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# 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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Running Title: Estimating $K_i$ from $IC_{50}$ values for P450 inhibition

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**ABBREVIATIONS:** AFE, average fold error; AUC, area under the plasma concentration-time curve; AUCR, plasma AUC ratio; $C_{max}$, maximum plasma concentration; $C_{max,ss}$, maximum plasma concentration at steady state; EMA, European Medicines Agency; FDA, US Food & Drug Administration; $fu_{inc,}$ fraction of unbound drug in the microsomal incubation; I or [I], concentration of inhibitor; $IC_{50}$, concentration of inhibitor causing 50% inhibition; $K_i$, inhibition constant for direct, reversible inhibition, $K_m$, Michaelis-Menten constant (the substrate concentration supporting half-maximum enzymatic rate); LC-MS/MS, liquid chromatography/tandem mass spectrometry; NMRSE, normalized root mean square error; RMSE, root mean square error; S or [S], substrate concentration; $V_{max}$, maximum rate of an enzymatic reaction.

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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 P450 inhibition could be reliably predicted by dividing the corresponding $IC_{50}$ values by two, based on the relationship that, for competitive inhibition, $K_i = IC_{50}/2$ when $[S] = K_m$. 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 min) 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 threefold range. In the case of noncompetitive inhibitors, $K_i$ values predicted from $IC_{50}/2$ values were overestimated by a factor of nearly two (AFE = 1.85; n = 13), which is to be expected because, for noncompetitive inhibition, $K_i = IC_{50}$ (not $IC_{50}/2$). The results suggest that, under appropriate experimental conditions with the substrate concentration equal to $K_m$, values of $K_i$ for direct, reversible inhibition can be reliably estimated from values of $IC_{50}/2$.

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**Introduction:**

When an investigational drug is evaluated in vitro as a direct, reversible inhibitor of human liver P450 enzymes based on the FDA's 2012 draft *Guidance for Industry* on drug interactions (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM292362.pdf), the need for a clinical drug interaction study is based on Equation 1:

$$AUCR = R_1 = 1 + \frac{[I]}{K_{i,unbound}} \qquad (1)$$

where [I] is the maximum total (bound + unbound) plasma concentration of drug at steady state ($C_{max,ss}$) and $K_{i,unbound}$ 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 plasma 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., $f_m$ = 1.0) (Ito et al., 1998). 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 Equation 2.

$$AUCR = \frac{1}{\frac{1}{1 + \frac{[I]}{K_{i,unbound}}} \cdot fm \cdot f_{m.enzyme} + \left(1 - fm \cdot f_{m.enzyme}\right)} \qquad (2)$$

A clinical drug interaction study is recommended for CYP enzymes other than CYP3A when the ratio $R_1$ > 1.1. The same criteria apply to significant circulating metabolites, defined by the FDA as metabolites whose plasma AUC is ≥25% of parent AUC following dosing to steady state with the maximum clinical dose. The 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 $[I]_{unbound}/K_{i,unbound} \geq 0.02$, where $K_{i,unbound}$ is as defined above 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 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 Equation 1 is defined as $[I]_{gut}$ = molar dose/250 mL. Values of $[I]_{gut}$ can greatly exceed values of [I] (total $C_{max,ss}$); accordingly, the FDA's cutoff value for inhibition of intestinal P450 enzymes is 11 (based on $1+[I]_{gut}/K_{i,unbound}$). The corresponding EMA cutoff is 10 (based on $[I]_{gut}/K_{i,unbound}$).

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 ($IC_{50}$) with a selective P450 probe at a substrate concentration approximately equal to $K_m$. Determining the mechanism of reversible inhibition (competitive, noncompetitive, mixed or uncompetitive) and measuring the value of $K_i$ requires an in vitro evaluation of the effects of multiple inhibitor concentrations *versus* multiple substrate concentrations, ideally with the former spanning $K_i$ and the latter spanning $K_m$. Determining values of $IC_{50}$ and $K_i$ based on the unbound concentration of inhibitor in the test system (typically human liver microsomes) requires knowledge of $fu_{inc}$ (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).

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The relationship between $K_i$ and values of $IC_{50}$ determined when [S] = $K_m$ 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, $K_i$ = $IC_{50}$. In the case of competitive and uncompetitive inhibition, $K_i$ = $IC_{50}$/2. In the case of mixed inhibition, $K_i$ values range from $IC_{50}$ to $IC_{50}$/2. The FDA's 2012 *Guidance for Industry* on drug interactions acknowledges that $K_i$ values are often estimated from values of $IC_{50}$/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 $K_i$ determinations to investigate whether experimentally determined $K_i$ values can in fact be reliably estimated from values of $IC_{50}$/2 when $IC_{50}$ values are determined under conditions of [S] ≈ $K_m$.

## 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 US Pharmacopeia (Rockville, Maryland). 8-Hydroxyefavirenz, 8-hydroxyefavirenz-$d_4$ (internal standard) and 6α-hydroxypaclitaxel-$d_5$ (internal standard) were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). The following chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri): coumarin, paclitaxel, bufuralol, 1'-hydroxy-bufuralol, chlorzoxazone, 6-hydroxychlorzoxazone, 6-hydroxychlorzoxazone-$d_2$ (internal standard), nifedipine and oxidized nifedipine. 7-Hydroxycoumarin was purchased from Cerilliant (Round Rock, Texas). 6α-Hydroxypaclitaxel, 7-hydroxycoumarin-$d_5$ (internal standard) and dehydronifedipine-$d_6$ (internal standard) were purchased from SynFine

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Research (Richmond Hill, Ontario, Canada). 10-Deacetyltaxol (internal standard) was purchased from A.F. Hauser, Inc. Pharmaceutical (Valparaiso, Indiana).

**Investigational drugs**

A total of 132 investigational drugs and drug metabolites were examined during the course of CYP inhibition studies sponsored by numerous pharmaceutical companies in the USA, 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 CYP inhibition) required neither knowledge of chemical structures nor physicochemical properties.

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**Test System**

Pooled human liver microsomes (n = 16 or n = 200; mixed gender) were prepared from non-transplantable livers and characterized at XenoTech, LLC (Lenexa, KS) as described previously (Parkinson et al., 2004; Pearce et al., 1996).

**Incubation Conditions**

$K_i$ and $IC_{50}$ values were determined in accordance with recommendations in FDA and EMA guidance documents and consensus papers (Tucker et al., 2001; Bjornsson et al., 2004). All experiments were performed under the following conditions: (1) the concentration of P450 marker substrate was approximately equal to $K_m$ for $IC_{50}$ determinations and spanned $K_m$ for $K_i$ determinations (i.e., [S] ranged from 0.25 times $K_m$ to 10 times $K_m$, solubility permitting); (2) the substrate incubation time was 5 min 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), $MgCl_2$ (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 LC-MS/MS methods on AB Sciex API 2000, 3000 or 4000 mass spectrometers (AB Sciex) with Shimadzu HPLC 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 AB Sciex Analyst data system, and metabolites were quantified by reference to a standard calibration curve based on back calculation of a weighted (1/x), linear, least-squares regression.

**Data Processing**

$IC_{50}$ data were processed with one of two validated software packages, namely Galileo-LIMS (Galileo version 3.3, Thermo Fisher Scientific Inc.) or XLfit (version 3.0.5, ID Business Solutions Ltd., Guildford, Surrey, UK, which is utilized 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

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damped least squares algorithm, to fit a non-linear regression (sigmoidal) curve to $IC_{50}$ data based on the following equation:

$$Fit = Min + \frac{Max - Min}{1 + \left(\frac{[Inhibitor]}{IC_{50}}\right)^{slope}} \qquad (3)$$

where Min = zero (no inhibition) and Max = 100 (complete inhibition). In XLfit, the terms Min and Max are called background and range, respectively. Both software programs have been validated for their ability to calculate $IC_{50}$ values only when they lie within the actual range of inhibitor concentrations tested. In other words, none of the $IC_{50}$ 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 (LIMS; 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-square analysis (with lower values indicating better fit) or by comparison of Akaike Information Criterion (AIC) values (with higher values indicating better fit), which provided an initial basis for identifying the mechanism of inhibition. Eadie-Hofstee plots (rate vs. rate/[S]) were inspected visually. At times, the

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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 $IC_{50}/2$ was assessed by determining the average fold error (AFE) according to Equation 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.

$$AFE = 10^{\left|\frac{1}{n}\sum log\frac{Predicted\ K_i}{Observed\ K_i}\right|} \qquad (4)$$

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

$$RMSE = \sqrt{\frac{1}{n}\sum(predicted\ K_i - observed\ K_i)^2} \qquad (5)$$

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

$$NRMSE = \frac{RMSE}{Max\ observed\ K_i - Min\ observed\ K_i} \cdot 100 \qquad (6)$$

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

**Results**

The 9 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 CYP enzyme (data not shown). The correlation 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 uncompetitive inhibitors (n = 5) were also represented in the dataset. The distribution of P450 enzymes analyzed as well as the marker substrates used are shown in Fig. 1 and Table 2. For the 7 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

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(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) *Ki* values and the predicted $K_i$ values (from $IC_{50}/2$) 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 4 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 two, 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

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(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 threefold range (the actual value was 3.41). A preliminary account of this work included a second case where the predicted $K_i$ value was more than threefold greater than the experimentally determined $K_i$ value (Haupt et al., 2011). However, in this case, the initial $IC_{50}$ value was below the lowest concentration of inhibitor tested and an interpolated $IC_{50}$ value was used in the analysis. This observation underscores the importance of predicting $K_i$ values only when the $IC_{50}$ value falls within the range of inhibitor concentrations tested.

It is important to note that other experimental conditions can affect the measured values of $IC_{50}$ and the kinetic constants $K_m$ and $K_i$ (Austin et al., 2002; Brown et al., 2007a,b; Di Marco et al., 2003; Hallifax and Houston, 2006; Howgate et al. 2006; McLure et al., 2000; Margolis and Obach, 2003; Obach 1996, 1997 and 1999; Ogilvie et al., 2011; Parkinson et al., 2011). In this study, all $K_i$ and $IC_{50}$ values were determined at 0.1 mg microsomal protein per mL or less with a short substrate incubation time (5 min) to minimize metabolic loss of the inhibitory drug, to reduce the possibility of metabolism-dependent inhibition, and to maximize the concentration of unbound inhibitor. Membrane partitioning (commonly but erroneously called non-specific binding [Nagar and Korzekwa, 2012]) decreases the unbound concentration of inhibitor, but this effect can be corrected by measuring or calculating $fu_{inc}$ (Austin et al. 2002, Hallifax and Houston, 2006) and expressing the inhibition constant as $K_{i,unbound}$, 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 microsomal protein), such that values of $fu_{inc}$ were the same for

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predicted and observed $K_i$ determinations. Accordingly, correcting for $fu_{inc}$ 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 approximately 200,000 fold, most of them (240 of 343) fell between 1 and 25 µM (with 61 greater than 25 µM and 42 less than 1 µM). In the majority of cases (287 of 343 determinations), the initial $IC_{50}$ value was 50 µM or less (hence, the predicted $K_i$ value was 25 µM or less), suggesting that the decision to measure $K_i$ values was biased towards those cases where the initial $IC_{50}$ determination suggested relatively strong P450 inhibition. There were 37 cases where the initial $IC_{50}$ was greater than 100 µM (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 relevant inhibitor concentration is molar dose/250 mL, not plasma $C_{max,ss}$. 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 where $K_i$ was determined when the initial $IC_{50}$ was greater than 100 µM.

Interestingly, uncompetitive inhibition, characterized by a decrease in both $V_{max}$ 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, namely midazolam, testosterone and nifedipine, are thought to bind to 3 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

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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 support theoretical considerations (Table 1) that, when determined under in vitro conditions of low protein concentration, short substrate incubation time and [S] ≈ $K_m$, 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 ~2 (AFE = 1.85), which is consistent with theoretical considerations (Table 1). However, noncompetitive inhibitors accounted for a relatively low percentage (~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, albeit 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 21): *For a drug that is a reversible 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 Ki 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 CYP 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

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fraction of unbound drug in the test system, such that $K_{i,u}$ is estimated from $(IC_{50}/2)\cdot fu_{inc}$, where $fu_{inc}$ is determined experimentally or calculated from logP (in the case of nonionic and basic drugs) or $logD_{7.4}$ (in the case of acidic and zwitterionic drugs), as described by Austin et al. (2002) and Hallifax and Houston (2006).

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**Authorship Contributions**

Participated in research design:

F. Kazmi, B.W. Ogilvie, D. Buckley, B. Paris and A. Parkinson

Conducted experiments:

L. Haupt, B. Smith, and S. Leatherman

Performed data analysis:

L. Haupt, F. Kazmi, B.W. Ogilvie, D. Buckley, B. Paris, O. Parkinson, and A. Parkinson

Wrote or contributed to the writing of the manuscript:

L. Haupt, F. Kazmi, B.W. Ogilvie, D. Buckley, O. Parkinson, B. Paris, and A, Parkinson

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**Footnotes**

Parts of this work were previously presented at the following meeting: Haupt L, Kazmi F, Ogilvie B, Buckley D, Smith B, Leatherman S, and Parkinson A. Can $K_i$ values for direct inhibition of CYP enzymes be reliably estimated from IC50 values? *Seventeenth North American Regional ISSX Meeting;* 2011, Oct 16-20; Atlanta, GA.

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**Figure Legends**

**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.

**Fig. 2.** Comparison of 343 observed (experimentally determined) $K_i$ values with predicted $K_i$ values from $IC_{50}/2$ when [S] ≈ $K_m$ . The line shown is a line of identity.

**Fig. 3.** Comparison of observed (experimentally determined) $K_i$ values with $K_i$ values estimated from values of $IC_{50}/2$ (when [S] ≈ $K_m$) as a function of the mechanism of reversible P450 inhibition. The line shown is a line of identity.

**Fig. 4.** Ratio of observed (experimentally determined) $K_i$ values with $K_i$ values estimated from values of $IC_{50}/2$ (when [S] ≈ $K_m$) as a function of the mechanism of reversible P450 inhibition. The line shown is a line of identity.

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TABLE 1

Relationship between $K_i$ and $IC_{50}$ (when [S] = $K_m$) for different mechanisms of reversible enzyme inhibition (adapted from Cheng and Prusoff, 1973; Brandt et al., 1987; Cer et al., 2009)

| **Type of inhibition** | **Modified Michaelis-Menten equation**[1] | **Relationship between $K_i$ and $IC_{50}$ when [S] = $K_m$** |
|---|---|---|
| Competitive | $v = \frac{Vmax \cdot [S]}{K_m \left(1 + \frac{[I]}{K_{i_\alpha}}\right) + [S]}$ | $K_i = \frac{IC_{50}}{2}$ |
| Uncompetitive | $v = \frac{Vmax \cdot [S]}{K_m + [S] \cdot \left(1 + \frac{[I]}{K_{i_\beta}}\right)}$ | $K_i = \frac{IC_{50}}{2}$ |
| Mixed | $v = \frac{Vmax \cdot [S]}{K_m \cdot \left(1 + \frac{[I]}{K_{i_\alpha}}\right) + [S] \cdot \left(1 + \frac{[I]}{K_{i_\beta}}\right)}$ | $K_i = IC_{50}\ to\ \frac{IC_{50}}{2}$ depending on the ratio of $K_{i_\alpha}\ to\ K_{i_\beta}$ |
| Noncompetitive | Same equation as above but this is a special case of mixed inhibition where $K_{i_\alpha} = K_{i_\beta}$ and $K_m$ remains unchanged. | $K_i = IC_{50}$ |

[1] $K_{i_\alpha} = \frac{[E][I]}{[EI]}$ (binding of inhibitor, *I*, to the enzyme, *E*)

$K_{i_\beta} = \frac{[ES][I]}{[ESI]}$ (binding of the inhibitor, *I*, to the enzyme-substrate complex, *ES*).

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TABLE 2

Distribution of the types of reversible inhibition observed for 343 $K_i$ determinations for nine different P450 enzymes in pooled human liver microsomes

| P450 enzyme | Substrate | Number | Mechanism of inhibition | | | |
|---|---|---|---|---|---|---|
| | | | Mixed | Competitive | Non-competitive | Un-competitive |
| CYP1A2 | Phenacetin | 21 (6%) | 4 | 17 | 0 | 0 |
| CYP2A6 | Coumarin | 6 (2%) | 4 | 1 | 1 | 0 |
| CYP2B6 | Efavirenz | 8 (2%) | 4 | 2 | 2 | 0 |
| | Bupropion | 14 (4%) | 13 | 1 | 0 | 0 |
| CYP2C8 | Amodiaquine | 28 (8%) | 24 | 4 | 0 | 0 |
| | Paclitaxel | 33 (10%) | 26 | 2 | 5 | 0 |
| CYP2C9 | Diclofenac | 47 (14%) | 40 | 7 | 0 | 0 |
| CYP2C19 | S-Mephenytoin | 44 (13%) | 35 | 9 | 0 | 0 |
| CYP2D6 | Bufuralol | 1 (<1%) | 0 | 1 | 0 | 0 |
| | Dextromethorphan | 41 (12%) | 22 | 19 | 0 | 0 |
| CYP2E1 | Chlorzoxazone | 2 (1%) | 2 | 0 | 0 | 0 |
| CYP3A4/5 | Midazolam | 57 (17%) | 29 | 25 | 3 | 0 |
| | Testosterone | 31 (9%) | 11 | 19 | 1 | 0 |
| | Nifedipine | 10 (3%) | 3 | 1 | 1 | 5 |
| **TOTAL** | | **343** | **217** (63.3%) | **108** (31.5%) | **13** (3.8%) | **5** (1.5%) |

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TABLE 3

Summary of the accuracy (AFE) and precision (RMSE and NRMSE) of predicting $K_i$ values for reversible cytochrome P450 inhibition from values of $IC_{50}/2$ when [S] ≈ $K_m$

| **Inhibition type** | **n** | **Ratio of observed versus predicted $K_i$** | | | | **Correlation coefficient** (r) | **AFE**[1] | **RMSE**[2] | **NRMSE**[3] (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | **Mean** | **Range** | **n < 0.5** | **n > 2.0** | | | | |
| Mixed | 217 | 1.13 | 0.481 - 2.95 | 1 | 9 | 0.926 | 1.07 | 74.2 | 3.90 |
| Competitive | 108 | 1.19 | 0.335 - 3.41 | 1 | 9 | 0.985 | 1.10 | 35.8 | 2.57 |
| Non-competitive | 13 | 1.90 | 1.26 - 2.79 | 0 | 6 | 0.994 | 1.85 | 79.2 | 17.2 |
| Uncompetitive | 5 | 1.35 | 0.725 - 2.55 | 0 | 1 | 0.951 | 1.24 | 3.85 | 33.5 |
| Mixed, competitive and uncompetitive | 330 | 1.15 | 0.335 – 3.41 | 2 | 19 | 0.941 | 1.08 | 63.5 | 3.34 |
| Total | 343 | 1.18 | 0.335 – 3.41 | 2 | 25 | 0.940 | 1.10 | 64.2 | 3.38 |

[1] AFE = Average fold error (see Equation 4)

[2] RMSE = Root mean square error (see Equation 5)

[3] NRMSE = Normalized root mean square error (see Equation 6)

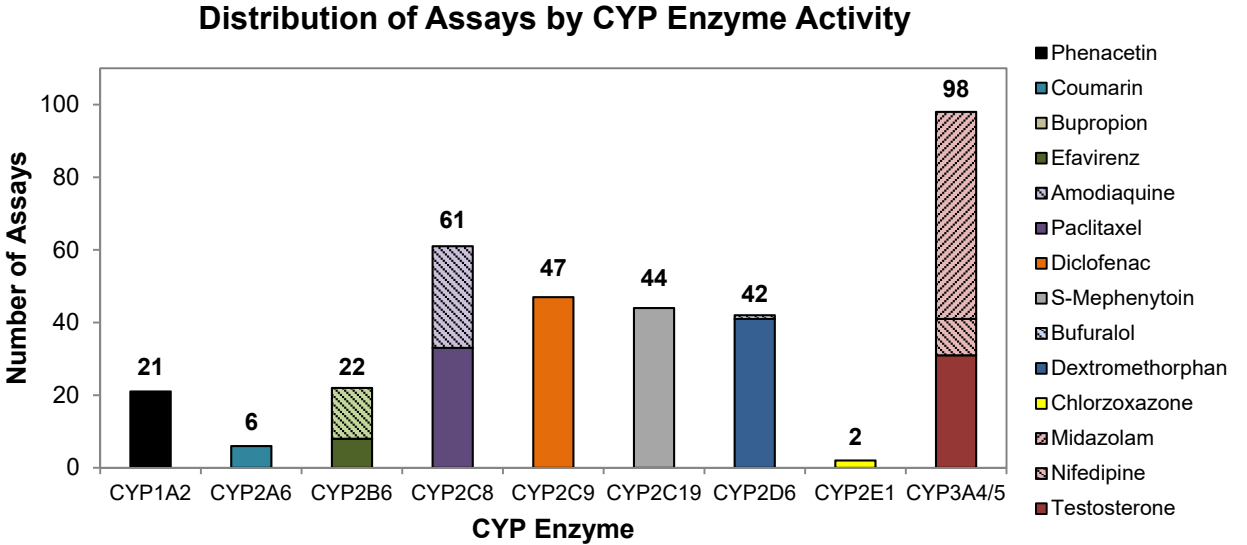
Distribution of Assays by CYP Enzyme Activity
Number of Assays
0
20
40
60
80
100
21
6
22
61
47
44
42
2
98
CYP1A2
CYP2A6
CYP2B6
CYP2C8
CYP2C9
CYP2C19
CYP2D6
CYP2E1
CYP3A4/5
CYP Enzyme
Phenacetin
Coumarin
Bupropion
Efavirenz
Amodiaquine
Paclitaxel
Diclofenac
S-Mephenytoin
Bufuralol
Dextromethorphan
Chlorzoxazone
Midazolam
Nifedipine
Testosterone

Figure 1

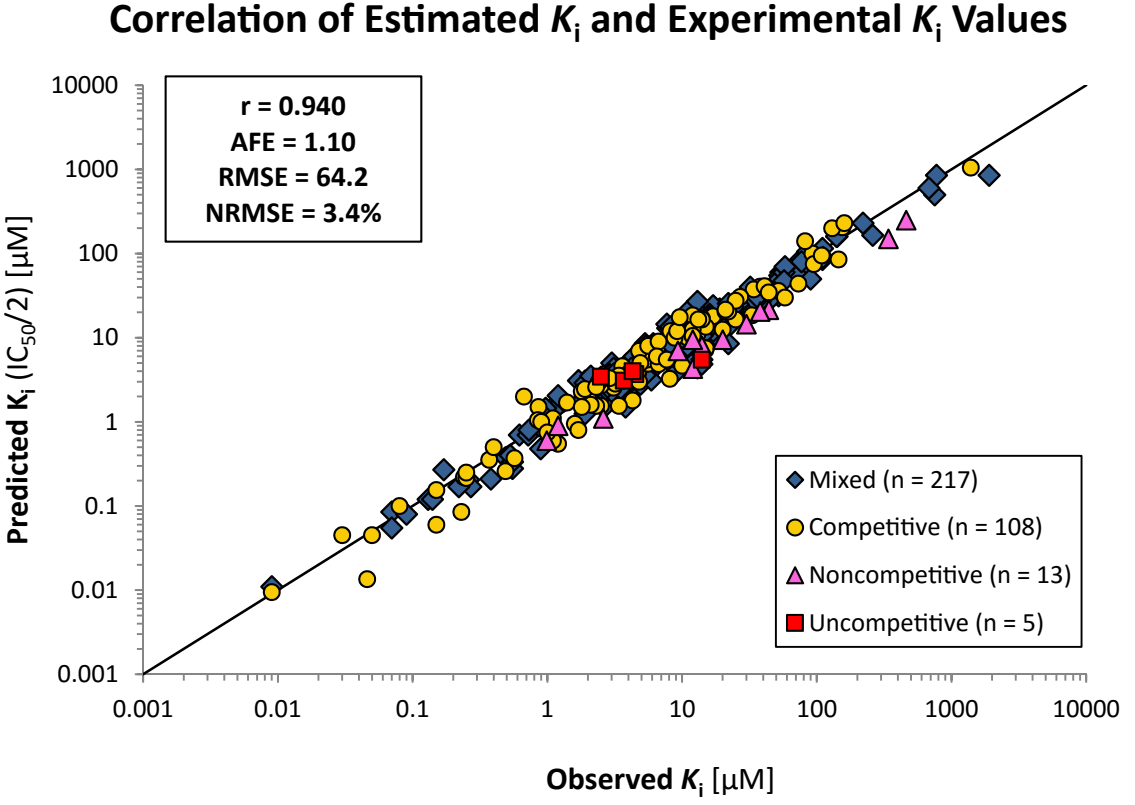
Correlation of Estimated $K_i$ and Experimental $K_i$ Values
r = 0.940
AFE = 1.10
RMSE = 64.2
NRMSE = 3.4%
Predicted $K_i$ ($IC_{50}$/2) [μM]
Observed $K_i$ [μM]
10000
1000
100
10
1
0.1
0.01
0.001
0.001
0.01
0.1
1
10
100
1000
10000
Mixed (n = 217)
Competitive (n = 108)
Noncompetitive (n = 13)
Uncompetitive (n = 5)

**Figure 2**

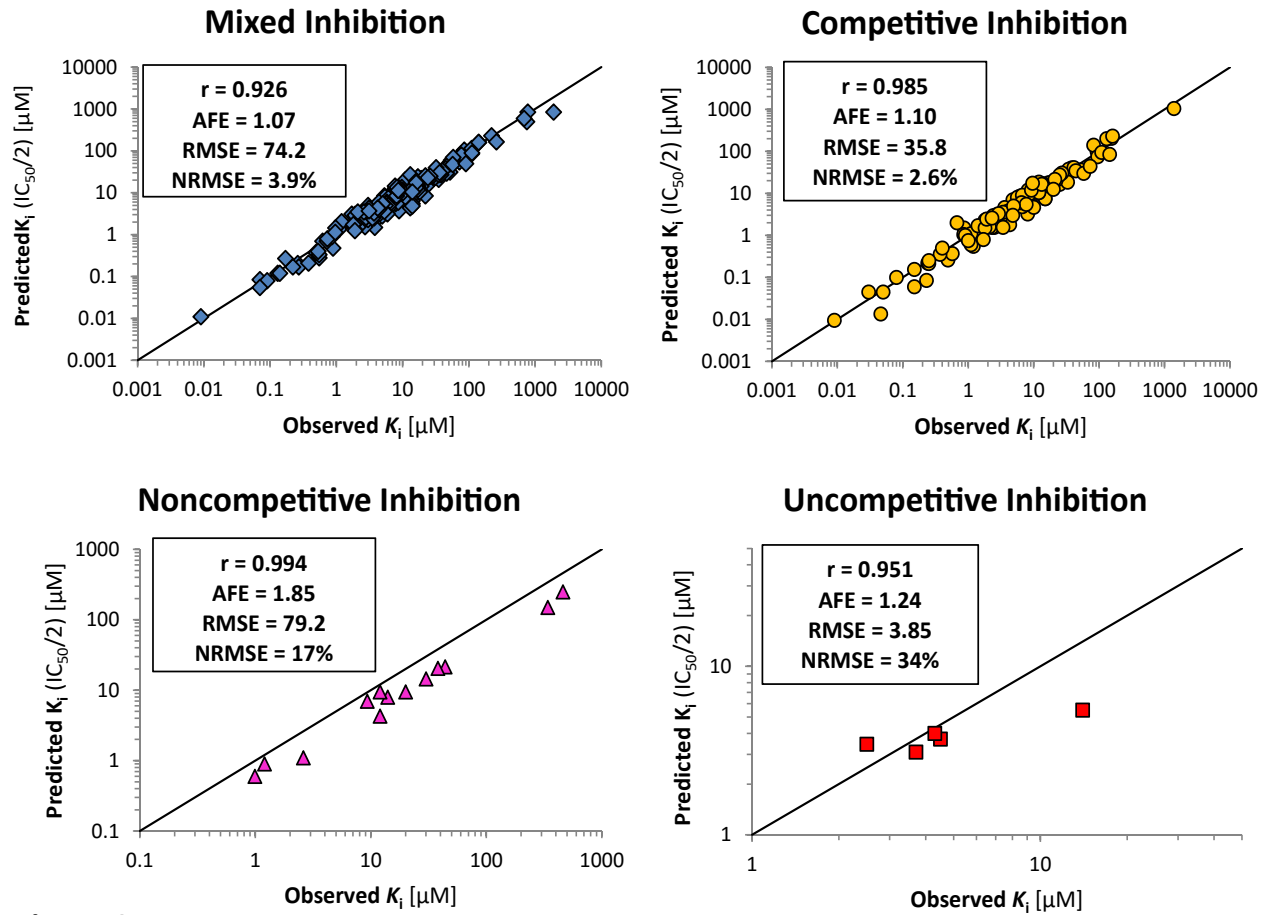
Mixed Inhibition
r = 0.926
AFE = 1.07
RMSE = 74.2
NRMSE = 3.9%
PredictedKi (IC50/2) [µM]
Observed Ki [µM]
Competitive Inhibition
r = 0.985
AFE = 1.10
RMSE = 35.8
NRMSE = 2.6%
Predicted Ki (IC50/2) [µM]
Observed Ki [µM]
Noncompetitive Inhibition
r = 0.994
AFE = 1.85
RMSE = 79.2
NRMSE = 17%
Predicted Ki (IC50/2) [µM]
Observed Ki [µM]
Uncompetitive Inhibition
r = 0.951
AFE = 1.24
RMSE = 3.85
NRMSE = 34%
Predicted Ki (IC50/2) [µM]
Observed Ki [µM]

**Figure 3**

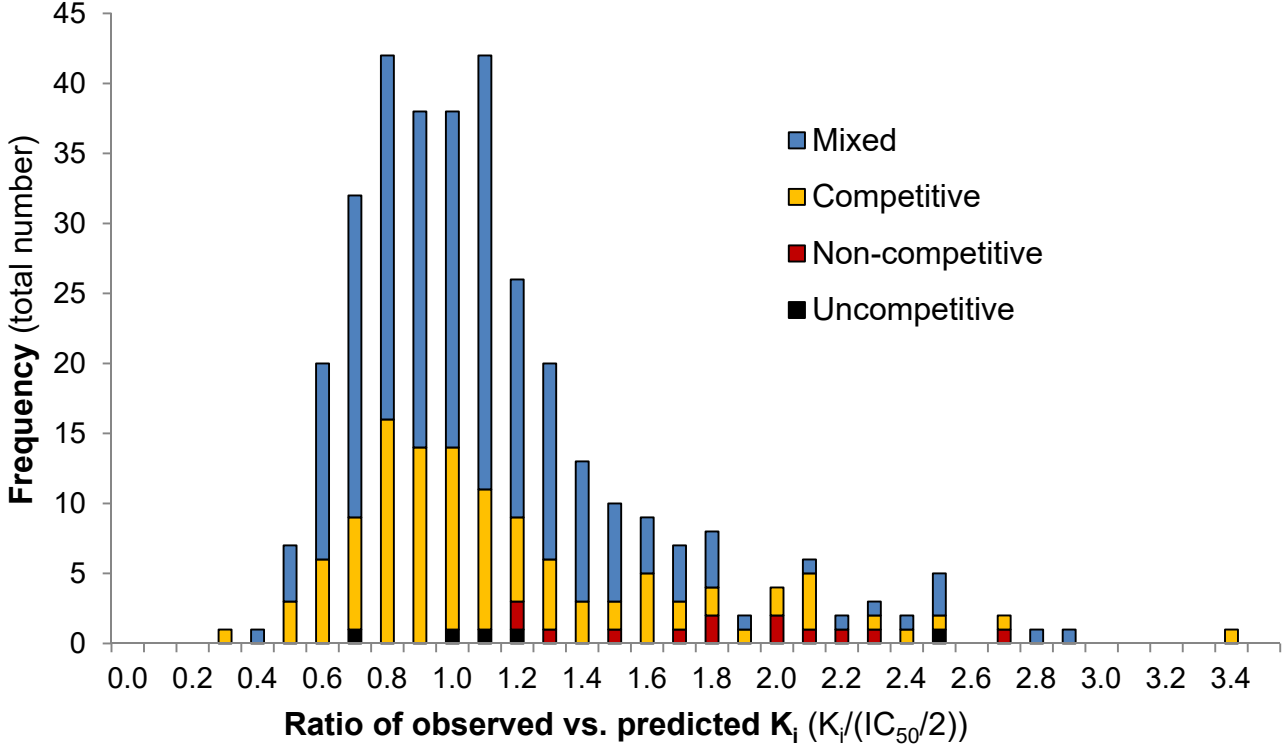
Frequency (total number)
45
40
35
30
25
20
15
10
5
0
Mixed
Competitive
Non-competitive
Uncompetitive
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
Ratio of observed vs. predicted $K_i$ ($K_i$/($IC_{50}$/2))

Figure 4