-hydroxymidazolam generated is further metabolized, it is estimated that 1.3% of the parent drug would be depleted during the 35-min incubation. This result would not significantly affect the assumed substrate nominal concentration of midazolam. For time-dependent inhibitors, the progressive loss of enzyme throughout the incubations would also serve to minimize the loss of inhibitor from the media. The molar concentration of the P450 form is small compared with the total drug concentration and relatively little loss of inhibitor from the plasma can completely inhibit the entire target P450.

In conclusion, the single time point HHSHP K$_{i\text{app}}$ provided relatively simple and accurate DDI predictions mediated by three major drug-metabolizing P450s for both TDI and reversible inhibitors. This method simplified the prediction process by use of the total average systemic plasma concentration as a universal concentration for inhibitors and by obviating the need to correct for the unbound fraction in human plasma.

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Participated in research design: Mao, Mohutsky, Harrelson, Wrighton, and Hall.
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Wrote or contributed to the writing of the manuscript: Mao, Mohutsky, Harrelson, Wrighton, and Hall.

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