

Intersystem Extrapolation Factors Are Substrate-Dependent for CYP3A4: Impact on Cytochrome P450 Reaction Phenotyping[§]

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ABSTRACT

The use of intersystem extrapolation factors (ISEF) is required for the quantitative scaling of drug metabolism data generated in individually expressed cytochrome P450 (CYP) enzymes when estimating fractional contribution (f_m) to metabolism by P450 enzymes in vivo. For successful prediction of f_m , ISEF values must be universal across all substrates for any individual enzyme. In this study, ISEF values were generated for ten CYP3A4 selective substrates using a common source of recombinant heterologously expressed CYP3A4 (rCYP) and a pool of human liver microsomes. The resulting ISEF values for CYP3A4 were substrate-dependent and ranged 8-fold, with the highest value generated from intrinsic clearance of midazolam depletion (0.36) and the lowest from quinidine depletion (0.044). Application of these ISEF values for estimation of the fractional contribution of CYP3A4 and CYP2C19 to omeprazole clearance yielded values that ranged from 0.21–0.63 and 0.37–0.79, respectively, as compared with back-extrapolated in vivo f_m values of 0.27 (CYP3A4) and 0.85 (CYP2C19) from clinical pharmacokinetic

data. For risperidone, estimated f_m values for CYP3A4 and CYP2D6 ranged from 0.87–0.98 and 0.02–0.13, respectively, as compared with in vivo values of 0.36 (CYP3A4) and 0.63–0.88 (CYP2D6), showing that the importance of CYP3A4 was overestimated, and the importance of CYP2D6 underestimated. Overall, these findings suggest that ISEF values for CYP3A4 can vary with the marker substrate used to derive them, thereby reducing the effectiveness of the approach of using metabolism data from rCYP3A4 with ISEF values for the prediction of fraction metabolized values in vivo.

SIGNIFICANCE STATEMENT

Intersystem extrapolation factors are utilized for assigning fractional contributions of individual enzymes to drug clearance (f_m) from drug metabolism data generated in recombinant P450s. The present data shows that intersystem extrapolation factors values for cytochrome P4503A4 vary with the substrate. This can lead to variable and erroneous prediction of f_m .

Introduction

Interindividual variability in drug pharmacokinetics can arise via a variety of mechanisms, among which are differences in activities of drug metabolizing enzymes. Such differences can be caused by environmental factors, such as drug-drug interactions (DDI) or effects of diet or dietary supplements on drug-metabolizing enzyme activities. They can also arise from inherent differences in expression of drug metabolizing enzymes among individuals based on genetic variation. An understanding of the mechanistic underpinnings of interindividual differences in the pharmacokinetics of any given drug first requires the identification of enzymes involved in the metabolism of the drug followed by quantifying the relative contribution of each enzyme.

Among the drug metabolizing enzyme families, cytochrome P450 (CYP) has the greatest involvement in drug clearance (Yu et al., 2014; Cerny, 2016; Guengerich, 2008). Hundreds of DDIs can be attributed to alterations of P450 activities, and pharmacogenetic differences in drug pharmacokinetics can be attributed to several of these enzymes. In vitro methods and reagents have been developed that enable the conduct of studies with the aim of assigning specific P450 enzymes in the

metabolism of a drug, also termed “reaction phenotyping.” (Rodrigues, 1999; Zhang et al., 2007; Lu et al., 2003; Zientek et al., 2015). These include human liver microsomes (HLMs) from individual donors that have been characterized for drug metabolism activities, compounds and antibodies which preferentially inhibit single P450 enzymes, and heterologously expressed individual P450 enzymes (rCYP) derived from recombinant DNA technology. Collectively, these reagents are used to develop knowledge of the P450 enzymes contributing to metabolism of a drug and the quantitative fraction of total metabolism each enzyme contributes, termed f_m (fraction metabolized).

The application of rCYP enzymes for quantitative reaction phenotyping has increased over time because of the ability to study isozyme activity in isolation from other contributing enzymes and an ability to employ higher enzyme concentrations affording enhanced sensitivity for detecting metabolism of low clearance compounds. When using rCYPs to quantitate f_m for distinct P450 isoforms, the data must be appropriately scaled to represent the expression levels and activities of the respective enzymes in the liver. This led to the concepts of relative activity factors and relative abundance factors wherein rate data for a drug metabolism reaction in an incubation with rCYP would be corrected by factors to account for natural differences in these enzymes' activities and expression levels, respectively. These concepts were further evolved to the intersystem extrapolation factor (ISEF; Chen, et al., 2011) that essentially combines relative activity factors and relative abundance factors to account for individual P450 enzymes having differences in *specific activity* between heterologous expression systems

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ABBREVIATIONS: CYP, cytochrome P450; DDI, drug-drug interactions; f_m , fraction metabolized; $f_{u,mic}$, unbound fraction in microsomes; HLM, human liver microsome; ISEF, intersystem extrapolation factor; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide-reduced form; rCYP, heterologously expressed individual P450 enzymes.

and human liver microsomes. The requirement for these scaling factors is determined by factors, such as modulation of P450 activity by accessory proteins such as NADPH, P450 oxidoreductase and cytochrome b₅, that have different relative expression levels in heterologous systems versus liver microsomes, as well as demonstrated effects of one P450 enzyme on the activity of another when present together in a natural system (Reed and Backes, 2012).

However, a cornerstone of developing and using ISEFs is that the reactions used to define them are highly selective for the P450 enzyme of interest such that when evaluated in a mixture of enzymes (i.e., liver microsomes), the entire measured activity can be attributed to that one enzyme. Over the course of years, such reactions have been identified and carefully characterized. Additionally, it must also be assumed that the ISEF is constant for every substrate for a given P450 enzyme. However, this assumption may not always hold true: it has been shown that for CYP3A4 there are different substrate properties wherein the same inhibitor can have different potencies to different substrates, which has been attributed to multiple binding sites on the CYP3A4 enzyme (Kapelyukh, et al., 2008; Ekroos and Sjogren, 2006). Thus, ISEF values generated using one substrate may not be appropriate for another substrate, even when these are metabolized by the same P450 enzyme. This represents an important shortcoming for the application of ISEF, relative activity factor, or relative abundance factor approaches in estimating f_m values as it would not be known, a priori, which marker substrate activity is most relevant for a metabolic reaction for a newly investigated drug.

The objective of the experiments reported herein is to determine the variation in ISEFs calculated for ten CYP3A4 substrates. This includes the use of midazolam, the most commonly employed CYP3A4 substrate, as a comparator. The substrates span a range of chemical structures and may bind to CYP3A4 in different fashions. The implications of the findings for the approach of scaling rate data from expressed P450 enzymes to predict f_m values are discussed.

Materials and Methods

Materials

Substrates, metabolite standards, internal standard (IS), and inhibitors were obtained from the following sources: amlodipine, vardenafil, vincristine, and sildenafil from Sequoia Research Products, Pangbourne, UK; nisoldipine, triazolam, midazolam, eletriptan, felodipine, lidocaine, omeprazole, quinidine, risperidone, ketoconazole, CYP3cide, itraconazole, NADPH, and diclofenac (IS) from Sigma-Aldrich, St. Louis, MO; PF-05218881 and [²H₄]1'-hydroxymidazolam (IS) were prepared at Pfizer (Groton, CT). Pooled HLM (lot HLM-102) prepared from 50 donors of mixed sex were obtained from BD Biosciences (Woburn, MA). Recombinant heterologously expressed CYP3A4 (rCYP3A4; 87 pmol CYP per mg protein) containing rabbit P450 oxidoreductase was prepared under contract by Panvera Corp. (Madison, WI).

Intrinsic Clearance Measurement in Pooled Human Liver Microsomes and Recombinant CYP3A4. Ten CYP3A selective substrates (1.0 μM) were selected for incubation with rCYP3A4 and HLM-102 for ISEF determination based on substrate depletion. The rCYP and HLM protein concentrations selected for each incubation were based on the degree of substrate lability. The substrates amlodipine, eletriptan, quinidine, triazolam, and vincristine were incubated with rCYP3A4 at 100 pmol/ml and microsomal protein at 1 mg/ml; midazolam, sildenafil and vardenafil with rCYP3A4 at 10 pmol/ml and microsomal protein at 0.1 mg/ml; and felodipine and nisoldipine incubated with rCYP3A4 1 pmol/ml and microsomal protein at 0.01 mg/ml. All incubations were conducted in 100 mM KH₂PO₄ (pH 7.4) containing MgCl₂ (3.3–5.0 mM for rCYP3A4 and HLM, respectively) and NADPH (1.2 mM) in a total volume of 0.5 ml. Incubations were commenced with the addition of NADPH and carried out in a 96-well heat block at a temperature of 37°C. At times ranging between 0 and 60 minutes, aliquots (0.05 ml) were quenched with 0.2 ml of CH₃CN containing IS (100 ng/

ml PF-05218881). Terminated incubation mixtures were centrifuged at 2,300 × g for 5 minutes and supernatants (0.15 ml) dried under a warm nitrogen stream. Residues were reconstituted in 10/90 CH₃CN/H₂O containing 0.2% formic acid (0.3 ml). Samples were analyzed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system as described in Supplemental Tables 1 and 2. Experiments were conducted on at least two separate days each with replicates of *N* = 2. The duplicate intrinsic clearance determinations were measured within experiments and then averaged across different experimental days, followed by correction for free fraction in the incubation.

Substrate Saturation Experiments. Midazolam (0.1 – 30 μM) was incubated with HLM (0.01 mg/ml) or rCYP3A4 (1 pmol/ml) in 100 mM KH₂PO₄ (pH 7.4) containing MgCl₂ (3.3 mM), and NADPH (1.2 mM) in a total volume of 0.2 ml. Reactions were initiated with the addition of substrate and conducted in a 96-well heat block at a temperature of 37°C. After 4 minutes, aliquots (0.05 ml) were quenched with 0.2 ml of CH₃CN containing IS (20 ng/ml [²H₄]1'-hydroxymidazolam), followed by centrifugation at 1440 × g for 10 minutes. Supernatants were dried under a warm nitrogen stream, and residues were reconstituted in 75/25 CH₃CN/H₂O containing 0.1% formic acid (0.2 ml). Prepared samples were analyzed for 1'-hydroxymidazolam formation by LC-MS/MS as described in Supplemental Tables 1 and 2. Two separate experiments were conducted on separate days each with replicates of *N* = 3. ISEF values were calculated within each experimental day and then averaged across days for a final value.

Chemical Inhibition of CYP3A in HLM-102. Substrates (1.0 μM) were incubated with HLM-102 at protein concentrations described above. Incubations were carried out in 100 mM KH₂PO₄ (pH 7.4) containing MgCl₂ (3.3 mM), NADPH (1.2 mM), and either ketoconazole (1 μM) or CYP3cide (1 μM) in a total volume of 0.5 ml. Incubations were commenced with the addition of NADPH (for incubations with ketoconazole), or with the addition of substrate (for incubations with CYP3cide) and carried out in a 96-well heat block at a temperature of 37°C. At times ranging between 0 and 60 minutes, aliquots (0.05 ml) were quenched with 0.2 ml of CH₃CN containing IS (100 ng/ml PF-05218881). Terminated incubation mixtures were centrifuged at 2,300 × g for 10 minutes and supernatants (0.1 ml) were dried under a warm nitrogen stream. Residues were reconstituted in 10/90 CH₃CN/H₂O (0.2 ml) and analyzed by LC-MS/MS as described in Supplemental Tables 1 and 2. Additionally, standards of each of the analytes were co-injected with the inhibitors on the same LC-MS/MS system to rule out the potential for interference of analyte response by ion suppression.

Estimation of CYP3A4 fm for Risperidone and Omeprazole. Risperidone (1 μM) was incubated with HLM-102 (1 mg/ml), rCYP3A4 (100 pmol/ml), and rCYP2D6 (10 pmol/ml), and omeprazole (0.25 μM) was incubated with HLM-102 (1 mg/ml), rCYP3A4 (10 pmol/ml), and rCYP2C19 (10 pmol/ml). Incubations were carried out in 100 mM KH₂PO₄ (pH 7.4) containing MgCl₂ (3.3 or 5.5 mM) and NADPH (1.2 mM). Incubations were commenced with the addition of substrate and carried out in a 96-well heat block at a temperature of 37°C. At times ranging between 0 and 60 minutes, aliquots (0.04 ml) were quenched with 0.16 ml of CH₃CN containing IS (25 ng/ml diclofenac). Terminated incubation mixtures were centrifuged at 2,300 × g for 5 minutes and supernatants diluted with 0.1% formic acid in H₂O (risperidone, 1:1 v/v) or 250 mM ammonium acetate in H₂O (omeprazole, 1:1 v/v) to prevent chemical degradation. LC-MS/MS method details for each substrate are included in Supplemental Tables 1 and 2. A single experiment was conducted with replicates of *N* = 3.

Data Analysis. LC-MS/MS chromatograms were quantified using area ratios (analyte peak area/IS peak area) using AB Sciex Analyst software (versions 1.4–1.7). The natural logarithm of percent remaining data were plotted against time and slopes determined using a linear regression model in GraphPad Prism (versions 6–9). Data point selection to establish linearity was assessed in GraphPad using comparison of best fit model by sequential elimination of the latter time points until the preferred fitting was to a linear model. Data points that represented < 15% of substrate remaining were also generally excluded.

For ISEF determination by enzyme kinetic analysis, 1'-hydroxymidazolam concentrations were quantified against a standard curve with an applied weighting of 1/*x*² using AB Sciex Analyst software (version 1.7). The coefficient of determination, *r*², was greater than 0.99 for the calibration curves.

TABLE 1

Summary of DDI for nine of the drugs selected for this analysis. The estimates for fractional metabolism represent lower limits with an assumption that the perpetrator drug causes complete inhibition of cytochrome P4503A4 in vivo

Drug	Clinical DDI (Fold-Increase in Exposure)	DDI Perpetrator	In vivo f_m	Reference for DDI Study
Vardenafil	49.1	ritonavir	0.98	LEVITRA Package Insert
Midazolam	26.4	ritonavir	0.96	Greenblatt, et al., 2009
Nisoldipine	25.3	ketoconazole	0.96	Heinig, et al., 1999
Triazolam	22.3	ketoconazole	0.96	Varhe, et al., 1994
Sildenafil	9.9	ritonavir	0.90	Muirhead, et al., 2000
Felodipine	6.3	itraconazole	0.84	Jalava, et al., 1997
Eletriptan	5.9	ketoconazole	0.83	RELPAK Package Insert
Amlodipine	3.0	telapravir	0.67	Lee, et al., 2011
Quinidine	2.4	itraconazole	0.58	Kaukonen, et al., 1997

Unbound intrinsic clearance ($CL_{int,u}$) using substrate depletion (and binding values listed in Supplemental Table 3) was determined for the various substrates using the following equation:

$$CL_{int,u} = \frac{0.693}{t_{1/2}} \times \frac{mL \text{ incubation}}{mg \text{ microsomal protein or pmol CYP enzyme}} \times \frac{1}{f_{u,mic}} \quad (1)$$

Since $f_{u,mic}$ was not measured at the same protein concentrations used in the intrinsic clearance determination experiments, the following equation was used to adjust the $f_{u,mic}$:

$$f_{u,2} = \frac{1}{\frac{C_2}{C_1} \left(\frac{1-f_{u,1}}{f_{u,1}} \right) + 1} \quad (2)$$

where C_1 is the protein concentration used in measured value, $f_{u,1}$ is the measured $f_{u,mic}$ value, C_2 is the protein concentration used in calculated value, and $f_{u,2}$ is the calculated $f_{u,mic}$ value (Austin, et al., 2002). Measured $f_{u,1}$ values were either determined in-house or obtained from literature.

Drug-specific CL_{int} ISEFs from substrate depletion analysis were calculated for the various substrates using the following equation (Chen, et al., 2011):

$$CL_{int} \text{ ISEF} = \frac{CL_{int,u} \text{ (HLM)}}{CL_{int,u} \text{ (rCYP)} \times \text{CYP3A4 abundance (HLM)}} \quad (3)$$

in which the value used for CYP3A4 abundance in HLM is 137 pmol/mg. Kinetic constants for midazolam 1-hydroxylase activity were determined from substrate saturation experiments in HLM and rCYP3A4 based on curve fitting to either the Michaelis-Menten (Eq. 4) or substrate inhibition model (Eq. 5), respectively, using GraphPad 9 for Windows:

$$Y = \frac{V_{max} \times X}{K_M + X} \quad (4)$$

$$Y = \frac{V_{max} \times X}{(K_M + X + (1 + X/K_i))} \quad (5)$$

where X is substrate concentration, Y is enzyme activity, V_{max} is maximum enzyme activity, K_M is Michaelis-Menten constant for enzyme activity, and K_i represents substrate inhibition at high concentrations. Intrinsic clearance was calculated from the enzyme kinetic parameters as V_{max}/K_M and corrected for free fraction in microsomal incubations.

The ISEF derived from enzyme kinetics parameters of 1'-hydroxymidazolam metabolite formation was calculated using the following equation (Chen, et al., 2011):

$$\text{Kinetics ISEF} = \frac{V_{max}/K_M \text{ (HLM)}}{V_{max}/K_M \text{ (rhCYP)} \times \text{CYP3A4 abundance (HLM)}} \quad (6)$$

Contribution by CYP3A4 from chemical inhibition studies was calculated using the following equations:

$$\% \text{ CYP3A Contribution} = \frac{CL_{int,control} - CL_{int,INH}}{CL_{int,control}} \quad (7)$$

where $CL_{int,INH}$ is the intrinsic clearance in the presence of inhibitor (ketoconazole or CYP3side).

CYP3A4, CYP2C19, and CYP2D6 abundance values in HLM were obtained from SimCYP Population-based Simulator Version 19 (SimCYP LTD, Sheffield,

UK). Physiologic scaling factors of 45 mg of microsomal protein per gram of liver and 20 g of liver per kg of body weight, in addition to applying ISEF and CYP abundance scaling, were used to scale intrinsic clearance between $CL_{int,u}$ and $CL_{int,sc,u}$ (units of mL/min/kg) (Houston, 1994).

The f_m values for risperidone and omeprazole were calculated using the following equation:

$$f_m = \frac{CL_{int,sc,u} \text{ CYP}_x}{CL_{int,sc,u} \text{ CYP}_{total}} \quad (8)$$

where CYP_x represents the ISEF-scaled value from a single CYP isoform, and CYP_{total} represents the sum of scaled values from all CYP isoforms.

Results

Ten drugs were selected for this evaluation. These included well-established and highly used CYP3A substrates such as midazolam, triazolam, and felodipine. These are also well-established as CYP3A4 cleared drugs through demonstration of DDI with well-known potent CYP3A inhibitors such as ketoconazole, itraconazole, and ritonavir (Table 1).

Enzyme Kinetics of Midazolam 1'-Hydroxylation and Derivation of ISEF by Metabolite Formation. The enzyme kinetic parameters and intrinsic clearance values describing the formation of 1'-hydroxymidazolam in HLM and rCYP3A4 are presented in Table 2, and substrate saturation plots are depicted in Fig. 1. A Michaelis-Menten or substrate inhibition model was used to estimate the kinetic parameters for midazolam 1'-hydroxylase in HLMs from two separate experiments. Values for K_M were 1.62 and 1.37 μM , for V_{max} were 852 and 646 pmol/min/mg, and for $CL_{int,u}$ were 525 and 472 mL/min/mg (based on the midazolam $f_{u,mic}$ of 1). In rCYP3A4, a substrate inhibition model best described the kinetics with estimated parameters of 0.412 and 0.448 μM for K_M , 6.73 and 5.35 pmol/min/pmol for V_{max} , and 16.3 and 11.9 mL/min/pmol for $CL_{int,u}$ for the two respective experiments. Using a CYP3A4 abundance value of 137 pmol/mg microsomal protein from SimCYP (version 19), ISEF values of 0.23 and 0.29 were calculated for the two separate experiments with an arithmetic mean of 0.26.

Intrinsic Clearance of Ten CYP3A Substrates and Estimation of ISEF Values by Substrate Depletion. Substrate depletion rates were measured for ten CYP3A substrates (1 μM) in HLM and rCYP3A4, shown in Fig. 2 and listed in Table 3. Intrinsic clearance values in HLM ranged from 12.8 (vincristine) to 7093 (nisoldipine) $\mu\text{L}/\text{min}/\text{mg}$ of microsomal protein. The rates correlated between HLM and rCYP3A4 ($r^2 = 0.869$) albeit the rank order was not exact. ISEF values were calculated for each and ranged from 0.044 for quinidine to 0.36 for midazolam (at a substrate concentration 1 μM). There appeared to be three clusters of ISEF values: high values of 0.26 to 0.36 (midazolam, nisoldipine, vardenafil, and midazolam 1'-hydroxylase from above), moderate values of 0.14 to 0.19 (triazolam, sildenafil, felodipine, and vincristine), and low values of 0.044 to 0.077 (quinidine, eletriptan, and amlodipine). A second study using a

TABLE 2

Enzyme kinetic parameters for midazolam 1'-hydroxylation in pooled human liver microsomes and heterologously expressed cytochrome P4503A4
Values in parenthesis represent the 95% confidence interval of the data fitting.

	Reagent	K_M (μM)	V_{max} ($\text{pmol}/\text{min}/\text{x}$) ^a	$CL_{\text{int,u}}$ ($\mu\text{L}/\text{min}/\text{x}$) ^b	CYP abundance (pmol/mg microsomal protein)	ISEF
Experiment 1	HLM	1.62 (1.34 - 1.98) ^c	852 (787 - 930)	525	137	0.23
	rCYP3A4	0.412 (0.359 - 0.473) ^c	6.73 (6.46 - 7.03)	16.3		
Experiment 2	HLM	1.37 (1.02 - 1.84)	646 (597 - 699)	472	137	0.29
	rCYP3A4	0.448 (0.362 - 0.552) ^c	5.35 (5.02 - 5.71)	11.9		
Arithmetic Mean						0.26

^a V_{max} units are $\text{pmol}/\text{min}/\text{mg}$ for human liver microsome and $\text{pmol}/\text{min}/\text{pmol}$ P450 for heterologously expressed individual P4503A4.

^b CL_{int} units are $\mu\text{L}/\text{min}/\text{mg}$ for human liver microsome and $\mu\text{L}/\text{min}/\text{pmol}$ P450 for heterologously expressed individual P4503A4; midazolam $f_{\text{mic}} = 1$ for both human liver microsome and heterologously expressed individual P4503A4.

^cData fit to substrate inhibition model, resulting substrate inhibition (K_i) values were $> 100 \mu\text{M}$ (3 times the highest substrate concentration tested).

reduced substrate concentration ($0.1 \mu\text{M}$) was conducted to ensure sub-saturating incubation conditions (below K_M) for compounds known to have low K_M values in the dataset. Assessed in the follow up experiment, the recalculated ISEFs for midazolam, quinidine, and nisoldipine following the reduction in substrate concentration were 0.15, 0.044, and 0.14, respectively. A summary of the resulting range in ISEF values is depicted in Fig. 3.

Verification of the Role of CYP3A in Metabolism of the Test Substrates. When using the overall substrate consumption to estimate ISEF values, it is critical that CYP3A4 is exclusively involved in the metabolism in HLM. If other enzymes were to meaningfully contribute, the resulting ISEF values would be erroneous. To establish this, the intrinsic clearance of these substrates was evaluated in HLM in the

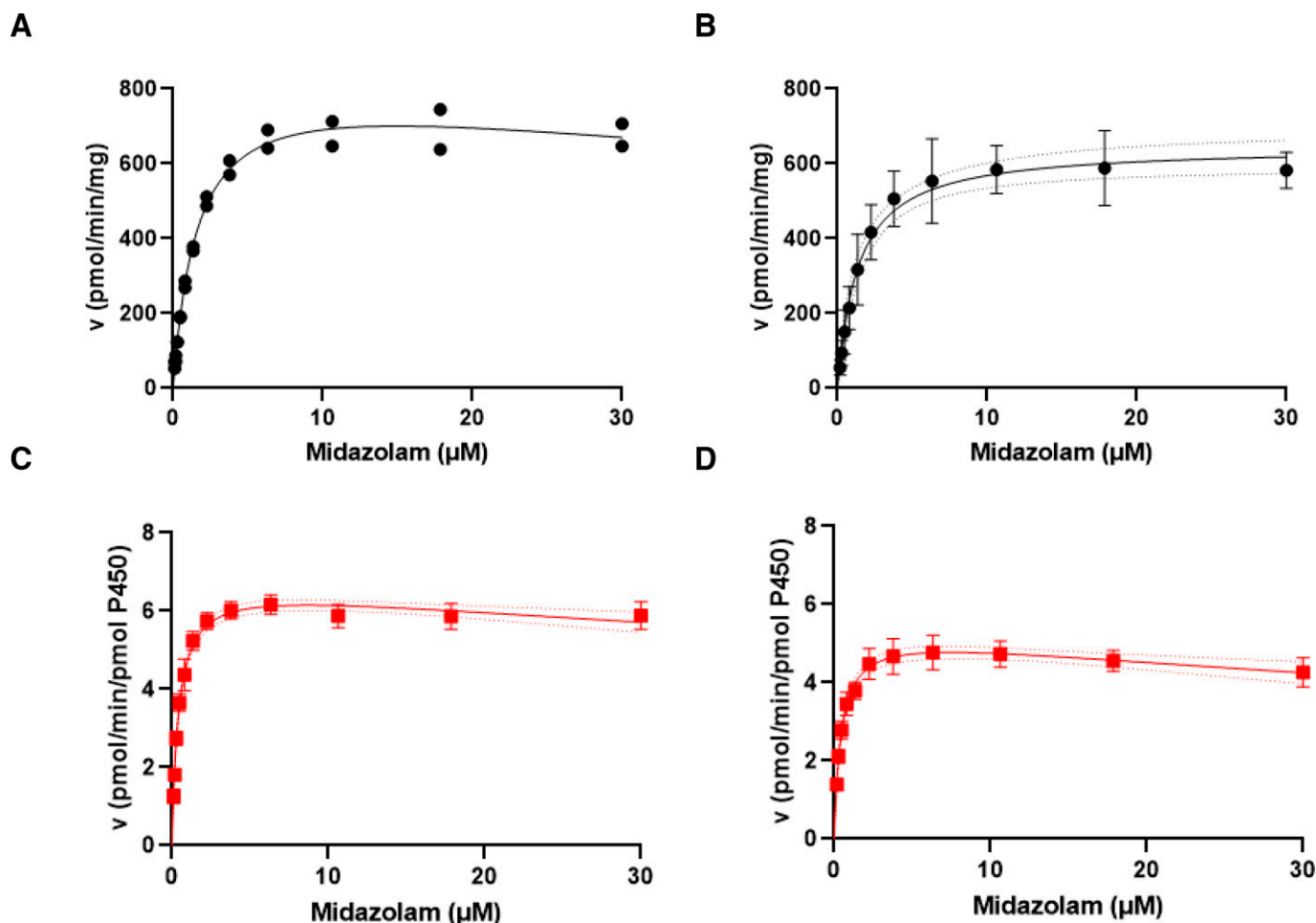


Fig. 1. Enzyme kinetics of midazolam 1'-hydroxylation in pooled human liver microsomes and recombinant heterologously expressed Cytochrome P4503A4. Panels A and B represent two separate experiments conducted in human liver microsomes and panels C and D represent two separate experiments conducted in heterologously expressed individual P4503A4. Error bars represent standard deviation and dotted lines represent 95% confidence intervals of the resulting curves.

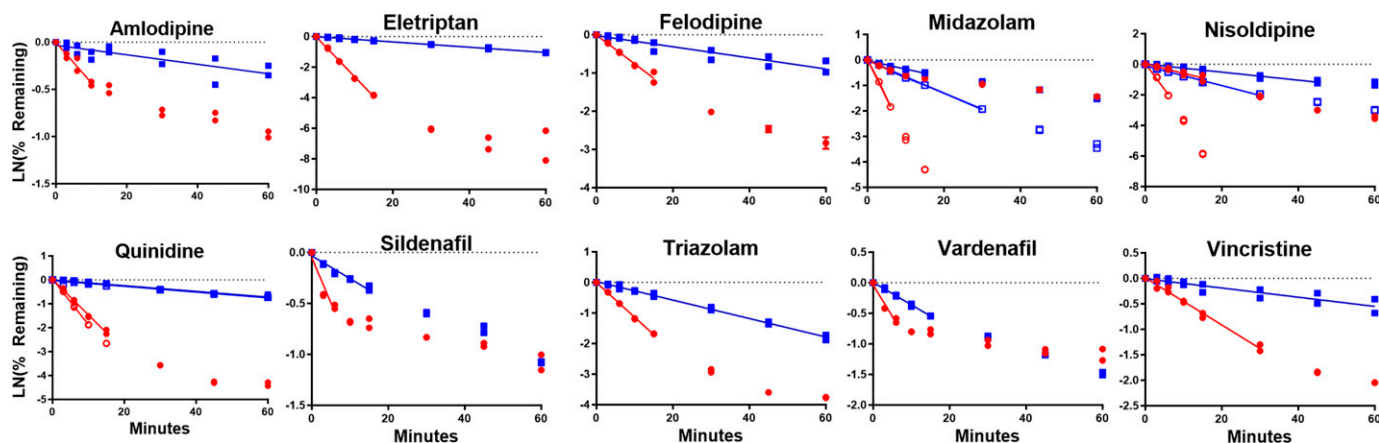


Fig. 2. Depletion of cytochrome P4503A4 substrates (1 μM) incubated at 0.01, 0.1, or 1 mg/ml of human liver microsomes (blue squares) or 1, 10, or 100 pmol/mL of heterologously expressed individual P4503A4 (red circles). Varying levels of protein were used to best capture first order kinetics of substrate decline. Compounds repeated at lower substrate (0.1 μM) are shown as open symbols.

presence and absence of ketoconazole (CYP3A4/5 inhibitor) and CYP3cide (CYP3A4 selective inactivator), shown in Fig. 4. For eight of ten compounds, ketoconazole caused marked inhibition such that depletion rates in the inhibited state could not be reliably measured. Felodipine consumption was inhibited 80% by ketoconazole, suggesting that the ISEF value of 0.17 derived from this drug should be lowered by 20%. Amlodipine depletion was too slow in the control experiment to estimate the extent of inhibition by ketoconazole. Instead, an evaluation was made of dehydroamlodipine formation in human hepatocytes pretreated with the CYP3A inactivator troleanomycin (25 μM) by monitoring the dehydroamlodipine peak in the high-performance liquid chromatography-UV trace. [Dehydroamlodipine is the only metabolite of amlodipine generated by CYP3A4 (Zhu, et al., 2014)]. The results indicated 97% inhibition of metabolite formation, supporting that amlodipine is almost entirely metabolized by CYP3A. As expected, based on the low CYP3A5 expression in the lot of HLM used, the resulting CYP3A4 inhibition by CYP3cide was similar, if not identical, to inhibition by ketoconazole. Overall, these findings support the use of these ten drugs for estimation of CYP3A4 ISEF values.

Case Examples: Estimation of CYP3A4 f_m for Risperidone and Omeprazole. ISEF values are used in P450 reaction phenotyping for scaling of data gathered in individually expressed P450 enzymes.

TABLE 3

Intrinsic clearance and calculated cytochrome P4503A4 intersystem extrapolation factor values for ten substrates
Units are $\mu\text{L}/\text{min}/\text{pmol}$ P450 for heterologously expressed individual P4503A4 and $\mu\text{L}/\text{min}/\text{mg}$ protein for human liver microsome.

Compound	$CL_{int,u}$		ISEF
	CYP3A4	HLM	
Midazolam (1.0 μM)	8.50	417	0.36
Nisoldipine (1.0 μM)	91.9	4129	0.32
Vardenafil	9.75	376	0.29
Midazolam (1'-hydroxymidazolam formation)	14.1	498	0.26
Triazolam	1.47	37.1	0.19
Sildenafil	9.89	239	0.18
Felodipine	97.9	2275	0.17
Midazolam (0.1 μM)	32.8	690	0.15
Nisoldipine (0.1 μM)	383	7093	0.14
Vincristine	0.659	12.8	0.14
Amlodipine	8.97	95.3	0.077
Quinidine (1.0 μM)	2.52	20.1	0.058
Elettriptan	4.29	29.2	0.050
Quinidine (0.1 μM)	3.30	19.8	0.044

When CYP3A4 is a contributor to the metabolism of a drug, but not the sole contributor, the accuracy of the ISEF value used can have an influence on the resulting f_m values for all enzymes involved. The exercise of applying ISEFs to estimating f_m values was applied to predictions for risperidone and omeprazole (Fig. 5). The drugs were selected for having CYP3A4/5 and one additional isoform as the two primary contributing enzymes. Risperidone is metabolized by CYP3A4 and CYP2D6 (Yasui-Furukori, et al., 2001), while omeprazole is metabolized by CYP3A4 and CYP2C19 (Yamazaki, et al., 1997; Abelo, et al., 2000). The estimated f_m values for risperidone were calculated using a range of ISEF values that were derived from the enzyme kinetics of midazolam 1'-hydroxylase activity (0.26), the high value determined from vardenafil intrinsic clearance (0.29), and the lowest value from quinidine intrinsic clearance (0.044; using a substrate concentration of 0.1 μM), while the ISEF values for CYP2D6 (derived from bufuralol 1'-hydroxylation) remained constant at 0.14 (Table 5). The resulting risperidone f_m values ranged from 0.87 to 0.98 for CYP3A4 and from 0.02 to 0.13 for CYP2D6, consistent with the sensitivity of this output to the values used for ISEF. Interestingly, CYP2D6 and CYP3A4 both generate the main risperidone metabolite, 9-hydroxyrisperidone, but of opposite stereochemistry (Yasui-Furukori, et al., 2001). Irrespective of the CYP3A4 ISEF used, the CYP3A4 contribution to risperidone clearance is consistently overestimated in comparison with the in vivo CYP2D6 PM

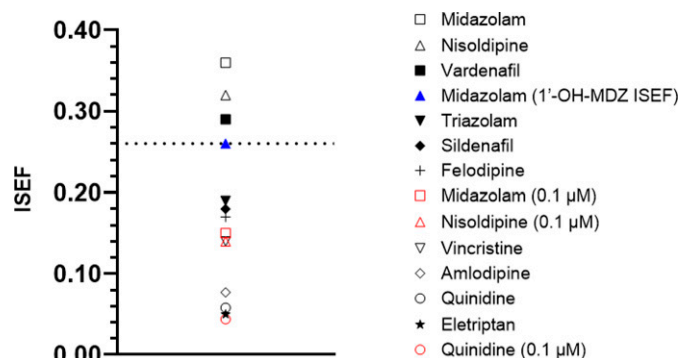


Fig. 3. The distribution of intersystem extrapolation factor values, calculated either by substrate depletion or 1'-hydroxymidazolam metabolite formation (blue triangle). Black symbols represent the compounds run at 1 μM and red symbols represent the three compounds repeated at 0.1 μM (quinidine, nisoldipine, and midazolam) to ensure substrate concentration was sufficiently below the Michaelis-Menten constant.

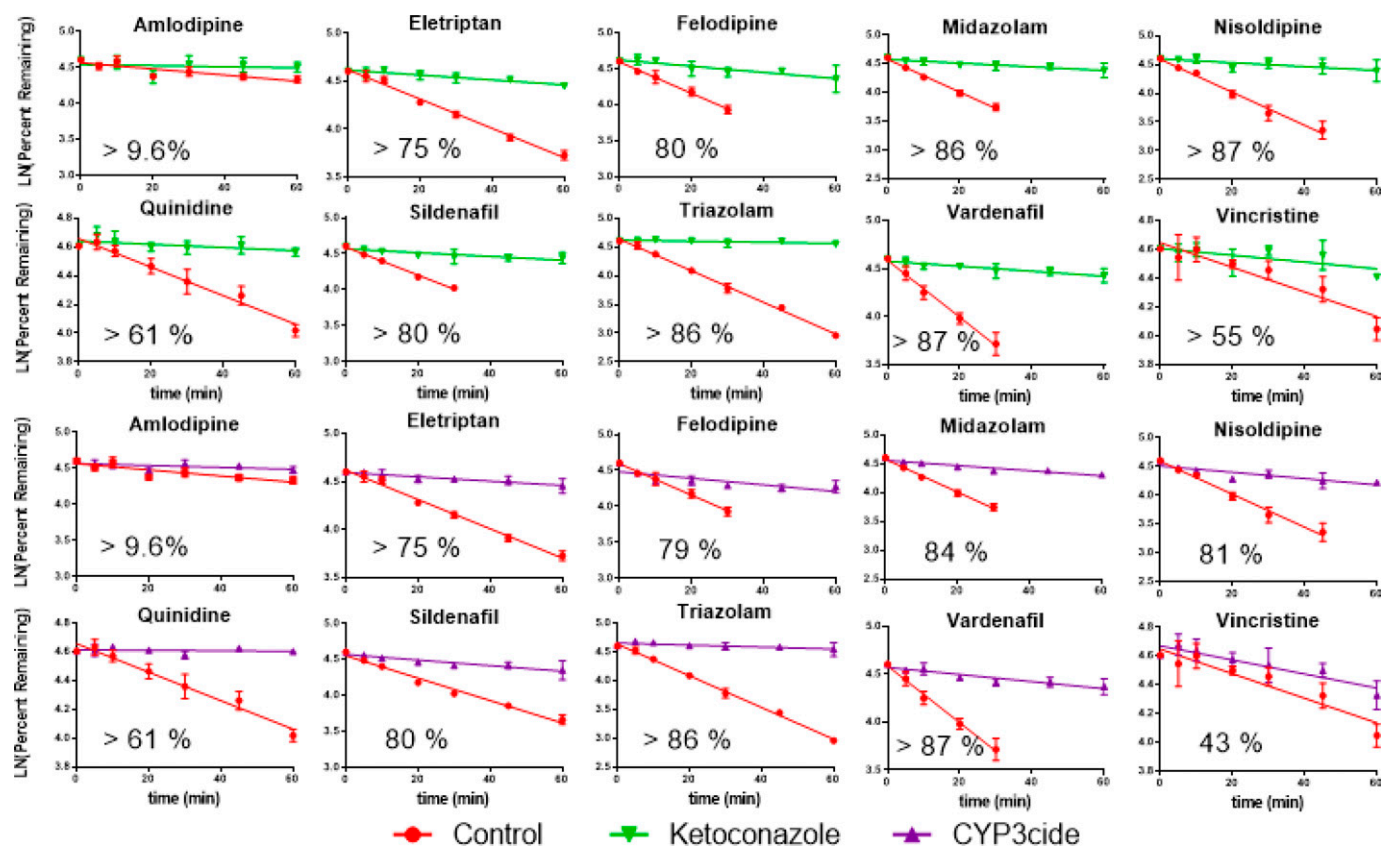


Fig. 4. Depletion of cytochrome P450 (CYP)3A4 substrates (1 μ M) incubated at 0.01, 0.1 or 1 mg/mL human liver microsomes in the presence of CYP3A4/5 competitive inhibitor ketoconazole (1 μ M) or CYP3A4 inactivator CYP3cide (1 μ M) compared with solvent control. Error bars represent standard deviation. Varying levels of protein were chosen to best capture first order kinetics of substrate decline. For amlodipine, the impact of CYP3A4 was difficult to discern due to a slow rate of substrate depletion. The fraction metabolized by CYP3A4 as measured by dehydroamlodipine formation was determined to be 0.97 in pooled human hepatocytes in the presence and absence of 25 μ M troleadomycin.

versus EM data and ketoconazole DDI data suggesting the clinically derived f_m values for these two enzymes is 0.36 for CYP3A4 and 0.63 to 0.88 for CYP2D6.

For omeprazole (Table 4), the same three ISEF values for CYP3A4 were applied in addition to the CYP2C19 ISEF of 0.50 derived from *S*-mephenytoin 4'-hydroxylase. The estimates of omeprazole f_m values ranged from 0.21 to 0.63 for CYP3A4 and from 0.37 to 0.79 for CYP2C19, reinforcing that f_m is sensitive to the values used for ISEF. Clinical estimates for CYP2C19 and CYP3A4 f_m values are 0.85 and 0.27, respectively, derived from CYP2C19 pharmacogenetic and ketoconazole DDI data. The CYP3A4 ISEF value derived from substrate depletion of quinidine (0.044) yielded the omeprazole CYP3A f_m that

best predicted the ketoconazole DDI results. Since f_m values are relative numbers, the impact or uncertainty of the ISEF for one P450 will impact the overall output, and hence conclusions, for involvement of other P450s to the metabolic clearance of a drug.

Discussion

Quantitative estimation of the relative contributions of individual drug metabolizing enzymes to the metabolic clearance of drugs *in vitro* (a.k.a. reaction phenotyping) is a standard activity in drug research. Data from such experiments are used to understand the potential for inter-patient variability in drug exposure due to phenomena, such as

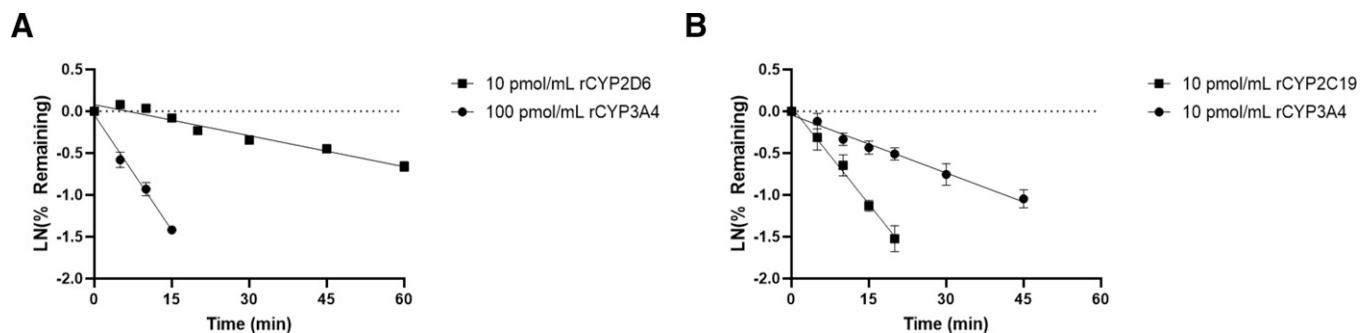


Fig. 5. Metabolism of risperidone and omeprazole by recombinant heterologously expressed cytochrome P450 enzymes. (A) Risperidone depletion in heterologously expressed individual P4503A4 (circles) and heterologously expressed individual P4502D6 (squares). (B) Omeprazole depletion in heterologously expressed individual P4503A4 (circles) and heterologously expressed individual P4502C19 (squares).

TABLE 4

Use of cytochrome P4503A4 intersystem extrapolation factors from three substrates in the estimation of fractional metabolism values for omeprazole

rCYP	Reaction	ISEF Parameters		CL _{int}		f _m	
		ISEF Value	CYP Abundance units of pmol/mg microsomal protein	rCYP CL _{int,u} (μL/min/pmol P450)	ISEF-derived HLM CL _{int,sc,u} (mL/min/kg) ^a	In Vitro f _m	In Vivo f _m
rCYP3A4	1'-Hydroxymidazolam Formation	0.26	137	2.35	75.4	0.60	0.27 ^b
	Vardenafil Depletion	0.29			84.1	0.63	
	Quinidine Depletion	0.044			12.8	0.21	
rCYP2C19	(S)-Mephenytoin 4'-Hydroxylation	0.50 ^c	14	7.84	49.4	0.37-0.79	0.85 ^d

^aBinding values were scaled using the equation described in Austin et al. (2002), from values generated via equilibrium dialysis. Measured unbound fraction in microsomes for omeprazole was 0.90 at 0.71 mg/ml, which scaled to f_{u,mic} of 0.98, 0.99, and 0.87 for heterologously expressed individual P4503A4, heterologously expressed individual P4502C19, and human liver microsome incubations, respectively.

^bCalculated from clinical data reported in Bottiger, et al., 1997.

^cCytochrome P450C19 intersystem extrapolation factor determined from mephenytoin 4'-hydroxylase activity (internal data on file, Pfizer, Inc.) This intersystem extrapolation factor has been used successfully for other CYP2C19 cleared drugs.

^dMedian fractional metabolism value calculated from fifty clinical studies in which omeprazole was dosed to cytochrome P450C19 extensive and poor metabolizers, obtained from the University of Washington Drug Interaction Database.

being victim to drug-drug interactions and pharmacogenetic differences, among others. One of the common approaches to conducting these investigations is the use of individual cytochrome P450 enzymes. Rates of metabolism of drugs are measured in individually expressed P450 enzymes, and these rates are converted to relative contributions using ISEF values. However, it has been our experience, and the experiences of others (Lindmark, et al., 2018; Wang, et al., 2019) that this approach can sometimes fail, or at least offer very different results as compared with the use of human liver microsomes or hepatocytes and P450-selective inhibitors.

In the present investigation, ISEF values for rCYP3A4 were derived from several substrates for purposes of comparing the impact of variability on f_m prediction. Midazolam metabolism, especially the 1'-hydroxylase activity, is a commonly employed CYP3A4 marker, and was used in these experiments to calculate three different ISEF values from three different conditions. Enzyme kinetics for 1'-hydroxylation yielded an ISEF of 0.26, while measurement of substrate consumption at two substrate concentrations yielded values of 0.36 (1.0 μM) and 0.15 (0.1 μM), representing over a 2-fold range for a single CYP3A substrate (Table 3). The differences in observed ISEF values derived from substrate consumption at 0.1 and 1.0 μM is likely due to the differences in K_M values between HLM and CYP3A4 being within this range (Table 2), illustrating the importance of using the correct, sub-K_M substrate concentration. When the derived ISEF values were based on activity of the other CYP3A-selective substrates, the values ranged over 8-fold (or 7-fold if the two highest ISEF values generated at higher substrate concentrations are excluded). One possible contributing factor to

the observed variability is the potential for non-CYP3A involvement in substrate metabolism of liver microsomes. However, the drugs selected in this study were all confirmed to be selective for CYP3A with metabolism shown to be completely inhibited by ketoconazole. Another reason for the observed variability is the potential for CYP3A5 to have meaningful and differential contributions to the metabolism of the drugs selected for this study. It is known that CYP3A5 can catalyze midazolam metabolism (Gibbs, et al., 1999; Tseng, et al., 2014). However, the pooled liver microsomes used in this analysis have been shown to possess very low activity for CYP3A5. CYP3A4 has also been shown to exhibit unusual behavior for an enzyme, with demonstration of inhibitor behavior that is dependent on which substrate is used as the marker activity (Kenworthy, et al., 1999) in addition to non-Michaelis-Menten enzyme kinetics for some substrates (Houston and Galetin, 2005). Also, it has been shown that P450 activities can be influenced by complexation with each other and by the relationship among the enzymes and their partner proteins, such as NADPH P450 oxidoreductase and cytochrome b₅ (Reed and Backes, 2012; Yamazaki, et al., 1996). Whether any of these phenomena are behind the observation of different ISEF values for CYP3A4 from different substrates is not known, but the concept of ISEF values is highly dependent on consistent behavior for an enzyme at a molecular level irrespective of the system in which it resides. The data generated in the present work was from rCYP3A4 in a baculovirus *Spodoptera frugiperda* expression system; however, the findings are not unique to this system since similar observations by others used rCYP3A4 from different sources (Lindmark, et al., 2018; Wang, et al., 2019; Siu et al., 2017).

TABLE 5

Use of cytochrome P4503A4 intersystem extrapolation factor from three substrates in the estimation of fractional metabolism values for risperidone

rCYP	Reaction	ISEF Parameters		CL _{int}		f _m	
		ISEF Value	CYP Abundance units of pmol/mg microsomal protein	rCYP CL _{int,u} (μL/min/pmol P450)	ISEF-derived HLM CL _{int,sc,u} (mL/min/kg) ^a	In Vitro f _m	In Vivo f _m
rCYP3A4	1'-Hydroxymidazolam Formation	0.26	137	1.62	51.9	0.98	0.36 ^b
	Vardenafil Depletion	0.29			57.9	0.98	
	Quinidine Depletion	0.044			8.79	0.87	
rCYP2D6	Bufuralol 1'-Hydroxylation	0.14 ^c	8	1.31	1.32	0.02-0.13	0.63-0.88 ^d

^aBinding values were scaled using equation described in Austin et al. (2002), from values generated via equilibrium dialysis. Measured unbound fraction in microsomes for risperidone in human liver microsomes was 0.860 at 0.71 mg/ml, which scaled to f_{u,mic} of 0.57, 0.95, and 0.60 for heterologously expressed individual P4503A4, heterologously expressed individual P4502D6, and human liver microsome incubations, respectively.

^bMahatthanatrakul et al., 2012.

^cCytochrome P450D6 intersystem extrapolation factor determined from bufuralol 1'-hydroxylase activity (internal data on file, Pfizer, Inc.) This intersystem extrapolation factor has been used successfully for other cytochrome P450D6 cleared drugs.

^dSpina et al., 2001; Gasso et al., 2014; Cabaliero et al., 2014; Bondolfi et al., 2001.

Obtaining different ISEF values using different marker substrates is highly detrimental to the approach of using data from individually expressed P450 enzymes for estimates of f_m values. This was illustrated in the examples of risperidone and omeprazole wherein it is known that CYP3A4 plays a partial role in drug clearance (Böttiger, et al., 1997; Mahatthanatrakul, et al., 2012). Omeprazole has been shown clinically to be mostly metabolized by CYP2C19, through demonstrated differences in exposure between CYP2C19 extensive and poor metabolizers, with only a minor contribution from CYP3A4 (Böttiger, et al., 1997). However, estimating f_m for CYP3A4 for omeprazole metabolism by using the higher ISEF values derived from 1'-hydroxymidazolam formation CL_{int} and vardenafil depletion CL_{int} , suggests that CYP3A4 is the major enzyme involved in its clearance (Table 5) with respective f_m values of 0.60 and 0.63; and since f_m values are relative, it then also suggests that CYP2C19 is only a minor contributing enzyme ($f_m < 0.40$). Use of the lowest ISEF value, 0.044 derived from quinidine intrinsic clearance, yields f_m values for omeprazole metabolism by CYP3A4 more closely reflecting the in vivo situation (0.21 versus 0.27, respectively). Prior to having in vivo data for a new drug, it would be difficult to know which ISEF value to use for f_m estimation. For risperidone, the case is more concerning. Risperidone is known to be primarily cleared by CYP2D6, as shown by comparing CYP2D6 extensive and poor metabolizers (Gasso, et al., 2014; Cabaleiro, et al., 2014). However, the use of metabolism data from recombinant CYP3A4 and CYP2D6 and the respective ISEF values suggest that CYP3A4 is the dominant enzyme in risperidone clearance (f_m of 0.87–0.98) regardless of which CYP3A4 ISEF value is employed. When used prospectively, this leads to a complete misassignment of the importance of CYP2D6 in risperidone clearance.

Overall, these findings, along with the findings of others (Lindmark, et al., 2018; Wang, et al., 2019; Siu et al., 2017), cast doubt on the quantitative reliability of the approach of using metabolism data from recombinant heterologously expressed P450 enzymes. This is because among all the P450 enzymes, CYP3A4 is the most frequent enzyme involved in the metabolism of drugs, but ISEF values calculated for CYP3A4 vary broadly with the substrate used. Successful employment of the ISEF approach is dependent on all marker substrates used to calculate ISEF values to be metabolized similarly in human liver microsomes and recombinant systems. Some experimental variability from substrate to substrate can be expected; however, the 8-fold difference observed for CYP3A4 is too high to be useful. For a new investigational compound that is metabolized by CYP3A4, it cannot be known ahead of time which of the model CYP3A4 substrates used in generating ISEF values would be most comparable, and thus which ISEF value to use when scaling for fraction metabolized. This phenomenon reduces the effectiveness of the approach of using metabolism data from rCYP3A4 with ISEF values for the prediction of f_m values in vivo. While heterologously expressed P450 enzymes can be useful in identifying which enzymes have the potential to be involved in metabolism, the quantitative assignment of individual enzymes will require confirmation using other approaches, such as the use of P450 selective inhibitors in human liver microsomes. Research into this is ongoing and will be reported in due course.

Authorship Contributions

Participated in research design: Dantonio, Doran, Obach.

Conducted experiments: Dantonio, Doran, Obach.

Performed data analysis: Dantonio, Doran, Obach.

Wrote or contributed to the writing of the manuscript: Dantonio, Doran, Obach.

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References

- Abelö A, Andersson TB, Antonsson M, Naudot AK, Skånberg I, and Weidolf L (2000) Stereoselective metabolism of omeprazole by human cytochrome P450 enzymes. *Drug Metab Dispos* 28:966–972.
- Austin RP, Barton P, Cockroft SL, Wenlock MC, and Riley RJ (2002) The influence of nonspecific microsomal binding on apparent intrinsic clearance, and its prediction from physicochemical properties. *Drug Metab Dispos* 30:1497–1503.
- Böttiger Y, Tybring G, Götharson E, and Bertilsson L (1997) Inhibition of the sulfoxidation of omeprazole by ketoconazole in poor and extensive metabolizers of S-mephenytoin. *Clin Pharmacol Ther* 62:384–391.
- Cabaleiro T, Ochoa D, López-Rodríguez R, Román M, Novalbos J, Ayuso C, and Abad-Santos F (2014) Effect of polymorphisms on the pharmacokinetics, pharmacodynamics, and safety of risperidone in healthy volunteers. *Hum Psychopharmacol* 29:459–469.
- Cemy MA (2016) Prevalence of non-cytochrome P450-mediated metabolism in Food and Drug Administration-approved oral and intravenous drugs: 2006–2015. *Drug Metab Dispos* 44:1246–1252.
- Chen Y, Liu L, Nguyen K, and Fretland AJ (2011) Utility of intersystem extrapolation factors in early reaction phenotyping and the quantitative extrapolation of human liver microsomal intrinsic clearance using recombinant cytochromes P450. *Drug Metab Dispos* 39:373–382.
- Ekkroos M and Sjögren T (2006) Structural basis for ligand promiscuity in cytochrome P450 3A4. *Proc Natl Acad Sci USA* 103:13682–13687.
- Gassó P, Mas S, Papagianni K, Ferrando E, de Bobadilla RF, Arnaiz JA, Bioque M, Bernardo M, and Lafuente A (2014) Effect of CYP2D6 on risperidone pharmacokinetics and extrapyramidal symptoms in healthy volunteers: results from a pharmacogenetic clinical trial. *Pharmacogenomics* 15:17–28.
- Gibbs MA, Thummel KE, Shen DD, and Kunze KL (1999) Inhibition of cytochrome P-450 3A (CYP3A) in human intestinal and liver microsomes: comparison of K_i values and impact of CYP3A5 expression. *Drug Metab Dispos* 27:180–187.
- Greenblatt DJ, Peters DE, Oleson LE, Harmatz JS, MacNab MW, Berkowitz N, Zinny MA, and Court MH (2009) Inhibition of oral midazolam clearance by boosting doses of ritonavir, and by 4,4-dimethyl-benziso-(2H)-selenazine (ALT-2074), an experimental catalytic mimic of glutathione oxidase. *Br J Clin Pharmacol* 68:920–927.
- Guengerich FP (2008) Cytochrome p450 and chemical toxicology. *Chem Res Toxicol* 21:70–83.
- Heinig R, Adelmann HG, and Ahr G (1999) The effect of ketoconazole on the pharmacokinetics, pharmacodynamics and safety of nisoldipine. *Eur J Clin Pharmacol* 55:57–60.
- Houston JB (1994) Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance. *Biochem Pharmacol* 47:1469–1479.
- Houston JB and Galetin A (2005) Modelling atypical CYP3A4 kinetics: principles and pragmatism. *Arch Biochem Biophys* 433:351–360.
- Jalava KM, Olkkola KT, and Neuvonen PJ (1997) Itraconazole greatly increases plasma concentrations and effects of felodipine. *Clin Pharmacol Ther* 61:410–415.
- Kapelyukh Y, Paine MJ, Maréchal JD, Sutcliffe MJ, Wolf CR, and Roberts GC (2008) Multiple substrate binding by cytochrome P450 3A4: estimation of the number of bound substrate molecules. *Drug Metab Dispos* 36:2136–2144.
- Kenworthy KE, Bloomer JC, Clarke SE, and Houston JB (1999) CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. *Br J Clin Pharmacol* 48:716–727.
- Kaukonen KM, Olkkola KT, and Neuvonen PJ (1997) Itraconazole increases plasma concentrations of quinidine. *Clin Pharmacol Ther* 62:510–517.
- Lee JE, van Heeswijk R, Alves K, Smith F, and Garg V (2011) Effect of the hepatitis C virus protease inhibitor telaprevir on the pharmacokinetics of amlodipine and atorvastatin. *Antimicrob Agents Chemother* 55:4569–4574.
- Lindmark B, Lundahl A, Kanebratt KP, Andersson TB, and Isin EM (2018) Human hepatocytes and cytochrome P450-selective inhibitors predict variability in human drug exposure more accurately than human recombinant P450s. *Br J Pharmacol* 175:2116–2129.
- Lu AYH, Wang RW, and Lin JH (2003) Cytochrome P450 in vitro reaction phenotyping: a re-evaluation of approaches used for P450 isoform identification. *Drug Metab Dispos* 31:345–350.
- Mahatthanatrakul W, Sriwiriyan S, Ridditid W, Boonleang J, Wongnawa M, Rujimamahsan N, and Pipatattanaseree W (2012) Effect of cytochrome P450 3A4 inhibitor ketoconazole on risperidone pharmacokinetics in healthy volunteers. *J Clin Pharm Ther* 37:221–225.
- Muirhead GJ, Wulff MB, Fielding A, Kleinermans D, and Buss N (2000) Pharmacokinetic interactions between sildenafil and saquinavir/ritonavir. *Br J Clin Pharmacol* 50:99–107.
- Reed JR and Backes WL (2012) Formation of P450 · P450 complexes and their effect on P450 function. *Pharmacol Ther* 133:299–310.
- Rodríguez AD (1999) Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem Pharmacol* 57:465–480.
- Siu YA and Lai WG (2017) Impact of probe substrate selection on cytochrome P450 reaction phenotyping using the relative activity factor. *Drug Metab Dispos* 45:183–189.
- Tseng E, Walsky RL, Luzietti Jr RA, Harris JJ, Kosa RE, Goosen TC, Zientek MA, and Obach RS (2014) Relative contributions of cytochrome CYP3A4 versus CYP3A5 for CYP3A-cleared drugs assessed in vitro using a CYP3A4-selective inactivator (CYP3A4i). *Drug Metab Dispos* 42:1163–1173.
- Varhe A, Olkkola KT, and Neuvonen PJ (1994) Oral triazolam is potentially hazardous to patients receiving systemic antimycotics ketoconazole or itraconazole. *Clin Pharmacol Ther* 56:601–607.
- Wang S, Tang X, Yang T, Xu J, Zhang J, Liu X, and Liu L (2019) Predicted contributions of cytochrome P450s to drug metabolism in human liver microsomes using relative activity factor were dependent on probes. *Xenobiotica* 49:161–168.
- Yamazaki H, Johnson WW, Ueng YF, Shimada T, and Guengerich FP (1996) Lack of electron transfer from cytochrome b5 in stimulation of catalytic activities of cytochrome P450 3A4.

- Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome b5. *J Biol Chem* **271**:27438–27444.
- Yamazaki H, Inoue K, Shaw PM, Checovich WJ, Guengerich FP, and Shimada T (1997) Different contributions of cytochrome P450 2C19 and 3A4 in the oxidation of omeprazole by human liver microsomes: effects of contents of these two forms in individual human samples. *J Pharmacol Exp Ther* **283**:434–442.
- Yasui-Furukori N, Hidestrand M, Spina E, Facciola G, Scordo MG, and Tybring G (2001) Different enantioselective 9-hydroxylation of risperidone by the two human CYP2D6 and CYP3A4 enzymes. *Drug Metab Dispos* **29**:1263–1268.
- Yu J, Ritchie TK, Mulgaonkar A, and Ragueneau-Majlessi I (2014) Drug disposition and drug-drug interaction data in 2013 FDA new drug applications: a systematic review. *Drug Metab Dispos* **42**:1991–2001.
- Zhang H, Davis CD, Sinz MW, and Rodrigues AD (2007) Cytochrome P450 reaction-phenotyping: an industrial perspective. *Expert Opin Drug Metab Toxicol* **3**:667–687.
- Zhu Y, Wang F, Li Q, Zhu M, Du A, Tang W, and Chen W (2014) Amlodipine metabolism in human liver microsomes and roles of CYP3A4/5 in the dihydropyridine dehydrogenation. *Drug Metab Dispos* **42**:245–249.
- Zientek MA and Youdim K (2015) Reaction phenotyping: advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. *Drug Metab Dispos* **43**:163–181.

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