The Reliability of Estimating $K_i$ Values for Direct, Reversible Inhibition of Cytochrome P450 Enzymes from Corresponding IC$_{50}$ Values: A Retrospective Analysis of 343 Experiments

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ABSTRACT

In the present study, we conducted a retrospective analysis of 343 in vitro experiments to ascertain whether observed (experimentally determined) values of $K_i$ for reversible cytochrome P450 (P450) inhibition could be reliably predicted by dividing the corresponding IC$_{50}$ values by two, based on the relationship (for competitive inhibition) in which $K_i = IC_{50}/2$ when [S] (substrate concentration) = $K_m$ (Michaelis-Menten constant). Values of $K_i$ and IC$_{50}$ were determined under the following conditions: 1) the concentration of P450 marker substrate, [S], was equal to $K_m$ (for IC$_{50}$ determinations) and spanned $K_m$ (for $K_i$ determinations); 2) the substrate incubation time was short (5 minutes) to minimize metabolism-dependent inhibition and inhibitor depletion; and 3) the concentration of human liver microsomes was low (0.1 mg/ml or less) to maximize the unbound fraction of inhibitor. Under these conditions, predicted $K_i$ values, based on IC$_{50}$/2, correlated strongly with experimentally observed $K_i$ determinations ($r = 0.940$; average fold error (AFE) = 1.10). Of the 343 predicted $K_i$ values, 316 (92%) were within a factor of 2 of the experimentally determined $K_i$ values, and only one value fell outside a 3-fold range. In the case of noncompetitive inhibitors, $K_i$ values predicted from IC$_{50}$/2 values were overestimated by a factor of nearly 2 (AFE = 1.85; $r = 0.940$; average fold error (AFE) = 1.10). Of the 343 predicted $K_i$ values, 0.02, where $R_1$ > 1.1 (where $R_1 = 1 + [I]/K_{unbound}$). The same criteria apply to significant circulating metabolites, defined by the FDA as metabolites whose plasma AUC is $\approx$25% of the parent AUC following dosing to steady state with the maximum clinical dose. The European Medicines Agency’s (EMA’s) 2012 Guideline on the Investigation of Drug Interactions (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf) is based on a similar type of ratio and cutoff value, namely, $[I]_{unbound}/K_{unbound} \approx 0.02$, where $K_{unbound}$ is as defined earlier and $[I]_{unbound}$ is the unbound (free) maximum concentration of drug in plasma (mean $C_{max}$ obtained following treatment with the highest recommended clinical dose). The EMA’s guideline also applies to significant circulating metabolites, defined as phase 1 metabolites whose plasma AUC is $>25$% of the parent drug and $>10$% of total circulating drug-derived material. To evaluate the potential for reversible inhibition of CYP3A enzymes in the intestine, the concentration of inhibitor in eq. 1 is defined as $[I]_{put} = \text{molar dose}/250 \text{ ml}$. Values of $[I]_{put}$ can greatly

Introduction

When an investigational drug is evaluated in vitro as a direct, reversible inhibitor of human liver cytochrome P450 (P450) enzymes based on the Food and Drug Administration’s (FDA’s) 2012 draft Guidance for Industry on drug interactions (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf), the need for a clinical drug interaction study is based on eq. 1:

$$AUCR = R_1 = 1 + \frac{[I]}{K_{unbound}}$$

(1)

where $[I]$ is the maximum total (bound + unbound) plasma concentration of drug at steady state ($C_{max,ss}$), and $K_{unbound}$ is the dissociation constant for the enzyme-inhibitor complex for direct, reversible inhibition based on the concentration of unbound drug in the in vitro test system. AUCR, the area under the plasma concentration-time curve (AUC) ratio, represents the fold increase in plasma AUC of a probe drug whose clearance is entirely determined by metabolism by the P450 enzyme inhibited by the investigational drug (i.e., fractional metabolism by an enzyme, or $f_m = 1.0$) (Ito et al., 1998; Rodrigues et al., 2001). When other pathways contribute to clearance of the probe drug, such that $f_m < 1$, the fold increase in plasma AUC of the probe drug in the presence of the inhibitory drug is given by eq. 2:

$$AUCR = \frac{1}{1 + \frac{[I]}{K_{unbound}} \cdot f_m \cdot f_{m,enzyme} + (1 - f_m \cdot f_{m,enzyme})}$$

(2)

A clinical drug interaction study is recommended for P450 enzymes other than CYP3A when the ratio $R_1 > 1.1$ (where $R_1 = 1 + [I]/K_{unbound}$). The same criteria apply to significant circulating metabolites, defined by the FDA as metabolites whose plasma AUC is $\approx 25$% of the parent AUC following dosing to steady state with the maximum clinical dose. The European Medicines Agency’s (EMA’s) 2012 Guideline on the Investigation of Drug Interactions (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf) is based on a similar type of ratio and cutoff value, namely, $[I]_{unbound}/K_{unbound} \approx 0.02$, where $K_{unbound}$ is as defined earlier and $[I]_{unbound}$ is the unbound (free) maximum concentration of drug in plasma (mean $C_{max}$ obtained following treatment with the highest recommended clinical dose). The EMA’s guideline also applies to significant circulating metabolites, defined as phase 1 metabolites whose plasma AUC is $>25$% of the parent drug and $>10$% of total circulating drug-derived material. To evaluate the potential for reversible inhibition of CYP3A enzymes in the intestine, the concentration of inhibitor in eq. 1 is defined as $[I]_{put} = \text{molar dose}/250 \text{ ml}$. Values of $[I]_{put}$ can greatly

ABBREVIATIONS: AFE, average fold error; AUC, area under the plasma concentration-time curve; EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; NRMSE, normalized root mean square error; P450, cytochrome P450; RMSE, root mean square error.
exceed values of [I] (total C\text{max}; accordingly, the FDA’s cutoff value for inhibition of intestinal P450 enzymes is 11 (based on 1 + [I]/K\text{IC50}/K\text{unbound}). The corresponding EMA cutoff is 10 (based on [I]/K\text{IC50}/K\text{unbound}).

Typically, inhibition of P450 enzymes is first evaluated in vitro by determining the concentration of investigational drug (or significant circulating metabolite) that causes 50% inhibition of P450 enzyme activity (IC50) with a selective P450 probe at a substrate concentration approximately equal to K\text{m} (Michaelis-Menten constant). Determining the mechanism of reversible inhibition (competitive, noncompetitive, mixed, or uncompetitive) and measuring the value of K\text{i} requires an in vitro evaluation of the effects of multiple inhibitor concentrations versus multiple substrate concentrations, ideally with the former spanning K\text{m} and the latter spanning K\text{m}. Determining values of IC50 and K\text{i} based on the unbound concentration of inhibitor in the test system (typically human liver microsomes) requires knowledge of f\text{unc} (the fraction of unbound drug in the microsomal incubation), which can be determined experimentally or estimated theoretically from the inhibitor’s logP or logD value and the concentration of microsomal protein (Austin et al., 2002; Hallifax and Houston, 2006).

The relationship between K\text{i} and values of IC50 determined when [S] (substrate concentration) \(= K_m\) depends on the mechanism of inhibition, as summarized in Table 1 (Cheng and Prusoff, 1973; Brandt et al., 1987; Cer et al., 2009). In the case of noncompetitive inhibition, K\text{i} = IC50/2. In the case of competitive and uncompetitive inhibition, K\text{i} = IC50/2. In the case of mixed inhibition, K\text{i} values range from IC50 to IC50/2. The FDA’s 2012 Guidance for Industry on drug interactions acknowledges that K\text{i} values are often estimated from values of IC50/2, based on the conservative assumption that the mechanism of reversible inhibition is competitive in nature. In the present study, we conducted a retrospective analysis of 343 in vitro K\text{i} determinations to investigate whether determined K\text{i} values can, in fact, be reliably estimated from values of IC50/2 when IC50 values are determined under conditions of [S] \(\approx K_m\).

### Materials and Methods

**Chemicals and Reagents.** The commercial sources of most substrates, metabolites, internal standards, and reagents have been described previously (Pearce et al., 1996; Paris et al., 2009; Parkinson et al., 2011). Efavirenz was purchased from U.S. Pharmacopeia (Rockville, MD). 8-Hydroxyefavirenz, 8-hydroxyefavirenz-d1 (internal standard), and 6-hydroxyefavirenz-d3 (internal standard) were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Coumarin, paclitaxel, bufuralol, 1-hydroxybufuralol, chloroxazone, 6-hydroxychloroxazone, 6-hydroxychloroxazone-d4 (internal standard), nifedipine, and oxidized nifedipine. 7-Hydroxycoumarin was purchased from Cerilliant (Round Rock, TX). 6o-Hydroxypaclitaxel, 7-hydroxycoumarin-d3 (internal standard), and dehydronifedipine-d6 (internal standard) were purchased from SynFinel Research (Richmond Hill, Ontario, Canada). 10-Deacetyltaxol (internal standard) was purchased from A.F. Hauser, Inc. Pharmaceutical (Valparaiso, IN).

**Investigational Drugs.** A total of 132 investigational drugs and drug metabolites were examined during the course of P450 inhibition studies sponsored by numerous pharmaceutical companies in the United States, Europe, and Japan. Unfortunately, because they are proprietary compounds and for reasons of confidentiality, we are not at liberty to disclose the identity of the structures of these compounds. The compounds represent a set of structurally diverse, small drug molecules under development for several different therapeutic indications. The design of the experiments and the interpretation of the results of this study (a comparison of two endpoints of P450 inhibition) required neither knowledge of chemical structures nor physicochemical properties.

**Test System.** Pooled human liver microsomes (n = 16 or 200; mixed gender) were prepared from nontransplantable livers and characterized at Xenotech, LLC (Lexena, KS) as described previously (Pearce et al., 1996; Parkinson et al., 2004).

**Incubation Conditions.** K\text{i} and IC50 values were determined in accordance with recommendations in the FDA and EMA guidance documents and consensus papers (Tucker et al., 2001; Bjornsson et al., 2003). All experiments were performed under the following conditions: 1) the concentration of P450 marker substrate was approximately equal to Km for IC50 determinations and spanned K\text{m} for K\text{i} determinations (i.e., [S] ranged from 0.25 times K\text{m} to 10 times K\text{m}; solubility permitting); 2) the substrate incubation time was 5 minutes to minimize metabolism-dependent inhibition and inhibitor depletion; and 3) the concentration of human liver microsomes was 0.1 mg/ml or less to maximize the unbound inhibitor concentration.

In general, incubations were conducted at 37°C in 200- or 400-μl incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM), NADPH-generating system, and human liver microsomes. Most of the P450 reactions examined have been described in detail elsewhere (Paris et al., 2009; Parkinson et al., 2011). The P450 substrates, analytes (metabolites measured), internal standards, and microsomal protein concentration for all of the P450 reactions examined are summarized in Supplemental Table 1.

**Analytical Methods.** All metabolites and their internal standards (usually isotopically labeled metabolites) were measured with validated liquid chromatography–tandem mass spectrometry methods on AB Sciex (Framingham, MA) API 2000, 3000, or 4000 mass spectrometers with Shimadzu (Kyoto, Japan) high-performance liquid chromatography pumps and autosampler systems according to methods described previously (Paris et al., 2009; Parkinson et al., 2011). Peak areas for all metabolites were integrated with reference to a standard calibration curve based on back-calculation of a weighted (1/x), linear, least-squares regression.

**Data Processing.** IC50 data were processed with one of two validated software packages, Galileo-Laboratory Information Management System (Galileo version 3.3; Thermo Fisher Scientific Inc., Grand Island, NY) or XLfit (version 3.0.5; ID Business Solutions Ltd., Guildford, Surrey, UK), which is

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\[ K_i = \frac{K_{IC50}}{2} \]

(binding of inhibitor, I, to the enzyme, E); \[ K_i = \frac{K_{IC50} \times K_m}{2} \]

(binding of the inhibitor, I, to the enzyme-substrate complex, ES).
used within a customized software program (DI IC50 LCMS Template version 2.0.3) for Microsoft Excel Office 2000 (version 9.0; Microsoft Inc., Redmond, WA). Both software programs use a Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963), also known as a damped least-squares algorithm, to fit a nonlinear regression (sigmoidal) curve to IC50 data based on the following equation:

\[
\text{Fit} = \frac{\text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{\text{Inhibitor}}{\text{IC}_{50}}\right)^{-\text{slope}}}}{2}
\]

where Min = zero (no inhibition) and Max = 100 (complete inhibition). In Excel, the terms Min and Max are called background and range, respectively. Both software programs have been validated for their ability to calculate IC50 values only when they lie within the actual range of inhibitor concentrations tested. In other words, none of the IC50 values reported here was extrapolated from data that fell above or below the highest or lowest concentration of inhibitor, respectively.

The data for \(K_i\) determinations were processed with one of two comparable methods. The first method used Microsoft Excel to calculate rates of metabolite formation, which were imported into GraFit (Erithacus Software Ltd., Horley, Surrey, UK) to perform nonlinear regression according to the Michaelis-Menten equations associated with each type of direct inhibition. The second method used a Galileo Laboratory Information Management System (Thermo Scientific, Waltham, MA) with Crystal Reports-SAP Business Objects (SAP, Newtown Square, PA). The data (i.e., reaction rates at all concentrations of inhibitor at all concentrations of P450 marker substrates) were fitted to the Michaelis-Menten equations for competitive, noncompetitive, uncompetitive, and mixed (competitive-noncompetitive) inhibition (see Table 1) by nonlinear regression analysis. The goodness of fit to each of the four inhibition equations was determined by \(\chi^2\) analysis (with lower values indicating better fit) or by comparison of Akaike information criterion values (with higher values indicating better fit), which provided an initial basis for identifying the mechanism of inhibition. Eadie-Hofstee plots (rate versus rate/[S]) were inspected visually. At times, the nonlinear regression lines did not correlate well with the data points depicted on the Eadie-Hofstee plot, and visual inspection of the kinetic plots was necessary to deduce the mechanism of inhibition. Both methods of data processing are validated to calculate \(K_i\) values only when they lie within the range of inhibitor concentrations tested.

**Statistical Analysis.** The accuracy of the prediction of observed \(K_i\) values from values of IC50/2 was assessed by determining the average fold error (AFE) according to eq. 4 (Obach et al., 1997). This method is based on absolute values of the logarithm of the ratio of predicted-to-observed values, meaning that all negative values are converted to positive values so that, for example, values 50% less and 100% more than the observed value both represent a 2-fold error. An AFE value of 1 represents a perfect prediction.

<table>
<thead>
<tr>
<th>P450 Enzyme</th>
<th>Substrate</th>
<th>Number</th>
<th>Mixed</th>
<th>Competitive</th>
<th>Noncompetitive</th>
<th>Uncompetitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>21 (6%)</td>
<td>4</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>6 (2%)</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Efavirenz</td>
<td>8 (2%)</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Bupropion</td>
<td>14 (4%)</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>Amodiaquine</td>
<td>28 (8%)</td>
<td>24</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Paclitaxel</td>
<td>33 (10%)</td>
<td>26</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Diclofenac</td>
<td>47 (14%)</td>
<td>40</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>S-mephenytoin</td>
<td>44 (13%)</td>
<td>35</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP2E6</td>
<td>Buturalol</td>
<td>1 (&lt;1%)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Dextromethorphan</td>
<td>41 (12%)</td>
<td>22</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Chlorozaalone</td>
<td>2 (1%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam</td>
<td>57 (17%)</td>
<td>29</td>
<td>25</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Testosterone</td>
<td>31 (9%)</td>
<td>11</td>
<td>19</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Nifedipine</td>
<td>10 (3%)</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>343</td>
<td>217 (63.3%)</td>
<td>108 (31.5%)</td>
<td>13 (3.8%)</td>
<td>5 (1.5%)</td>
</tr>
</tbody>
</table>

**Fig. 1.** Distribution of P450 enzymes and substrates evaluated in the determination of 343 \(K_i\) values for reversible P450 inhibition in human liver microsomes with 132 investigational drugs.
Correlation of Estimated $K_i$ and Experimental $K_i$ Values

$$r = 0.940$$
$$\text{AFE} = 1.10$$
$$\text{RMSE} = 64.2$$
$$\text{NRMSE} = 3.4\%$$

The precision of the prediction was assessed by calculating the root mean square error (RMSE) according to eq. 5 (Sheiner and Beal, 1981):

$$\text{RMSE} = \sqrt{\frac{1}{n} \sum (\text{predicted } K_i - \text{observed } K_i)^2}$$

The normalized RMSE (NRMSE; expressed as a percentage) was calculated as follows:

$$\text{NRMSE} = \frac{\text{RMSE}}{\text{Max observed } K_i - \text{Min observed } K_i} \times 100$$

In all cases, the observed $K_i$ is the experimentally determined value, and the predicted $K_i$ corresponds to IC$_{50}$/2.

Results

The nine P450 enzymes and 14 marker substrates examined in the current study, and the distribution of the types of reversible inhibition (i.e., competitive, noncompetitive, mixed, and uncompetitive inhibition) observed in vitro with human liver microsomes are summarized in Fig. 1 and Table 2. The inhibitors were 132 structurally diverse investigational drugs, some of which were examined as reversible inhibitors of more than one P450 enzyme (data not shown). The correlations between the 343 observed (experimentally determined) $K_i$ values and those predicted from the corresponding values of IC$_{50}$/2 (when [S] $\approx K_m$) are shown in Fig. 2 and Table 3. The correlations between observed and predicted $K_i$ values as a function of the mechanism of P450 inhibition are shown in Fig. 3. The ratios of the observed-to-predicted $K_i$ values as a function of the mechanism of P450 inhibition are shown in Fig. 4 and Table 3. Measures of the accuracy (AFE) and precision (RMSE and NRMSE) of predicting $K_i$ values from IC$_{50}$/2 when [S] $\approx K_m$ are shown in Figs. 2 and 3, and summarized in Table 3.

Discussion

A total of 343 $K_i$ values for reversible inhibition of various P450 enzymes was determined experimentally (from studies with multiple inhibitor concentrations versus multiple substrate concentrations) with 132 structurally diverse investigational drugs (some of which were examined with more than one P450 enzyme) after determining IC$_{50}$ values in human liver microsomes under conditions where [S] $\approx K_m$. Table 2 shows the distribution of the four mechanisms of reversible inhibition for the 343 $K_i$ determinations. The compounds were predominantly (~95%) mixed ($n = 217$) or competitive ($n = 108$) P450 inhibitors; however, noncompetitive inhibitors ($n = 13$) and

<table>
<thead>
<tr>
<th>Inhibition Type</th>
<th>$n$</th>
<th>Mean</th>
<th>Range</th>
<th>$n &lt; 0.5$</th>
<th>$n &gt; 2.0$</th>
<th>Correlation coefficient (r)</th>
<th>AFE $^a$</th>
<th>RMSE $^b$</th>
<th>NRMSE $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed</td>
<td>217</td>
<td>1.13</td>
<td>0.481–2.95</td>
<td>1</td>
<td>9</td>
<td>0.926</td>
<td>1.07</td>
<td>74.2</td>
<td>3.90</td>
</tr>
<tr>
<td>Competitive</td>
<td>108</td>
<td>1.19</td>
<td>0.335–3.41</td>
<td>1</td>
<td>9</td>
<td>0.985</td>
<td>1.10</td>
<td>35.8</td>
<td>2.57</td>
</tr>
<tr>
<td>Noncompetitive</td>
<td>13</td>
<td>1.90</td>
<td>1.26–2.79</td>
<td>0</td>
<td>6</td>
<td>0.994</td>
<td>1.85</td>
<td>79.2</td>
<td>17.2</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>5</td>
<td>1.35</td>
<td>0.725–2.55</td>
<td>0</td>
<td>1</td>
<td>0.951</td>
<td>1.24</td>
<td>3.85</td>
<td>33.5</td>
</tr>
<tr>
<td>Mixed, competitive, and uncompetitive</td>
<td>330</td>
<td>1.15</td>
<td>0.335–3.41</td>
<td>2</td>
<td>19</td>
<td>0.941</td>
<td>1.08</td>
<td>63.5</td>
<td>3.34</td>
</tr>
<tr>
<td>Total</td>
<td>343</td>
<td>1.18</td>
<td>0.335–3.41</td>
<td>2</td>
<td>25</td>
<td>0.940</td>
<td>1.10</td>
<td>64.2</td>
<td>3.38</td>
</tr>
</tbody>
</table>

$^a$See eq. 4.

$^b$See eq. 5.

$^c$See eq. 6.
uncompetitive inhibitors \((n = 5)\) were also represented in the data set. The distribution of P450 enzymes analyzed as well as the marker substrates used are shown in Fig. 1 and Table 2. For the seven drug-metabolizing P450 enzymes listed in the FDA and EMA guidance documents on drug interactions, the number of \(K_i\) determinations ranged from 21 (CYP1A2) to 94 (CYP3A4/5). Relatively few \(K_i\) values were determined with CYP2A6 \((n = 6)\) and CYP2E1 \((n = 2)\). Four P450 enzymes (CYP2B6, 2C8, 2D6, and 3A4/5) were assayed with more than one substrate.

The correlation between the observed (experimentally determined) \(K_i\) values and the predicted \(K_i\) values (based on \(IC_{50}/2\)) for the entire data set is shown in Fig. 2. The experimentally determined \(K_i\) values ranged 200,000-fold from 9 nM to 1.9 mM. Overall, predicted values of \(K_i\) (based on \(IC_{50}/2\)) correlated well \((r = 0.940)\) with observed values of \(K_i\), and the prediction was both accurate \((AFE = 1.10)\) and precise \((RMSE = 64.2; NRMSE = 3.38\%\)).

When the data were segregated by inhibition type, the observed and predicted \(K_i\) values were highly correlated for all four types of inhibition \((r\) values ranged from 0.926 to 0.994), as shown in Table 3 and Fig. 3. Values of \(IC_{50}/2\) served as accurate predictors of observed \(K_i\) values for competitive inhibition \((AFE = 1.10)\) and uncompetitive inhibition \((AFE = 1.24)\). This was as expected because, in theory, \(K_i\) should equal \(IC_{50}/2\) for these types of inhibition (Table 1). Values of \(IC_{50}/2\) also accurately predicted \(K_i\) values for mixed inhibition \((AFE = 1.07)\), where, in theory, \(K_i\) values can range from \(IC_{50}\) to \(IC_{50}/2\) (Table 1). However, \(IC_{50}/2\) was a less accurate predictor of \(K_i\) values for noncompetitive inhibition. In this case, the AFE value of 1.85 indicates that the prediction was off by a factor of nearly 2, which is consistent with the equation in Table 1, indicating that, for noncompetitive inhibition, \(K_i = IC_{50}\) not \(IC_{50}/2\). Omitting the 13 \(K_i\) values for noncompetitive inhibition (where \(IC_{50}/2\) was a less accurate predictor of \(K_i\) values for noncompetitive inhibition. In this case, the AFE value of 1.85 indicates that the prediction was off by a factor of nearly 2, which is consistent with the equation in Table 1, indicating that, for noncompetitive inhibition, \(K_i = IC_{50}\) not \(IC_{50}/2\). Omitting the 13 \(K_i\) values for noncompetitive inhibition (where theoretically \(K_i = IC_{50}\) and not \(IC_{50}/2\) resulted in a negligible improvement in overall AFE; the value decreased from 1.10 to 1.08, and NRMSE decreased slightly from 3.38 to 3.34\%, as shown in Table 3.

The ratios of observed-to-predicted \(K_i\) values are shown in Fig. 4 and summarized in Table 3. Of the 343 predicted \(K_i\) values, 316 (92\%) were within a factor of 2 of the experimentally determined \(K_i\) value. Two predicted values were less than 0.5 (but not less than 0.33) and 25 values were greater than 2, only one of which was greater than 3. In other words, of the 343 predicted \(K_i\) values, 316 were within a factor of 2 and 342 were within a factor of 3, meaning only one value fell outside a 3-fold range (the actual value was 3.41). A preliminary account of this work included a second case in which the predicted \(K_i\) value was more than 3-fold greater than the experimentally determined \(K_i\) value (Haupt et al., 2011). However, in this case, the initial \(IC_{50}\) value was below the
that, in these cases, the decision to perform a
inhibitory drug, to reduce the possibility of metabolism-dependent
binding (Nagar and Korzekwa, 2012) decreases the unbound concen-
tration of inhibitor, but this effect can be corrected by measuring or
calculating \( f_{\text{u,inc}} \) (Austin et al., 2002; Hallifax and Houston, 2006) and
expressing the inhibition constant as \( K_{i,\text{unbound}} \), now recommended by
both the FDA and EMA. This same approach can be applied to
measurements of \( K_i \) based on IC\(_{50}/2\). It should be noted that, in
the current study, IC\(_{50}\) and \( K_i \) values for a given P450 enzyme were
determined under identical experimental conditions (i.e., the
same concentration of micromolar protein), such that values of \( f_{\text{u,inc}} \) were
the same for predicted and observed \( K_i \) determinations. Accordingly,
correcting for \( f_{\text{u,inc}} \) would have had no effect on estimates of the ratio
of the prediction-to-observed \( K_i \) values or the accuracy and precision of
predicting \( K_i \) values from IC\(_{50}/2\).

Although the experimentally determined \( K_i \) values varied approxi-
mately 200,000-fold, most of them (240 of 343) fell between 1 and
25 \( \mu \)M (with 61 greater than 25 \( \mu \)M and 42 less than 1 \( \mu \)M). In the majority of
cases (287 of 343 determinations), the initial IC\(_{50}\) value was 50 \( \mu \)M or
less (hence, the predicted \( K_i \) value was 25 \( \mu \)M or less), suggesting
that the decision to measure \( K_i \) values was biased toward those cases in
which the initial IC\(_{50}\) determination suggested relatively strong P450
inhibition. There were 37 cases in which the initial IC\(_{50}\) was greater than
100 \( \mu \)M (i.e., the inhibition was relatively weak). It might be expected
that, in these cases, the decision to perform a \( K_i \) determination was
biased toward CYP3A4/5 because of its intestinal location, where the
receptor inhibitor concentration is molar dose/250 ml, not plasma
C\(_{\text{max,ss}}\). However, of the 37 cases of weak inhibition, \( K_i \) values were
determined for a wide variety of P450 enzymes: 13 for CYP3A4/5, 6 for
CYP2C9, 6 for CYP2C19, 5 for CYP2D6, 3 for CYP2B6, 2 for
CYP1A2, 1 for CYP2A6, and 1 for CYP2C8. Therefore, CYP3A4/5
represented only 35% of the cases in which \( K_i \) was determined when the
initial IC\(_{50}\) was greater than 100 \( \mu \)M.

Interestingly, uncompetitive inhibition, characterized by a decrease
in both \( V_{\text{max}} \) and \( K_m \), was observed only when nifedipine was the
substrate. Of the 10 assays performed with nifedipine, 5 exhibited
uncompetitive inhibition. The CYP3A substrates used in the current
study, midazolam, testosterone, and nifedipine, are thought to bind to
three distinct regions within the substrate-binding site and exhibit
distinct enzyme kinetics characterized by hyperbolic (typical
Michaelis-Menten) kinetics in the case of midazolam, substrate
activation (sigmoidal kinetics due to homotropic cooperativity) in the
case of testosterone, and substrate inhibition in the case of nifedipine.
The high prevalence of uncompetitive inhibition observed with
nifedipine, which suggests some inhibitors bind to the CYP3A-
nifedipine (enzyme-substrate) complex, is consistent with previous
studies documenting the so-called stand-alone properties of nifedipine
as a CYP3A4/5 substrate (Kenworthy et al., 1999; Wang et al., 2000;
Galetin et al., 2002, 2003; Foti et al., 2010).

Overall, our analysis of 343 determinations supports theoretical
considerations (Table 1) that, when determined under in vitro
conditions of low protein concentration, short substrate incubation
time, and [S] \( \approx K_m \), values of IC\(_{50}/2\) provide an accurate prediction
(generally within a factor of 2) of experimentally determined \( K_i \) values
for all types of reversible, direct inhibition of P450 enzymes. In the
case of noncompetitive inhibitors, values of \( K_i \) estimated from IC\(_{50}/2\) were
off by a factor of \( \sim 2 \) (AFE = 1.85), which is consistent with theoretical
considerations (Table 1). However, noncompetitive inhibitors
accounted for a relatively low percentage (\( \sim 4\% \)) of the types of
inhibition observed (13 of 343 determinations). In conclusion, the
results of our analysis suggest that, under appropriate experimental
conditions, \( K_i \) values for direct, reversible inhibition can be reliably,
although somewhat conservatively, estimated from values of IC\(_{50}/2\).

The following excerpt is from the FDA’s Guidance for Industry on
drug interactions (from footnote 2 on page 1): “For a drug that is
a competitive inhibitor, \( R = 1+[(I/K_i) (K_i)] \) is the unbound inhibition
constant determined in vitro. Sometimes inhibitor concentration
causing 50% inhibition (IC\(_{50}\)) is determined, and \( K_i \) can be calculated
as IC\(_{50}/2\) by assuming competitive inhibition.” The results of our
analysis suggest that measuring IC\(_{50}\) is sufficient to estimate \( K_i \) for
the purpose of evaluating the potential of an investigational drug to
cause clinically relevant P450 inhibition, with two stipulations. First,
IC\(_{50}\) is determined under appropriate experimental conditions, as
described in the previous paragraph. Second, the \( K_i \) value estimated
from IC\(_{50}/2\) is corrected for the fraction of unbound drug in the test
system, such that \( K_{i,u} \) is estimated from (IC\(_{50}/2\)) \( f_{\text{u,inc}} \), where \( f_{\text{u,inc}} \) is
determined experimentally or calculated from log P (in the case of
nonionic and basic drugs) or log D\(_{7.4}\) (in the case of acidic and
zwitterionic drugs), as described by Austin et al. (2002) and
Hallifax and Houston (2006).

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References
specific micromolar binding on apparent intrinsic clearance, and its prediction from physico-
chemical properties. Drug Metab Dispos 30:1497–1503.
Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G,
and Ni L, et al.; Pharmaceutical Research and Manufacturers of America (PhRMA) Drug
Metabolism/Clinical Pharmacology Technical Working Group;; FDA Center for Drug Eval-
studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. Drug
Metab Dispos 31:815–832.
Brandi RB, Laux HJ, and Yates SW (1987) Calculation of inhibition constant \( K_i \) and inhibitor type from
the concentration of inhibitor for 50% inhibition for Michaelis-Menten enzymes. Biochem Med
Brown HS, Chadwick A, and Houston JB (2007a) Use of isolated hepatocyte preparations for
cytochrome P450 inhibition studies: comparison with microsomes for \( K_i \) determination. Drug
Metab Dispos 35:2119–2126.
as an alternative in vitro system to microsomes for the prediction of metabolic clearance. Drug
Metab Dispos 35:293–301.
converting IC\(_{50}\) to Ki values for inhibitors of enzyme activity and ligand binding. Nucleic
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (\( K_i \)) and the
concentration of inhibitor which causes 50 per cent inhibition (\( E_0 \)) of an enzymatic reaction.