A Novel Model for the Prediction of Drug-Drug Interactions in Humans Based on In Vitro Cytochrome P450 Phenotypic Data

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ABSTRACT:

Ketoconazole has generally been used as a standard inhibitor for studying clinical pharmacokinetic drug-drug interactions (DDIs) of drugs that are primarily metabolized by CYP3A4/5. However, ketoconazole at therapeutic, high concentrations also inhibits cytochromes P450 (P450) other than CYP3A4/5, which has made the predictions of DDIs less accurate. Determining the in vivo inhibitor concentration at the enzymatic site is critical for predicting the clinical DDI, but it remains a technical challenge. Various approaches have been used in the literature to estimate the human hepatic free concentrations of this inhibitor, and application of those to predict DDIs has shown some success. In the present study, a novel approach using cryopreserved human hepatocytes suspended in human plasma was applied to mimic the in vivo concentration of ketoconazole at the enzymatic site. The involvement of various P450s in the metabolism of compounds of interest was quantitatively determined (reactive phenotyping). Likewise, the effect of ketoconazole on various P450s was quantitated. Using this information, P450-mediated change in the area under the curve has been predicted without the need of estimating the inhibitor concentrations at the enzyme active site or the $K_i$. This approach successfully estimated the magnitude of the clinical DDI of an investigational compound, MLX, which is cleared by multiple P450-mediated metabolism. It also successfully predicted the pharmacokinetic DDIs for several marketed drugs (theophylline, tolbutamide, omeprazole, desipramine, midazolam, alprazolam, cyclosporine, and loratadine) with a correlation coefficient ($r^2$) of 0.992. Thus, this approach provides a simple method to more precisely predict the DDIs for P450 substrates when coadministered with ketoconazole or any other competitive P450 inhibitors in humans.

Ketoconazole is an antifungal medicine and is also a potent CYP3A4/5 (CYP3A4) inhibitor in humans. Ketoconazole is widely used in vitro as a selective CYP3A4 inhibitor at low concentrations (Pelkonen et al., 1998; Li et al., 1999). However, at high concentrations it also inhibits cytochrome P450 (P450) isoymes other than CYP3A4 (Venkatakrishnan et al., 2001; Stresser et al., 2004) and UDP glucuronosyltransferase (Yong et al., 2005), as well as transporters such as sodium taurocholate cotransporting polypeptide, P-glycoprotein (Pgp), breast cancer resistance protein, and multidrug resistance-associated protein 2 (Azer et al., 1995; Salphati and Benet 1998, Achira et al., 1999; Xia et al., 2005b). For the purpose of overcoming possible drug resistance, clinical usage of ketoconazole involves much higher concentrations (~25-fold) than its in vitro efficacious concentration (Schaef-Korting et al., 1984). For studying the worst-case scenario of CYP3A4-mediated drug-drug interaction (DDI) potential on a compound, dosing of ketoconazole at 200 mg b.i.d. is recommended by the Pharmaceutical Research and Manufacturers of America (Bjornsson et al., 2003). At such a high dose, the maximum plasma concentration ($C_{max}$) could reach as high as 20 μM (Rochlitz et al., 1988; Hsu and Chen, 1997). Other dose regimens, such as 200 mg/day, have also been reported. These doses showed plasma $C_{max}$ ranging from 3.2 to 10 μM with most of the values about 10 μM (Schaefer-Korting et al., 1984; Ito et al., 1998; Pelkonen et al., 1998; Hardman et al., 2001; Blanchard et al., 2004). Ketoconazole treatment has been found to completely inhibit CYP3A4 activity in vivo (Obach et al., 2006), but the cross-inhibition to P450s other than CYP3A4 has not yet been thoroughly shown in vivo as it has been in vitro. The cross-P450 isozyme inhibition by ketoconazole in vivo is usually overlooked when predicting DDIs between ketoconazole and CYP3A4 substrates, and ketoconazole is still treated as a selective CYP3A4 inhibitor in vivo.

P450-reactive phenotyping is a quantitative measurement of the relative contributions of each P450 to the overall metabolism of a drug, when this drug is primarily metabolized by P450s. Several methods are commonly used in the determination of the reactive phenotyping that include the relative activity factor (Crespi, 1995; Venkatakrishnan et al., 2001), chemical inhibitors (Newton et al., 1995; Bourrie et al., 1996; Lu et al., 2003), and monoclonal antibodies (mAbs) (Gelboin et al., 1999; Shou et al., 2000; Soars et al., 2003). In

ABBREVIATIONS: CYP3A4, CYP3A4/5; P450, cytochrome P450; Pgp, P-glycoprotein; DDI, drug-drug interaction; mAb, monoclonal antibody; AUC, area under the curve; KHB, Krebs-Henseleit buffer; LC/MS/MS, liquid chromatography/tandem mass spectrometry; HPLC, high-performance liquid chromatography; $f_a$, fraction of activity remaining of a given enzyme in the presence of inhibitor; $f_{M,P450}$, fraction of metabolism by a given enzyme; MLX, Millennium investigational compound X.
one recent report, all three methods were applied to determine the relative P450 contributions to the metabolism of a proteasome inhibitor, Velcade (Millennium Pharmaceuticals, Cambridge, MA) (Uttamsingh et al., 2005). With the introduction of the potent and selective CYP2C19 and CYP3A4 inhibitors benzylvinanol and azamulin, respectively (Walsky and Obach 2003; Stresser et al., 2004), using chemical inhibitors to determine the reactive phenotyping has become an easy and cost-effective choice.

A major and important effort in the pharmaceutical industry is predicting the clinical DDI from in vitro data. It is especially important for drugs that are primarily metabolized by CYP3A4 because many drugs on the market are CYP3A4 inhibitors/substrates. Besides the pharmacokinetic properties of the substrate drug, two factors that dictate the DDI potential are the inhibitor’s inhibition constant ($K_i$) and the enzyme site free concentration of that inhibitor. The $K_i$ usually can be determined from in vitro microsomal incubation assays with the consideration of protein binding. But the enzyme site inhibitor concentration cannot readily be assessed. Vast efforts have been focused on defining the enzyme site inhibitor concentration, such as use of the peripheral vein concentration, the portal vein concentration, the steady-state systemic plasma concentration, a multiple (such as 10X) of the plasma concentration, the maximum plasma concentration, the maximum hepatic input concentration, or the liver concentration (Blanchard et al., 2004; Cook et al., 2004; Ito et al., 2004; Bachmann 2006; Obach et al., 2006). For each possible concentration, there is always a choice of using the total concentration or the free concentration. Choosing one way to estimate the enzyme site inhibitor concentration may provide a better DDI prediction for certain classes of drugs. However, there is no consensus on which concentration represents the enzyme site concentration and therefore should be used for the DDI prediction of all the classes of drugs.

In the present study, human hepatocytes were incubated in human plasma in the presence of various concentrations of ketoconazole to determine the ketoconazole cross-reactivity or cross-inhibition on five major P450s: CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Reactive phenotyping was determined for the investigational compound MLX using chemical inhibitors and mAbs. Without the need to consider the enzyme site inhibitor concentration or the $K_i$, the DDI potential [the area under the curve (AUC) changes] was predicted using the combined information from the ketoconazole cross-reactivity in human hepatocytes and the reactive phenotyping of MLX. This method was also applied to a set of marketed drugs and showed a good correlation between the predicted DDI and the observed clinical DDI. Thus, this method can help predict DDIs early in the preclinical development stage (Balani et al., 2005).

Materials and Methods

**Reagents.** Pooled human liver microsomes from 50 donors were purchased from XenoTech LLC (Kansas City, KS). Cryopreserved human hepatocytes were purchased from In Vitro Technologies, Inc. (Baltimore, MD) and AP Sciences Inc. (Baltimore, MD). 4-Hydroxybutyrate, 4-hydroxyphenytoin, dextrophorin, 1’-hydroxymidazolam, benzylvinanol, and azamulin were purchased from BD Gentest (Woburn, MA). Phenacetin, acetaminophen, tolbutamide, dextromethorphan, testosterone, 6-hydroxytestosterone, furafylline, sulfaphenazole, quinidine, ketoconazole, NADPH, and MgCl$_2$ were purchased from Sigma (St. Louis, MO). Human plasma was purchased from Bioreclamation Inc. (Hicksville, NY). S-Mephytonin was purchased from BIOMOL Research Labs., Inc. (Plymouth, PA). Ascorbic acids containing mAbs to CYP1A2 (mAb 26-7-5), CYP2C9 (mAb 763-15-5), CYP2C19 (mAb 1-7-4-8), CYP2D6 (mAb 50-1-3), CYP3A4 (3-29-9), and hen egg white lysozyme (control mAb HyHel-9) were gifts from Dr. Harry V. Gelboin (National Cancer Institute, Bethesda, MD).

**Ketoconazole P450 Inhibition Determination in Human Hepatocytes.** This study was performed in parallel in human plasma and Krebs-Henseleit buffer (KHB) fortified with 2 mM salicylamide. The salicylamide was used to reduce possible phase II conjugation to the precursor phase I metabolites, which are the analytes in this study (Lu and Li, 2001). Serially diluted ketoconazole stocks were prepared with the final concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.6, and 0 μM. Human hepatocytes (pooled from two female and two male donors) were thawed and prepared as described previously (Li et al., 1999). The hepatocytes (25 μL, final concentration of 1.0 × 10$^8$ hepatocytes/mL, viability >80%) were mixed with 25 μL of ketoconazole solution at various concentrations at room temperature for 20 min, followed by a prewarm period of 10 min at 37°C. The P450 isozyme-specific substrates (50 μL, final concentration: 30 μM phenacetin, 150 μM tolbutamide, 100 μM S-mephenytoin, 8 μM dextromethorphan, or 5 μM midazolam) were added to start the P450 activity assays. The incubations were carried out in a 37°C CO$_2$ (5%) incubator for 45 min for phenacetin, tolbutamide, dextromethorphan, and midazolam in KHB, and for phenacetin, dextromethorphan, and midazolam in human plasma. However, the incubations for tolbutamide in human plasma and S-mephenytoin in both KHB and human plasma were extended to 90 min because of the substrates’ low turnover rate. All the incubation conditions were in a predetermined linear range. The reactions were stopped by adding one equal or 2 volumes of acetonitrile containing 1 μM carbamidine (internal standard) to the KHB or human plasma samples, respectively. The samples were kept in a refrigerator for 30 min and then centrifuged at 3000g for 10 min. The supernatants were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the amount of metabolite formed. The percentage of metabolic activity remaining was calculated by comparing the P450 activities in samples with various concentrations of ketoconazole with their vehicle controls.

**Determination of Extracellular Ketoconazole Concentrations.** After the equilibrium period of ketoconazole in human hepatocytes of 20 min at room temperature and 10 min at 37°C, the hepatocytes were separated from the buffer or plasma via a centrifugation method described previously (Shitara et al., 2003). Ketoconazole concentrations in these extracellular media were analyzed using LC/MS/MS with standard curves prepared in the same matrices. To determine whether accountable metabolism occurred during this equilibrium period, two sets of ketoconazole-hepatocyte samples were prepared. The reaction-terminating solution, acetonitrile containing 1 μM carbamidine, was added to one set to 0 min and the second set after the equilibrium period. The percentage of ketoconazole remaining was determined using LC/MS/MS. The LC/MS/MS system used to determine ketoconazole and P450 substrate metabolites consisted of an Agilent 1100 high-performance liquid chromatograph (HPLC), a Leap CTC PAL autosampler, and a SCIEX API 4000 detector. Metabolite separation was achieved on a Phenomenex Synergi 4μ C18 80 Å 2.7μm 3.5 x 75 mm column with a gradient consisting of 0.1% formic acid/water (mobile phase A) and 0.1% formic acid/acetonitrile (mobile phase B) at a flow rate of 1.0 ml/min. Specifically, 5% of mobile phase B was applied for 0.5 min after injection and increased linearly to 95% B from 0.5 to 3.5 min. Mobile phase B was held at 95% from 3.5 to 3.6 min, and the column was re-equilibrated to 5% B from 3.6 to 5.0 min. A positive ion spray in the multiple-reaction monitoring mode was applied with a predetermined parent/product mass transition ion pairs for ketoconazole and P450 probe substrate metabolites.

**Reactive Phenotyping of MLX Using P450 Selective Chemical Inhibitors.** In 96-well plates, microsomes (0.5 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4) were prewarmed in duplicate with [1$^4$C]MLX (10 μM) and P450 selective inhibitors (predetermined concentrations) for 5 min at 37°C. The reactions were initiated by the addition of NADPH (2.0 mM)/MgCl$_2$ (3 mM) and incubated for 15 min. The reactions were terminated by the addition of equal volumes of acetonitrile. After storing for 30 min in a refrigerator, the sample plates were centrifuged at 3000g for 10 min, and the supernatants were analyzed using reverse-phase HPLC with an on-line $\beta$-RAM radiochemical detector. P450 selective substrates were incubated in parallel with microsomes in the presence of P450 selective inhibitors to show optimal inhibition. This information was then used to correct the partial inhibition and cross-reactivity of these inhibitors on the MLX metabolism. The final concentrations of P450...
selective inhibitors and probe substrates were 100 \mu M furafylline and 30 \mu M phenacetin for CYP1A2, 5 \mu M sulfaphenazole and 150 \mu M tolbutamide for CYP2C9, 20 \mu M benzylxirvanol and 100 \mu M S-mephenytoin for CYP2C19, 5 \mu M quinidine and 8 \mu M dextromethorphan for CYP2D6, and 2 \mu M azamulin and 50 \mu M testosterone for CYP3A4. The analyses of the P450 substrate metabolites were the same as described earlier. The analysis of MLX metabolites was performed using an HPLC system consisting of an Agilent 1100 binary pump (Palo Alto, CA) coupled to an In/US  \beta- \text{RAM} detector (In/US Systems, Inc., Tampa, FL). A 60-min gradient with 1.0 ml/min flow rate was applied to separate the metabolites from their parent peak using a Synergi Fusion-RP column (4.6 x 250 mm, 4 \mu m, 80 Å) purchased from Phenomenex Inc. (Torrance, CA). The peak areas of MLX and its metabolites were detected using an on-line \beta- \text{RAM} detector with 1:1 (v/v) infusion of the In-Flow Scintillator (In/US Systems, Inc.). The sample injection volume was 100 \mu l. Mobile phases A and B were water and acetonitrile, respectively, each supplemented with 0.1% v/v formic acid.

Reactive Phenotyping of MLX Using Anti-P450 mAbs. Similar to the chemical inhibitor assay, predetermined dilutions of mAbs were preincubated with microsomes (0.5 mg/ml) at 37°C for 10 min. The [14C]MLX (10 \mu M) and NADPH (2.0 mM)/MgCl2 (3 mM) were then added to start the incubations at 37°C for 15 min. The hen egg white lysozyme (control protein in the same -fold of dilutions) was included in the study as control for the 100% P450 activities. The dilutions of mAbs that generated the optimal inhibition were mAb 1-7-4-8, 1:240, CYP2D6 (mAb 50-1-3, 1:40), and CYP3A4 (3-29-9, 1:20). Percent inhibition at these dilutions was determined in an earlier study (mAb 1-7-4-8, 1:20). Percent inhibition at these dilutions was determined in an earlier study (mAb 1-7-4-8, 1:20).

Calculation of AUC Changes from In Vitro Data. Because the P450 contents in the gut are only a small fraction of that in the liver (Obach et al., 2006), the contribution of metabolism by gut to the overall metabolism in humans may generally be considered as low. Thus, Fg/Fg factor (Ito et al., 2005; Galetin et al., 2006), which is hardly available for the compounds of interest, was not considered for this study. Equation 4 (see under Discussion for details) was used to calculate AUC changes, assuming linear pk and a Cmax of 10 \mu M at 200 mg b.i.d. or q.d. dose of ketoconazole (Chien et al., 2006). For example, for omeprazole, with a ketoconazole dose of 200 q.d. and a Cmax of 10 \mu M, the fA3A4 and fA2C19 were calculated to be 0.006 and 0.604, respectively (Table 2). Together with fA3A4 of 0.40 and fA2C19 of 0.60 from literature, and assuming clearance only caused by metabolism (i.e., m/hep = 1), a prediction value of 2.74-fold increase in AUC was calculated. A similar calculation was done for another omeprazole study in which a low ketoconazole dose of 50 mg q.d. was reported (University of Washington DDI database, 2004). This predicted for a 1.74-fold change in AUC (Table 5). For theophylline and alprazolam, the renal clearance had marginal contribution to the total clearance of theophylline and alprazolam, but the information on ketoconazole’s effect on renal clearance was not available. Therefore, a range of values was predicted such that the low value considers the renal clearance contribution in the calculation, whereas the high value does not. One can imagine the low value is similar to the situation in which renal clearance is not affected by ketoconazole, whereas the high value is similar to the situation in which renal clearance is totally blocked by ketoconazole.

Results
The inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 by ketoconazole in human hepatocytes in KHB or human plasma is presented in Tables 1 and 2. Figure 1 shows the plot of extracellular ketoconazole concentration versus the P450 activity remaining from the data in Table 2. For both Tables, column 1 represents the concentrations in the incubation—the initial concentration of ketoconazole. The “Keto Extracellular” (column 2) represents the extracellular concentration of ketoconazole remaining in the KHB buffer or plasma after the equilibrium period. Com-

### Table 1

<table>
<thead>
<tr>
<th>Ketone in Incubation</th>
<th>Ketone in Extracellular</th>
<th>fA (%)*</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 \mu M</td>
<td>19.3</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>0</td>
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<tr>
<td>50 \mu M</td>
<td>9.23</td>
<td>16</td>
<td>4</td>
<td>36</td>
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<tr>
<td>25 \mu M</td>
<td>3.98</td>
<td>41</td>
<td>17</td>
<td>60</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>12.5 \mu M</td>
<td>1.48</td>
<td>55</td>
<td>31</td>
<td>78</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>6.25 \mu M</td>
<td>0.640</td>
<td>63</td>
<td>54</td>
<td>88</td>
<td>3</td>
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<tr>
<td>3.13 \mu M</td>
<td>0.263</td>
<td>77</td>
<td>72</td>
<td>101</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 \mu M</td>
<td>0.153</td>
<td>91</td>
<td>80</td>
<td>101</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

* fA (%) = fraction of enzyme activity remaining in the presence of ketoconazole inhibition.

### Table 2

<table>
<thead>
<tr>
<th>Ketone in Incubation</th>
<th>Ketone in Extracellular</th>
<th>fA (%)*</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 \mu M</td>
<td>71.8</td>
<td>79</td>
<td>60</td>
<td>32</td>
<td>96</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>50 \mu M</td>
<td>34.0</td>
<td>81</td>
<td>71</td>
<td>42</td>
<td>84</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>25 \mu M</td>
<td>14.1</td>
<td>83</td>
<td>72</td>
<td>57</td>
<td>99</td>
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</tr>
<tr>
<td>12.5 \mu M</td>
<td>7.30</td>
<td>89</td>
<td>75</td>
<td>63</td>
<td>91</td>
<td>1</td>
<td></td>
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<tr>
<td>6.25 \mu M</td>
<td>4.04</td>
<td>92</td>
<td>80</td>
<td>83</td>
<td>99</td>
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<tr>
<td>3.13 \mu M</td>
<td>2.22</td>
<td>92</td>
<td>84</td>
<td>90</td>
<td>100</td>
<td>11</td>
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<tr>
<td>1.6 \mu M</td>
<td>0.857</td>
<td>97</td>
<td>79</td>
<td>89</td>
<td>97</td>
<td>12</td>
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<td>100</td>
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</tbody>
</table>

* fA (%) = fraction of enzyme activity remaining in the presence of ketoconazole inhibition.
where A, B, C, D, and E are the percentage contributions of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively, to the metabolism of MLX.

In contrast to the incubation in KHB, it was noticed that at high concentrations, ketoconazole tended to cause more inhibition of CYP2C19 than CYP2C9. Within the concentration range tested in this study, dose-dependent inhibition of ketoconazole on CYP2D6 was not observed in the presence of plasma. As expected, ketoconazole was found to be stable in the hepatocyte incubations in KHB or plasma during the 30-min period of equilibration (data not shown); therefore, the lower extracellular concentrations of ketoconazole concentrations were caused by binding instead of metabolism. Table 3 shows the reactive phenotyping results for MLX using chemical inhibitors, suggesting MLX is primarily metabolized by CYP3A4, followed by CYP2D6 and to a lesser extent by CYP1A2. These results were similar to those generated using mAbs, except the contribution of CYP2D6 in the chemical inhibitor assay was much higher. In our experience, using quinidine and dextromethorphan tended to overestimate CYP2D6 contribution compared with that using the relative activity factor or the mAb method (Uttamsingh et al., 2005). A hypothesis that may help explain this observation is that quinidine at low concentrations is known to stimulate CYP3A4 metabolism, but at high concentrations it appears to show inhibition (Ludwig et al., 1999; Ngui et al., 2000). This stimulation by quinidine could reduce the estimates for net CYP3A4 inhibition, thereby leading to underestimation of the cross-reactivity of quinidine on CYP3A4, which in turn would lead to an overestimation of CYP2D6 contribution. Table 4 presents the cross-reactivity of the P450 inhibitors used in this study and a method of correcting for this cross-reactivity for obtaining the reactive phenotyping results of MLX. For example, the observed inhibition of MLX metabolism by furafylline (Table 4, the rightmost column) is attributed to the sum of partial inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 by furafylline, measured using P450 probe substrates. An equation can then be derived for each inhibitor. By solving the five equations simultaneously, the contribution of the five P450s (the five variables) to the metabolism of MLX can be solved, and the results are shown in Table 3. Table 5 lists DDI predictions for the investigational compound (MLX) and a set of marketed compounds. Also included is a comparison between the values predicted from this study and the values observed in clinical trials. A correlation coefficient (r²) of 0.992 (Fig. 2) was derived from the data in Table 5. A slope close to unity (0.980) suggested a good prediction of clinical DDIs from the in vitro data.

**Discussion**

Cryopreserved hepatocyte suspension is a valid model for studying drug metabolism and DDIs. Hepatocyte incubations are usually carried out in KHB, which is an essential but minimum salt buffer

**TABLE 3**

Relative contributions of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 to the metabolism of MLX in human liver microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
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</thead>
<tbody>
<tr>
<td>Phenacetin</td>
<td>4.8</td>
<td>0</td>
<td>3.2</td>
<td>3.2</td>
<td>63</td>
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<tr>
<td>Tolbutamide</td>
<td>0.531</td>
<td>0.224</td>
<td>0.436</td>
<td>0.666</td>
<td>0.531</td>
</tr>
<tr>
<td>S-Mephenytoin</td>
<td>0.138</td>
<td>0.436</td>
<td>0.970</td>
<td>0.105</td>
<td>0.195</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>0.531</td>
<td>0.436</td>
<td>0.970</td>
<td>0.105</td>
<td>0.195</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.195</td>
<td>0.105</td>
<td>0.138</td>
<td>0.531</td>
<td>0.224</td>
</tr>
<tr>
<td>MLX</td>
<td>89.9</td>
<td>30.4</td>
<td>3.57</td>
<td>77.6</td>
<td>30.4</td>
</tr>
</tbody>
</table>

**Discussion**

Cryopreserved hepatocyte suspension is a valid model for studying drug metabolism and DDIs. Hepatocyte incubations are usually carried out in KHB, which is an essential but minimum salt buffer.

**TABLE 4**

Substrate specificity of prototypic P450 inhibitors in human liver microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
<th>Average % Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furafylline</td>
<td>85.5</td>
<td>6.2</td>
<td>53.1</td>
<td>7.5</td>
<td>5.3</td>
<td>8.54</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>5.4</td>
<td>92.8</td>
<td>13.8</td>
<td>19.5</td>
<td>15.0</td>
<td>3.57</td>
</tr>
<tr>
<td>Benzylnarvanol</td>
<td>0.2</td>
<td>43.6</td>
<td>97.0</td>
<td>10.5</td>
<td>33.3</td>
<td>23.4</td>
</tr>
<tr>
<td>Quinidine</td>
<td>-44.5</td>
<td>22.4</td>
<td>28.3</td>
<td>66.6</td>
<td>23.2</td>
<td>30.4</td>
</tr>
<tr>
<td>Azamulin</td>
<td>-75.3</td>
<td>15.3</td>
<td>17.6</td>
<td>7.5</td>
<td>89.9</td>
<td>77.6</td>
</tr>
</tbody>
</table>

The relative contribution of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 to the metabolism of MLX was calculated by the following equations:

\[
\begin{align*}
8.54 & = 0.855x_A + 0.062x_B + 0.531x_C + 0.075x_D + 0.053x_E \\
3.57 & = 0.054x_A + 0.928x_B + 0.138x_C + 0.195x_D + 0.15x_E \\
23.4 & = 0.x_A + 0.436x_B + 0.970x_C + 0.105x_D + 0.333x_E \\
30.4 & = 0.x_A + 0.224x_B + 0.283x_C + 0.666x_D + 0.232x_E \\
77.6 & = 0.x_A + 0.153x_B + 0.176x_C + 0.075x_D + 0.899x_E \\
\end{align*}
\]

where A, B, C, D, and E are the percentage contributions of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively, to the metabolism of MLX.
for hepatocytes to maintain their metabolic function. Several researchers (Shibata et al., 2002; Bachmann et al., 2003) have tried to add human serum into hepatocyte incubations to better mimic the in vivo conditions. Cryopreserved hepatocytes are known to preserve the metabolic function. Several researchers (Shibata et al., 2002; Bachmann et al., 2003) have tried to add human serum into hepatocyte incubations to closely mimic the in vivo plasma concentration in vivo, the concentration of the inhibitor at the enzyme site in the in vitro model is also comparable with that of the enzyme site in vivo. In this way, estimating the actual enzyme site inhibitor concentration becomes unnecessary. This approach should apply to most reversible P450 inhibitors. The effect of inhibitors and test compounds as substrates and inhibitors of transporters is yet to be fully evaluated. The current approach works with the CYP3A inhibitor ketoconazole, which is not a substrate but an inhibitor of Pgp. Also, if a test compound is not a substrate but an inhibitor of Pgp. Also, if a test compound is an inhibitor, eq. 1 can be rewritten as:

\[
CL = f_m (f_{m, A2}CL + f_{m, C9}CL + f_{m, A2}CL + f_{m, A4}CL + ...)
+ f_{other, CL} \tag{1}
\]

where \(f_m\) is the fraction of clearance by metabolism, which often attributes to gut and liver metabolism \((f_m = f_{m, gut} \times f_{m, hep})\), and \(f_{other}\) is the fraction of clearance by other routes such as renal or biliary excretion \((f_m + f_{other} = 1)\). The \(f_{m, A2}\) is the fraction of metabolic clearance by CYP1A2, and so on. The “...” in eq. 1 refers to the enzymes other than the major five P450s. The \(f_m\) could be simplified as a fraction of hepatic metabolism \((f_{m, hep})\) if the substrate is administered i.v. or metabolism by intestinal or other organs is insignificant compared with that of the liver. Similar to what was proposed by Ito et al. (2005), in the presence of an inhibitor, eq. 1 can be rewritten as:

\[
CL_i = f_{m, hep} \left( \frac{f_{m, A2}CL}{1 + K_{A2}^{A4}} + \frac{f_{m, C9}CL}{1 + K_{C9}^{C9}} + ... \right) + f_{other, CL} \frac{1}{1 + K_{other}} \tag{2}
\]

The DDI potential is usually expressed as the ratio of AUC_i (AUC with the inhibitor) over the AUC of control (without inhibitor), which

<table>
<thead>
<tr>
<th>Compound</th>
<th>Predicted</th>
<th>Observed</th>
<th>(f_{\text{total}})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLX</td>
<td>8.33</td>
<td>*</td>
<td>0.18</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Theophylline</td>
<td>1.13–1.16</td>
<td>1.11</td>
<td>0.70</td>
<td>Obach 2005^d,e</td>
</tr>
<tr>
<td>Desipramine</td>
<td>1.00</td>
<td>1.02</td>
<td>&lt;0.01</td>
<td>Obach 2005^d,e</td>
</tr>
<tr>
<td>Midazolam</td>
<td>16.7</td>
<td>17.0</td>
<td>0.001</td>
<td>Obach 2005^d,e</td>
</tr>
<tr>
<td>Tolbutfamide</td>
<td>1.43</td>
<td>1.77</td>
<td>&lt;0.01</td>
<td>Soars 2003^e</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>1.74–2.74</td>
<td>1.36–2.05</td>
<td>&lt;0.01</td>
<td>Soars 2003^e</td>
</tr>
<tr>
<td>Loratadine</td>
<td>2.48</td>
<td>3.47</td>
<td>Negligible</td>
<td>U of Washington DDI database;^g Galetin 2006^f</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>3.45</td>
<td>4.39</td>
<td>&lt;0.01</td>
<td>U of Washington DDI database;^g Galetin 2006^f</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>2.75–4.91</td>
<td>3.98</td>
<td>0.20</td>
<td>U of Washington DDI database;^g Galetin 2006^f</td>
</tr>
</tbody>
</table>

^a Predictions are based on our model assuming 200 mg b.i.d. doses of ketoconazole have plasma \(C_{max}\) of 10 \(\mu\)M and linear pK.
^b Information on renal clearance was from Hardman et al. (2001), except that of desipramine was from Physicians Desk Reference (2001).
^c Assume substrates are selective (theophylline, desipramine for CYP1A2 and CYP2D6, respectively).
^d References for observed AUC changes.
^e The ketoconazole inhibition to the metabolism routes other than CYP3A4 was not considered because the information was not available.
^f Within 10% of the predicted value.

**Fig. 2.** Correlation of the observed clinical and the predicted DDI (-fold of AUC change) using eq. 4. The data are from Table 5. Average values of theophylline, omeprazole, and alprazolam were used in the plot instead of a range.
has an inverse relationship with the clearance ratio in the absence or presence of the inhibitor.

$$\frac{AUC_1}{AUC} = \frac{CL}{CL_1} = \frac{1}{f_{m,hep} \left( \frac{f_{A3A4}}{1 + K_{A3A4}} + \frac{f_{A2C9}}{1 + K_{A2C9}} + \ldots \right) + \frac{f_{other}}{1 + K_{other}}}$$ (3)

Thus, the AUC ratio for a compound primarily cleared by metabolism can be expressed as a function of the remaining activity of each enzyme toward the metabolism of this compound, or the remaining clearance routes.

$$\frac{AUC_1}{AUC} = \frac{CL}{CL_1} = \frac{1}{f_{m,hep}(f_{A3A4}/f_{A3A4} + f_{A2C9}/f_{A2C9} + \ldots) + f_{other}/f_{other}}$$ (4)

where \( f_{m,P450} \) is again the relative contribution of an enzyme to the total metabolism of a compound (without inhibitor), and \( f_A \) is the fraction of enzyme activity remaining in the presence of an inhibitor \( i.e., f_{A3A4} = 1/(1 + 1/K_{A3A4}) \). Whereas \( f_{m,P450} \) could be determined in several ways (Uttamsingh et al., 2005), including using the selective chemical inhibitors (Table 3), the \( f_{CH} \) in vivo can be estimated from the \( f_A \) obtained from the in vitro data such as those in Table 2. Thus, DDIs can be predicted by combining the information from the reactive phenotyping study and the inhibitor’s cross-reactivity study (eq. 4 if a compound is primarily cleared by metabolism or the other clearance route is known).

The \( C_{max} \) of ketoconazole at the steady state can be used to assess the worst-case inhibitory effect, although the \( C_{max} \) may vary from person to person because of interindividual variability in \( pK \) or from study to study because of different dosing regimens. For an individual \( C_{max} \) or a comparable in vitro concentration \( x \) in our model, the fraction of enzyme activity remaining \( f_A \) can be calculated from the titration curves (Fig. 1 or Table 2). Theoretically, the \( f_A \) and \( x \) follow a sigmoidal relationship:

$$f_A = \frac{\text{Minimum activity} + \frac{\text{Maximum activity} - \text{Minimum activity}}{1 + 10^{(\log EC50+yHill\ slope)}}}{1}$$ (5)

Practically, to determine the percentage of enzyme activity remaining \( f_A \) at an inhibitor concentration \( x \) requires a well fitted sigmoidal curve. From our data, within the physiological concentration range, ketoconazole only partially inhibits common P450s in human hepatocytes in the presence of human plasma. Thus, a linear model is more practical for estimating the percentage remaining of enzyme activity \( f_A \) at an inhibitor concentration \( x \). This linear model assumes that within a narrow range (one concentration above and one concentration below the targeted \( x \), \( C_{max} \)), the inhibition response is linear. Therefore, the P450 activity remaining can be interpreted between these points:

$$f_A = A_1 - (x - C_1)(A_1 - A_0)/(C_1 - C_L)$$ (6)

where \( A_1, A_2 \), and \( C_1, C_2 \) are the activities and concentrations above and below the target concentration \( x \), respectively.

MLX is an investigational compound. Through qualitative enzyme mapping and metabolite profiling studies, MLX was found to be metabolized mainly by P450 isozymes. Furthermore, quantitative P450 phenotyping also found that this compound was mainly metabolized by CYP3A4 with some contributions from CYP2D6 and CYP1A2. Considering that CYP2D6 contribution may be overestimated, ketoconazole at a concentration of 10 to 20 \( \mu M \) inhibits 87% of the CYP3A4 activity (\( \sim 100% \) inhibition of the 87% CYP3A4 contribution) (Tables 2 and 3) and 1% of the CYP1A2 contribution (\( \sim 20% \) cross-reactivity of the 4.8% CYP1A2 contribution) (Tables 2 and 3). Thus, using the approach of DDI prediction illustrated in eq. 4, an 8.33-fold increase in AUC was predicted for MLX coadministration with ketoconazole. A clinical DDI study using 200 mg b.i.d. of ketoconazole showed an increase of mean AUC of MLX within 10% of the predicted 8.33-fold (unpublished data).

There is limited literature available on compounds with information on both clinical DDIs with ketoconazole and quantitative reactive phenotyping. Table 5 lists several compounds found in recent literature (University of Washington DDI database, 2004; Galetin et al., 2006; Obach et al., 2006) with the observed clinical DDI. Although there is no reactive phenotyping information available for theophylline and desipramine, these two compounds have been treated as prototypical substrates for CYP1A2 and CYP2D6 for retroactive prediction based on our method. Midazolam is widely considered as an exclusive CYP3A4 substrate. Omeprazole was found to be metabolized by CYP2C19 (60%) and CYP3A4 (40%), whereas tolbutamide was found to be metabolized by CYP2C9 (72%) and CYP2C19 (28%) (Soars et al., 2003). CYP3A4 was reported to contribute 71, 80, and 60% to the metabolism of cyclosporine, alprazolam, and loratadine, respectively (Galetin et al., 2006), but the rest of the clearance routes have not been documented. It is also known that cyclosporine is partially cleared renally and that CYP2D6 contributes toward the metabolism of loratadine (Yumibe et al., 1996). In this study, the prediction of these three compounds was based on the assumption that ketoconazole does not affect the non-CYP3A4 metabolic clearance routes. In vivo human plasma ketoconazole concentration values vary in the literature, most being around 10 \( \mu M \) with 20 \( \mu M \) being the highest at 200 mg b.i.d.; in practice, it is assumed to be 10 \( \mu M \). The observed clinical values were adapted from Obach et al. (2006) and the DDI database (handout) from the University of Washington (2004). Using this information and the ketoconazole cross-reactivity data in Table 4, the prediction of AUC changes is presented in Table 5. It is noteworthy that these predictions compare very closely with the observed clinical values (\( r^2 = 0.992 \) and slope of 0.980) (Fig. 2).

For most of the compounds, renal clearance plays a minor role (1% or less) (Table 5); thus, renal clearance contribution was not included in the calculation. For theophylline and alprazolam, predictions were made with and without considering the renal clearance contribution. Because the effect of ketoconazole on the renal clearance of these two compounds is unknown, a range of predictions was reported such that the low value shows ketoconazole has no effect on renal clearance, whereas the high value shows that ketoconazole totally blocked the renal clearance. For Fig. 2, average values were used for data presented as ranges in Table 5. Desipramine was treated as a selective CYP2D6 substrate. Ketoconazole was predicted to have no effect on its metabolic clearance based on our hepatocyte cross-reactivity study. Prediction of ketoconazole effect on its renal clearance was not explored. For loratadine, cyclosporine, and alprazolam, only \( f_{m,P450} \) information was available (Galetin et al., 2006); hence, the interaction could be underestimated.

In summary, the DDI prediction approach presented in this study involves using two in vitro determinations: the reactive phenotyping (the relative contributions of enzymes to the overall metabolism of a drug, an intrinsic property of the drug) and the P450 cross-reactivity of an inhibitor at close to the physiological condition (hepatocyte...
incubation in human plasma). Ketoconazole is used in this study as an example. However, most inhibitors of interest, except those that are efflux pump substrates that can be pumped out of hepatocytes leading to reduced hepatocyte concentration and hence reduced potency, can be applied. In most cases, the pharmaceutical industry likes to show the worst-case scenario for DDI potential for drugs that are mainly metabolized by a few isozymes; therefore, a handful of known inhibitors, such as ketoconazole for CYP3A4 and quinidine for CYP2D6, are often used. Thus, this approach provides a simple way of predicting the DDI early in the preclinical development stage, with the main advantage being not have to estimating the actual inhibitor concentration at the enzyme site.

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References

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PREDICTION OF CLINICAL DRUG-DRUG INTERACTIONS

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