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Impact of Probe Substrate Selection on Cytochrome P450 Reaction Phenotyping Using the Relative Activity Factor[S]

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ABSTRACT

Accurately assessing the contribution of cytochrome P450 (P450) isoforms to overall metabolic clearance is important for prediction of clinical drug-drug interactions (DDIs). The relative activity factor (RAF) approach in P450 reaction phenotyping assumes that the interaction between P450-selective probes and testing systems is the same as the interaction of drug candidate with those systems. To test this assumption, an intersystem clearance ratio (ICR) was created to evaluate the difference in values between RAF-scaled intrinsic clearance (CL_{int}) and measured CL_{int} in human liver microsomes (HLMs). The RAF value for CYP3A4 or CYP2C9 derived from a particular P450-selective probe reaction was applied to calculate RAF-scaled CL_{int} for other probe reactions of the same P450 isoform in a crossover manner and compared with the measured HLM CL_{int}. When RAF derived from midazolam or nifedipine was used for CYP3A4, the ICR for testosterone 6β-hydroxylation was 31 and 25, respectively, suggesting significantly diverse interactions of CYP3A4 probes with the testing systems. Such ICR differences were less profound among probes for CYP2C9. In addition, these RAF values were applied to losartan and meloxicam, whose metabolism is mostly CYP2C9 mediated. Only using the RAF derived from testosterone for CYP3A4 produced the expected CYP2C9 contribution of 72%–87% and 47%–69% for metabolism of losartan and meloxicam, respectively. RAF derived from other CYP3A4 probes would have attributed predominantly to CYP3A4 and led to incorrect prediction of DDIs. Our study demonstrates a significant impact of probe substrate selection on P450 phenotyping using the RAF approach, and the ICR may provide a potential solution.

Introduction

Cytochrome P450 (P450) enzymes are well known for their importance in the metabolism of the majority of drugs (Coon, 2005; Guengerich, 2006). In the drug development process, quantification of contributions of P450 isoforms toward the overall metabolism of a drug candidate is necessary for evaluating the risk of the drug candidate as a potential victim in drug-drug interactions (DDIs). A commonly used method for such assessment is the relative activity factor (RAF) approach using recombinant human cytochrome P450 (rP450) and human liver microsomes (HLMs). In this approach, the intrinsic clearance (CL_{int}) of a P450-selective probe reaction is assessed in both systems (rP450 and HLMs) to establish the RAF for each P450 isoform. Determination of the RAF is highly dependent on the HLM batch, rP450 expression quality, and incubation conditions. Therefore, RAF values are highly diverse among laboratories and are specific to the experimental conditions applied in each laboratory, as illustrated by several investigators (Venkatakrishnan et al., 2001; Soars et al., 2003; Uttamsingh et al., 2005; Emoto and Iwasaki, 2007). However, once RAF values are established, the RAF of each P450 isoform can be subsequently applied to the CL_{int} of a potential drug candidate measured in the rP450 system to assess each isoform's relative contribution to metabolism in HLMs, as long as the conditions are kept consistent (Harper and Brassil, 2008; Bohnert et al., 2016). In this process, it is assumed that scaling CL_{int} from rP450 to HLMs is consistent between the P450-selective probe reaction and the metabolism of the drug candidate by that P450 isoform. However, to our knowledge, no study has yet tested this assumption. Furthermore, multiple binding sites with diverse substrate selectivity have been recognized for several P450 isoforms, especially CYP2C9 and CYP3A4, which are important in drug metabolism (Galetin et al., 2003; Kumar et al., 2006). Based on this rather unique property of P450, it is conceivable that a drug candidate may not always interact with its binding site in the same way as the probe from which the RAF was derived. In this study, diverse probe substrates were used to establish RAF values for CYP3A4 (midazolam, testosterone, and nifedipine) and CYP2C9 (*S*-warfarin, diclofenac, and tolbutamide). The RAF value generated from a particular probe was exploited to generate the RAF-scaled CL_{int} from rP450 to HLMs (RAF-scaled CL_{int}) for the other probe reactions of the same P450 isoform in a crossover manner. The RAF-scaled CL_{int} values from rP450 were then compared with the measured CL_{int} in HLMs (HLM CL_{int}), and an intersystem clearance ratio (ICR) was created to gauge the difference between these two values. In addition, RAF values were applied to three model drugs to determine the relative contributions of CYP3A4 versus CYP2C9 to their overall metabolic clearance. This study aimed to demonstrate the impact of RAF probe substrate selection on determination of the fractional contributions of enzymes involved in overall metabolic clearance (f_m) of P450 isoforms. The potential application of the ICR as a tool to identify the

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ABBREVIATIONS: CL_{int}, intrinsic clearance; $CL_{int,u}$, unbound intrinsic clearance; DDI, drug-drug interaction; f_m, fractional contribution of the enzyme involved in overall metabolic clearance; f_u, unbound fraction; HLM, human liver microsome; HPLC, high-performance liquid chromatography; ICR, intersystem clearance ratio; IS, internal standard; LC, liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; *m/z*, mass-to-charge ratio, P450, cytochrome P450; PBPK, physiologically based pharmacokinetic; PPB, potassium phosphate buffer; RAF, relative activity factor; rP450, recombinant human cytochrome P450; TOFMS, time-of-flight mass spectrometry.

appropriate probe for the RAF approach in P450 phenotyping was also explored.

Materials and Methods

Materials. Probe substrates and metabolites, losartan, (*R*)-propranolol, and sildenafil were purchased from Sigma-Aldrich (St. Louis, MO). Meloxicam was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mixed-sex pooled HLMs were purchased from XenoTech (Lenexa, KS). rP450 isoforms (Supersomes) and potassium phosphate buffer (PPB) were purchased from Corning Life Sciences (Tewksbury, MA). Reduced β-NADPH was purchased from Oriental Yeast Company (Andover, MA). All other chemicals, reagents, and solvents used in the analytical process were of either analytical or high-performance liquid chromatography (HPLC) grade.

Analytical Methods. Analyses of probe reactions were performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). A 4000 QTrap triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) was interfaced with Shimadzu HPLC systems including LC-10AD binary pumps and an SIL-HTC autosampler (Shimadzu Corporation, Kyoto, Japan). A Zorbax XDB-C18 5μ HPLC column was used (2.1 × 50 mm; Agilent, Santa Clara, CA). HPLC resolution was achieved with a gradient consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient consisted of the following steps: t = 0 minutes, %B = 1; t = 3 minutes, %B = 80; t = 4 minutes, %B = 99; t = 4.1 minutes, %B = 1; and a total run time of 7.5 minutes. The flow rate was 500 μl/min and the injection volume was 5–10 μl. The mass spectrometer was operated in positive ion mode using a turbospray ionization source. The ionization spray voltage was set at 5000 V and the source temperature was maintained at 650°C. The P450-selective probe reactions were assessed in multiple reaction monitoring mode with the following transitions: mass-to-charge ratio (m/z) 152 → 110 for acetaminophen, m/z 312 → 231 for 4′-hydroxydiclofenac, m/z 287 → 171 for 4-hydroxytolbutamide, m/z 325 → 179 for (*S*)-7-hydroxywarfarin, m/z 235 → 150 for (*S*)-4′-hydroxymephenytoin, m/z 278 → 186 for 1′-hydroxybufuralol, m/z 342 → 203 for 1′-hydroxymidazolam, m/z 345 → 284 for dehydronifedipine, and m/z 305 → 269 for 6β-hydroxytestosterone. The analytes were quantitated using a standard curve containing known amounts of metabolites of the probe reactions. Data processing was conducted using AB Sciex Analyst 1.6 software.

Parent depletion of losartan, meloxicam, and sildenafil was analyzed using liquid chromatography (LC) coupled to time-of-flight mass spectrometry (TOFMS) consisting of an SIL-30AC autosampler, a DGU-20A5R degasser, two LC-30AD pumps, a CTO-30A column oven (Shimadzu Corporation), and a TripleTOF 5600 mass spectrometer (AB Sciex). A Sunfire C18 5μ HPLC column was used (2.1 × 150 mm; Waters, Milford, MA). The mass spectrometer was calibrated using APCI Positive Calibration Solution (AB Sciex). HPLC resolution was achieved with a gradient consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient consisted of the following steps: t = 0 minutes, %B = 1; t = 3.5 minutes, %B = 1; t = 4 minutes, %B = 99; t = 4.1 minutes, %B = 1; and a total run time of 6 minutes. The flow rate was 600 μl/min and the injection volume was 10 μl. The samples were analyzed by using the protonated molecular ion with m/z values of 423.1695, 352.0426, and 475.2122 for losartan, meloxicam, and sildenafil, respectively. Peak area ratios of analyte to internal standard (IS) were calculated based on each peak area obtained from LC-TOFMS analysis. Peak area calculation and integration of analytes, IS, and positive control were processed by MultiQuant 2.0.2 (AB Sciex).

Protein Binding. Final concentrations of probe substrates were set to the apparent substrate concentration at which the reaction rate is half of the maximal velocity (K_m), and those of model drugs were 0.5 μM. Probe substrates or model drugs were spiked with 0.5 mg/ml HLMs or 50 pmol/ml rP450 isoforms. Three-hundred microliters of each sample was added to the dialysis membrane side of the rapid equilibrium devices in duplicate; 500 μl PPB (100 mM, pH 7.4) was added to the outer well of the rapid equilibrium devices. The plate was then covered with a Breathe-Easy (Diversified Biotech, Dedham, MA) sealing membrane and placed in a CO_2 incubator at 37°C with shaking at approximately 200 rpm for 4 hours. After the incubation, 50 μl was removed from each side of the membrane and was matrix matched. The resulting samples were extracted and analyzed by LC-TOFMS. The percentage of protein binding in the matrix was determined by comparing the peak area ratio of analyte from each side of the membrane.

RAF Determination. The incubations were carried out in deep-well 96-well plates containing 0.5 mg/ml HLMs or 50 pmol/ml rP450 isoforms, probe substrates, and PPB (100 mM, pH 7.4) at a final volume of 200 μl. The concentration ranges for the probe substrates were as follows: phenacetin (5–200 μM), diclofenac (1–40 μM), tolbutamide (7.81–1000 μM), *S*-warfarin (0.78–100 μM), *S*-mephenytoin (5–200 μM), bufuralol (2–70 μM), midazolam (5–200 μM), nifedipine (0.78–100 μM), and testosterone (0.78–100 μM). After preincubation in a water bath at 37°C with gentle shaking for 2 minutes, the reactions were initiated by adding an NADPH solution (2 mg/ml final concentration) and continued for 1, 2, 5, 10, and 15 minutes for nifedipine and testosterone and 2, 5, 10, 20, and 30 minutes for all other probe substrates. At the end of the incubations, the reaction samples were mixed with an equal volume of acetonitrile/methanol (1:1, by volume) containing IS. The IS solutions were prepared and diluted in acetonitrile/methanol (1:1, by volume) at the final concentrations of 10 μg/l. (*R*)-Propranolol was used as the IS for quantification of acetaminophen (CYP1A2), 4′-hydroxydiclofenac (CYP2C9), (*S*)-7-hydroxywarfarin (CYP2C9), (*S*)-4′-hydroxymephenytoin (CYP2C19), and 1′-hydroxymidazolam (CYP3A4). $^2H_9$-Hydroxytolbutamide, $^2H_9$-1′-hydroxybufuralol, dehydronifedipine-d_6, and 6β-hydroxytestosterone-d_7 were used as the IS for quantification of 4-hydroxytolbutamide (CYP2C9), 1′-hydroxybufuralol (CYP2D6), dehydronifedipine (CYP3A4), and 6β-hydroxytestosterone (CYP3A4), respectively. After mixing and centrifugation at 1500g for 10 minutes, the supernatant was analyzed by LC-MS/MS for the formation of respective P450-selective metabolites. The experiment was conducted in duplicate (n = 2).

Metabolism of Losartan, Meloxicam, and Sildenafil. The incubations were carried out in deep-well 96-well plates containing 0.5 mg/ml HLMs or 50 pmol/ml rP450 isoforms, 0.5 μM losartan, meloxicam, sildenafil, or positive controls, and PPB (100 mM, pH 7.4) at a final volume of 200 μl. The rP450 isoforms tested were CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The positive controls were 1 μM phenacetin, 1 μM diclofenac, 1 μM *S*-mephenytoin, 1 μM bufuralol, 1 μM midazolam, and 0.5 μM loperamide for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and HLMs, respectively. After preincubation in a water bath at 37°C with gentle shaking for 2 minutes, the reactions were initiated by adding an NADPH solution (2 mg/ml final concentration) and incubated up to 30 minutes. At the designated incubation times of 2, 5, 15, and 30 minutes, the reaction samples were quenched by adding 200 μl acetonitrile/methanol (1:1, by volume) containing the IS of 0.05 μM (*R*)-propranolol. After mixing and centrifugation at 1500g for 10 minutes at 4°C, the supernatant was analyzed by LC-TOFMS. The experiment was conducted in duplicate (n = 2).

Data Analysis and Calculations. For each P450-selective probe reaction, the K_m and V_{max} values were measured following typical Michaelis–Menten kinetics. The CL_{int} for each system was calculated from the measured V_{max} and K_m. The K_m value was corrected using the unbound fraction (f_u) of the substrate in both rP450 and HLM systems. Based on these measured values, the unbound intrinsic clearance ($CL_{int,u}$) for each probe was calculated.

The RAF for each P450 isoform was established based on the CL_{int} obtained in rP450 and HLMs (eq. 1):

$$\mathrm{RAF} = \frac{V_{max,\ HLM}/K_{m,\ u,\ HLM}}{V_{max,\ rP450}/K_{m,\ u,\ rP450}} \quad (1)$$

The ICR was defined as follows (eq. 2):

$$\mathrm{ICR} = \frac{\text{RAF-scaled } CL_{int,\ u}}{\text{Measured HLM } CL_{int,\ u}} = \frac{\left(V_{max,\ rP450}/K_{m,\ u,\ rP450}\right) \times \mathrm{RAF}}{V_{max,\ HLM}/K_{m,\ u,\ HLM}} \quad (2)$$

The relative contribution of a P450 isoform to total P450-mediated clearance was calculated as reported in the literature (eq. 3) (Bohnert et al., 2016; Supplemental Material):

$$\text{Contribution of a P450 to HLM } CL_{int,\ u} = \frac{\left(V_{max,\ rP450}/K_{m,\ u,\ rP450}\right) \times \mathrm{RAF}}{\sum\left[\left(V_{max,\ rP450}/K_{m,\ u,\ rP450}\right) \times \mathrm{RAF}\right]} \times 100 \quad (3)$$

Results

Establishment of RAF. P450-selective probe reactions are shown in Table 1. For CYP2C9 and CYP3A4, three probe reactions were used.

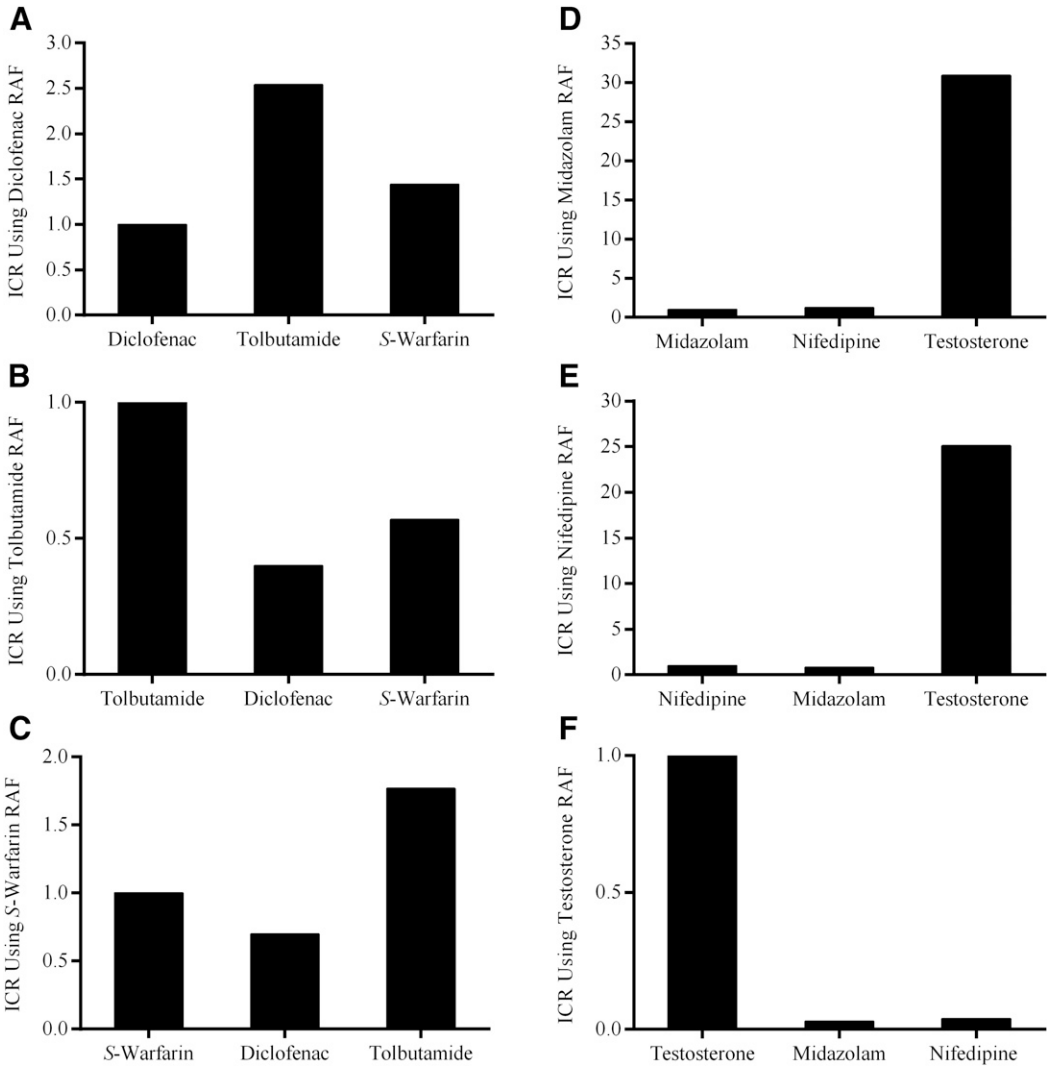

Fig. 1. Crossover analysis for ICR of typical probe substrates of CYP2C9 and CYP3A4 based on the RAF derived from a single probe of diclofenac (A), tolbutamide (B), and *S*-warfarin (C) for CYP2C9 and midazolam (D), nifedipine (E), and testosterone (F) for CYP3A4.

For CYP1A2, CYP2C19, and CYP2D6, a single probe reaction was used. The RAF value for each probe was generated based on the ratio of $CL_{int,u}$ in rP450 and HLMs using eq. 1. The establishment of RAF values corrected for protein binding is summarized in Table 1. The RAF values of CYP2C9 and CYP3A4 vary significantly with the selection of diverse probe reactions.

Cross-System Comparison of $CL_{int,u}$ for Probe Reactions of CYP2C9 and CYP3A4. For each probe reaction of CYP2C9 or CYP3A4, the unbound HLM CL_{int} was compared with the unbound RAF-scaled CL_{int} obtained from the measured $CL_{int,u}$ and the established RAF in each rP450. When the RAF generated from a probe reaction (e.g., tolbutamide for CYP2C9 or midazolam for CYP3A4) was

TABLE 1

P450-selective probe reactions and their parameters that derive the RAF values for various P450 isoforms

P450 Isoform	Probe Reaction	HLMs			rP450			RAF
		$K_{m,u}$	V_{max}	f_u	$K_{m,u}$	V_{max}	f_u	
		μM	*μM/min*		*μM*	*μM/min*		
CYP1A2	Phenacetin *O*-deethylation	66.3	0.514	1.00	5.7	0.733	1.00	0.061
CYP2C9	Diclofenac 4′-hydroxylation	22.4	0.040	0.80	7.9	0.034	0.84	0.415
	Tolbutamide 4-hydroxylation	236.5	0.058	0.95	76.7	0.114	0.92	0.164
	S-Warfarin 7-hydroxylation	10.2	0.002	0.83	6.9	0.004	0.95	0.289
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	56.8	0.039	0.91	19.8	0.887	0.95	0.015
CYP2D6	Bufuralol 1′-hydroxylation	5.4	0.060	0.57	6.7	1.135	0.78	0.066
CYP3A4	Midazolam 1′-hydroxylation	4.8	3.878	0.60	3.3	2.981	0.70	0.904
	Nifedipine dehydrogenation	8.4	0.754	0.58	6.3	0.770	0.77	0.734
	Testosterone 6β-hydroxylation	10.2	0.668	0.78	8.0	17.960	0.82	0.029

TABLE 2

Intrinsic clearance and protein binding values of losartan, meloxicam, and sildenafil measured in rP450 and HLM systems

Testing System	Losartan		Meloxicam		Sildenafil	
	$CL_{int,u}$	f_u	$CL_{int,u}$	f_u	$CL_{int,u}$	f_u
	min^{-1}		min^{-1}		min^{-1}	
rCYP1A2	0.0007	0.80	0.0029	0.81	0	0.49
rCYP2C9	0.0068	0.90	0.0039	0.91	0.0417	0.58
rCYP2C19	0.0006	0.91	0.0073	0.95	0	0.61
rCYP2D6	0	0.83	0.0010	0.91	0.0128	0.49
rCYP3A4	0.0134	0.82	0.0133	0.84	1.2000	0.51
HLMs	0.0042	0.88	0.0004	0.97	0.2663	0.34

applied to the probe itself, the ICR between unbound RAF-scaled CL_{int} and HLM CL_{int}, as calculated using eq. 2, was exactly equal to the unity value (1) as expected. However, when a particular RAF generated from a probe substrate was applied to the other probes of the same P450 isoform, ICR values showed significant deviation from the unity value (Fig. 1). For example, when the RAF value derived from tolbutamide was applied to other CYP2C9-selective probes such as diclofenac and *S*-warfarin, the ICR values were 0.4 and 0.6, respectively (Fig. 1B). Such deviations of ICR from unity were more marked among the CYP3A4 probes, especially testosterone. The ICR value was 31 and 25 when the RAF value generated from midazolam and nifedipine, respectively, was applied to testosterone 6β-hydroxylation (Fig. 1, D and E).

Impact of Selection of Probe Substrate Combination on P450 Phenotyping Assessments. RAF values were applied to three model drugs (losartan, meloxicam, and sildenafil) to determine the relative contributions of P450 isoforms to their overall metabolic clearance. For each of the model drugs, the $CL_{int,u}$ and f_u values were measured in HLMs and in each rP450 isoform; the results are shown in Table 2. The $CL_{int,u}$ of each rP450 was then adjusted with the established RAF and summed to calculate unbound RAF-scaled CL_{int}, which was compared with the unbound HLM CL_{int} to generate the ICR value. Since three diverse probe substrates were used to generate RAF values for CYP2C9 and CYP3A4, there were a total of nine different combinations of the unbound RAF-scaled CL_{int} and ICR for each model drug (Table 3). Applying the values obtained from each combination to eq. 3, the f_m of each P450 isoform was determined for losartan, meloxicam, and sildenafil (Figs. 2, 3, and 4, respectively). The estimated f_m values, specifically for CYP2C9 and CYP3A4, varied considerably depending on the RAF values derived from various combinations of P450-selective probes. Generally, the most significant impact on f_m occurred when various CYP3A4 probes were applied, whereas less impact was demonstrated among the CYP2C9 probes. In the case of losartan, with RAF derived from testosterone that was fixed for CYP3A4, the assessment of relative f_m by P450 isoforms did not vary greatly based on the selection of probes for CYP2C9. Using RAF generated from diclofenac, tolbutamide, and *S*-warfarin for CYP2C9, the results consistently showed the predominance of a CYP2C9 contribution, with f_m values of 87%, 72%, and 82%, as well as a minor CYP3A4 contribution, with f_m values of 12%, 25%, and 16%, respectively (Fig. 2C; Supplemental Table 1). In contrast, when CYP3A4 RAF values derived from midazolam and nifedipine were applied to losartan, the results estimated a much smaller contribution of CYP2C9, with f_m values varying from 8% to 22%, and a predominant CYP3A4 contribution, with f_m values ranging from 78% to 91% (Fig. 2, A and B; Supplemental Table 1). Similar results were also observed with meloxicam. When RAF derived from testosterone was fixed for CYP3A4, combined with the RAF for CYP2C9 derived from diclofenac, tolbutamide, and *S*-warfarin, the f_m value of CYP2C9 was assessed as 69%, 47%, and 61% and the f_m value of CYP3A4 was assessed as 16%, 28%, and 21%, respectively (Fig. 3C; Supplemental Table 2). Applying RAF values from other CYP3A4 probes would remarkably underestimate the contribution of CYP2C9 for meloxicam metabolism, with f_m values ranging from 5% to 14%, which were significantly minor relative to the CYP3A4 contribution ranging from 83% to 92% (Fig. 3, A and B; Supplemental Table 2). Among all combinations of probe substrates, the ICR values closest to the unity value were associated with using RAF derived from testosterone for CYP3A4 and diclofenac for CYP2C9 for losartan and using RAF derived from testosterone for CYP3A4 and tolbutamide for CYP2C9 for meloxicam (Table 3). In the case of sildenafil, any combination of probes assigned the predominant contribution of CYP3A4 over CYP2C9 in its overall metabolism (Fig. 4; Supplemental Table 3).

Discussion

For drug safety concerns, risk assessments for potential metabolism-based DDIs are mandated by regulatory agencies before the initiation of advanced clinical trials (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf). One of the crucial aspects of DDIs is the potential for a drug to become the victim of a coadministered drug capable of modulating its metabolic clearance. Inhibition

TABLE 3

RAF-scaled CL_{int} and ICR values of losartan, meloxicam, and sildenafil using RAF values derived from various combinations of probe substrate for CYP2C9 and CYP3A4

Drug	CYP3A4 Probe Substrate	CYP2C9 Probe Substrate								
		Diclofenac			Tolbutamide			*S*-Warfarin		
		RAF-Scaled CL_{int} [a]	Measured CL_{int} [b]	ICR[c]	RAF-Scaled CL_{int}	Measured CL_{int}	ICR	RAF-Scaled CL_{int}	Measured CL_{int}	ICR
		min^{-1}	min^{-1}		min^{-1}	min^{-1}		min^{-1}	min^{-1}	
Losartan	Midazolam	0.0149	0.0042	3.5	0.0132	0.0042	3.1	0.0141	0.0042	3.3
	Nifedipine	0.0127	0.0042	3.0	0.0110	0.0042	2.6	0.0118	0.0042	2.8
	Testosterone	0.0032	0.0042	0.8	0.0015	0.0042	0.4	0.0024	0.0042	0.6
Meloxicam	Midazolam	0.0140	0.0004	39.1	0.0130	0.0004	36.3	0.0135	0.0004	37.7
	Nifedipine	0.0117	0.0004	32.8	0.0107	0.0004	30.0	0.0112	0.0004	31.4
	Testosterone	0.0024	0.0004	6.6	0.0014	0.0004	3.9	0.0019	0.0004	5.2
Sildenafil	Midazolam	1.1028	0.2663	4.1	1.0923	0.2663	4.1	1.0975	0.2663	4.1
	Nifedipine	0.8988	0.2663	3.4	0.8883	0.2663	3.3	0.8936	0.2663	3.4
	Testosterone	0.0530	0.2663	0.2	0.0425	0.2663	0.2	0.0477	0.2663	0.2

[a]RAF-scaled $CL_{int} = \sum(CL_{int,u} \times RAF)$; based on $CL_{int,u}$ values of rP450 in Table 2 and RAF values in Table 1.
[b]Measured CL_{int} based on $CL_{int,u}$ values of HLMs in Table 2.
[c]ICR = RAF-scaled CL_{int}/measured CL_{int}.

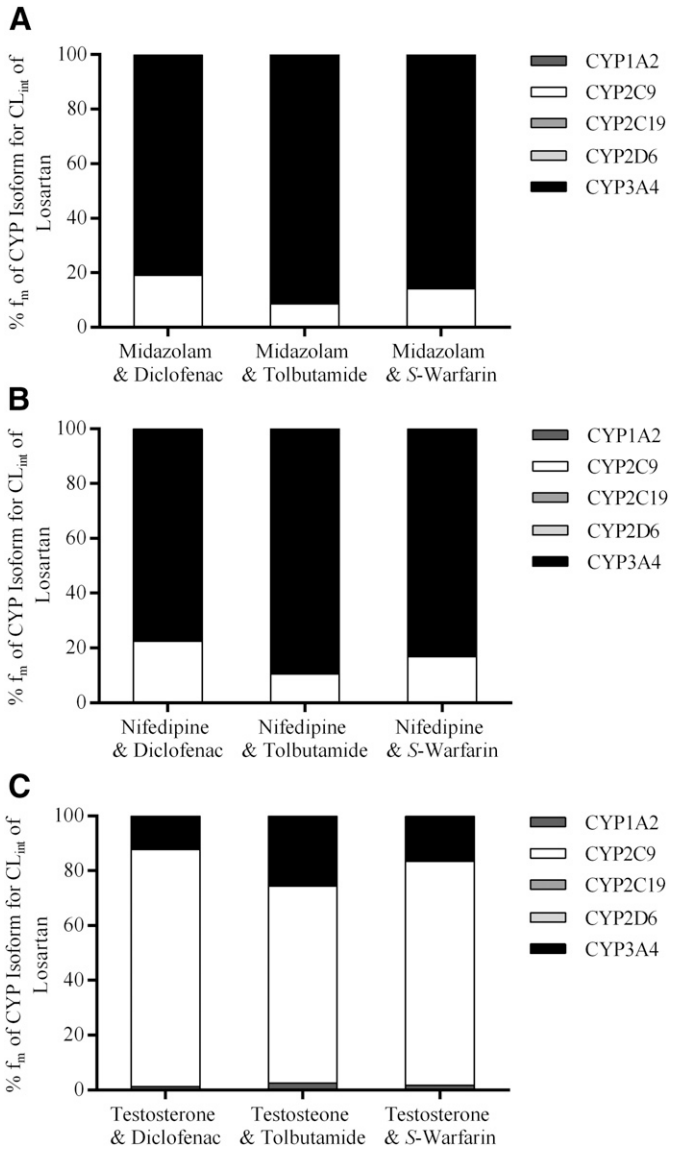

Fig. 2. Comparison of the relative percentage of contributions of P450 isoforms to the overall metabolic clearance of losartan using RAF values derived from multiple combinations of selective probes of CYP2C9 and CYP3A4: midazolam for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (A); nifedipine for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (B); and testosterone for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (C).

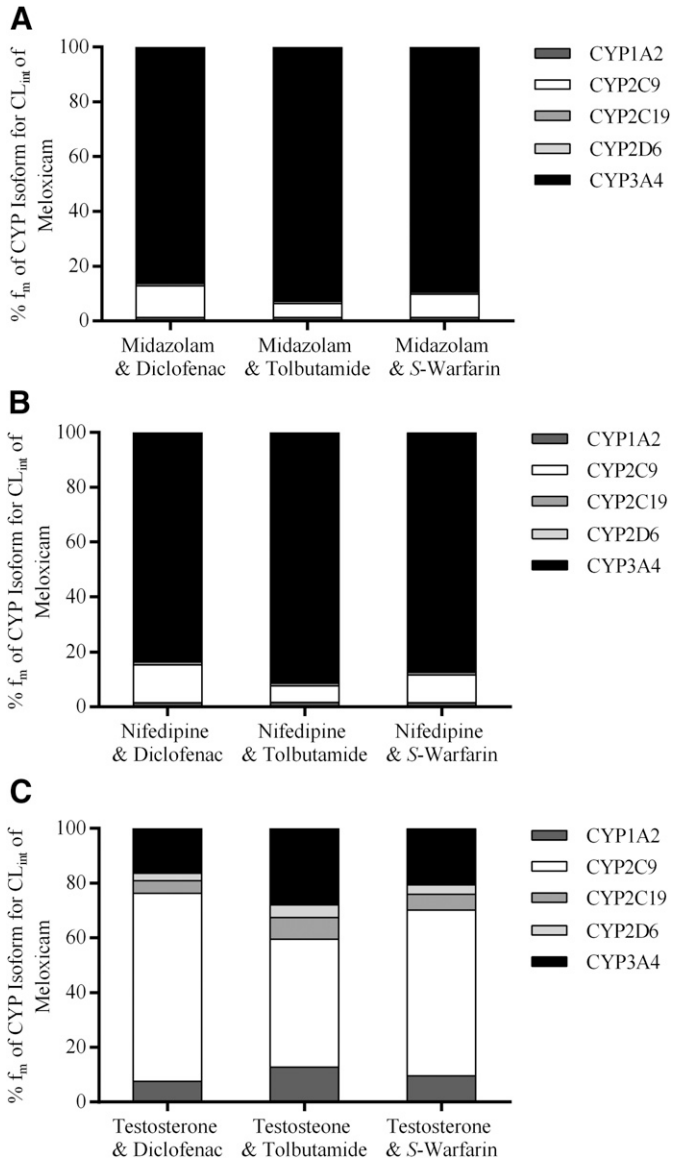

Fig. 3. Comparison of the relative percentage of contributions of P450 isoforms to the overall metabolic clearance of meloxicam using RAF values derived from multiple combinations of selective probes of CYP2C9 and CYP3A4: midazolam for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (A); nifedipine for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (B); and testosterone for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (C).

or induction of the enzymes involved in metabolic clearance can potentially increase or decrease the intended exposure of the target drug and lead to toxicity or lack of efficacy. To avoid such undesired consequences, assessment for a DDI requires an accurate measurement of f_m of the victim drug. Theoretically, for a victim drug that undergoes substantial metabolism, the magnitude of impact on its systemic exposure by a perpetrator of a metabolic enzyme is governed by the f_m for the enzyme (Zhang et al., 2007). Since the prediction of a DDI for a victim drug is so sensitive to the f_m value, it is desirable to obtain the best estimate for any enzymes involved in overall metabolic clearance (Bohnert et al., 2016). However, P450 enzymes often present significant challenges to phenotyping assessments that determine the f_m value of each isoform. One of such challenges is frequently encountered during application of the RAF approach with rP450 and HLM systems.

In this study, we challenged the assumption that the RAF value generated using a particular P450-selective probe reaction can always accurately scale the CL_{int} from rP450 to HLMs. The RAF value derived from a particular probe reaction was applied to other probe reactions of the same P450 isoform. The RAF-scaled CL_{int} was then compared with the measured HLM CL_{int} to generate an ICR. In an ideal scenario, the ICR would always be equal to the unity value for the original P450 probe from which the RAF was derived. However, when applying the RAF to other probes of the same P450, there were significant deviations of the ICR from the unity value (Fig. 1), suggesting the inadequacy of a specific RAF for scaling CL_{int} of other probes that might bind to the same enzyme but at different binding sites. Although the potential cause of such deviation is still unknown and is beyond the scope of this study, it is clear that protein binding in rP450 or HLM systems played an insignificant role, because the CL_{int} values used in our study were

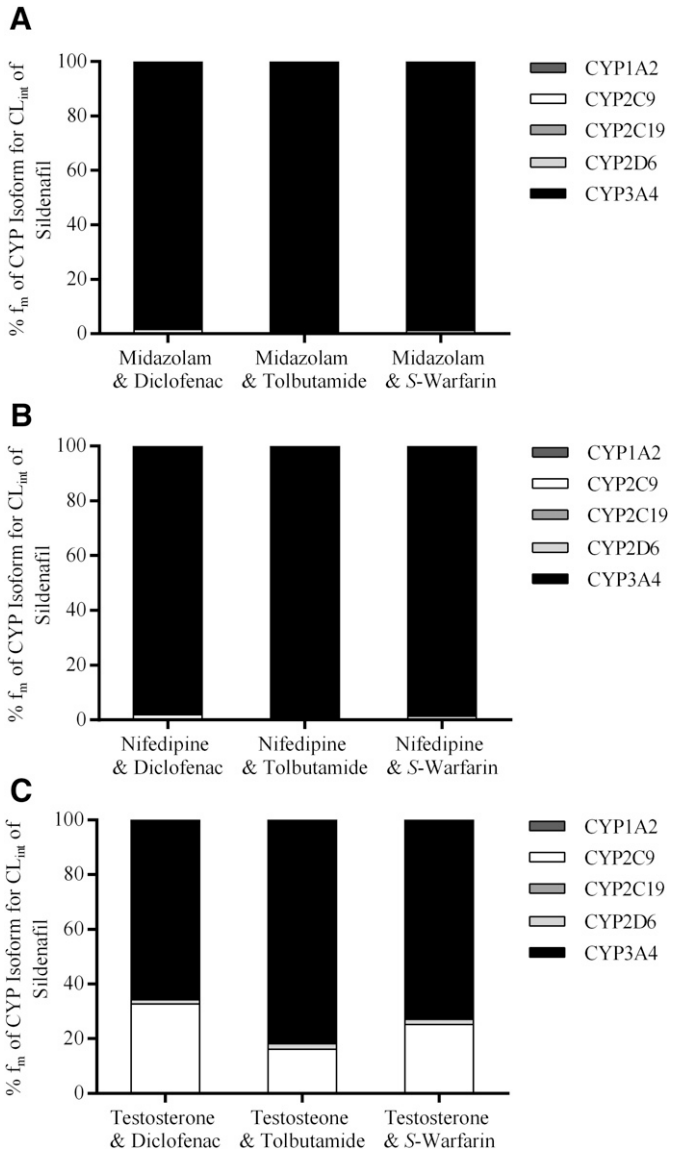

Fig. 4. Comparison of the relative percentage of contributions of P450 isoforms to the overall metabolic clearance of sildenafil using RAF values derived from multiple combinations of selective probes of CYP2C9 and CYP3A4: midazolam for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (A); nifedipine for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (B); and testosterone for CYP3A4 and diclofenac, tolbutamide, and *S*-warfarin for CYP2C9 (C).

already corrected for the unbound fraction of each probe substrate in each system (Table 1). The results indeed demonstrate the limitations of the commonly used RAF approach with a single probe for each P450 isoform. Since the CL_{int} scaled from rP450 has been widely used for the assessment of f_m of the P450 to the overall clearance in HLMs, our results further highlight the impact of probe substrate selection on the outcome of P450 reaction phenotyping using the RAF approach.

To further illustrate the impact on P450 phenotyping results, we selected losartan, meloxicam, and sildenafil as model drugs. CYP3A4 and CYP2C9 have been reported as the enzymes involved in the oxidative metabolism of these drugs (Hyland et al., 2001; Gates et al., 2005; Sica et al., 2005). In these case studies, the ICR was also generated by comparing the sum of RAF-scaled CL_{int} values obtained from rP450 and from direct measurement in HLMs (HLM CL_{int}). Ideally, if the interaction between the P450-selective probe and the enzyme systems completely represents the interaction between the drug candidate and the same enzyme systems, an ICR equal to the unity value would be expected. However, deviation of the ICR from unity has frequently been observed in P450 phenotyping using RAF or similar approaches. An ICR that is less than unity (ICR $<$ 1) is often attributed to potential involvement of drug metabolism enzymes other than the P450 isoforms included in the study. On the other hand, an ICR that is greater than unity (ICR $>$ 1) is also frequently observed. Our results imply that the selection of probe substrates for establishing the RAF could contribute to both observations, as shown in the examples of all three model drugs (Table 3). Furthermore, assessing an ICR close to the unity value may assist the selection of the most appropriate probe substrates for drug candidate phenotyping studies. This potential application was demonstrated with both losartan and meloxicam. Significantly higher contributions of CYP2C9 over CYP3A4 were reported for both losartan (Yasar et al., 2001; Sica et al., 2005) and meloxicam (Türck et al., 1996; Chesné et al., 1998) based on in vitro P450-selective inhibitor approaches and clinical observations. In our study, ICR values were observed to be closer to the unity value for these drugs when we selected testosterone for CYP3A4 and diclofenac or tolbutamide for CYP2C9 as the probe substrates to derive RAF values. For these two model drugs, such combinations of probes for RAF produced assessments of relative contributions of CYP2C9 and CYP3A4 that were consistent with results previously reported using other approaches (Figs. 2C and 3C). In contrast, other combinations of probes associated with much greater ICR values would have attributed predominant contributions of their metabolic clearances to CYP3A4. Such results would strongly contradict previous reports on losartan and meloxicam (Figs. 2, A and B, and 3, A and B). The significant impact of probe substrate selection for the RAF on the assessment of f_m was also demonstrated in the case of sildenafil, in which CYP3A4 was reported as the predominant contributor to metabolic clearance compared with CYP2C9 (Muirhead et al., 2000; Hyland et al., 2001). Although all combinations of probes confirmed that CYP3A4 was the major isoform contributing to the metabolism of sildenafil, f_m of CYP2C9 was much higher when RAF values derived from testosterone were used as the probe for CYP3A4 (Fig. 4). To our knowledge, this is the first systematic investigation to explore the impact of P450 probe substrate selection on the results of P450 reaction phenotyping using the RAF approach. As demonstrated in these case studies, appropriate selection of a P450 probe substrate can be critical in obtaining an accurate assessment of the relative contribution of a P450 isoform to the overall metabolism of the drug of interest. In practice, there is minimum impact on overall resources once all of the RAF values are established with various probes for a given P450 isoform. Subsequently, they can be applied to a drug candidate to generate the P450 f_m values that associate with a set of ICR values. The closeness of the ICR to unity may then serve as a reference to guide the selection of appropriate f_m values as demonstrated in the case studies presented, although further testing in the drug development process would be necessary to verify this potential application.

Physiologically based pharmacokinetic (PBPK) modeling and simulation has evolved as an important tool for drug development and regulatory submission (Zhao et al., 2012; Jones et al., 2015). Before extensive clinical trials, this tool can help improve predictions of pharmacokinetics and DDIs in humans based on data generated from appropriately designed and conducted in vitro studies. The results obtained from such predictions are used not only for decision making during drug development but also during the regulatory review process (Zhao et al., 2011). The outcome of DDI predictions from PBPK modeling and simulation can impact critical decisions, such as whether to conduct or waive clinical DDI studies, and inclusions of drug labeling (Zhao et al., 2011; Jones et al., 2015). However, the quality of PBPK modeling and simulation is highly dependent on the quality of input data.

Therefore, the generation of reliable assessments of P450 contribution to metabolic clearance is essential to cultivating confidence in PBPK modeling and simulation prior to clinical trials. This study demonstrates a significant impact of probe substrate selection on P450 phenotyping studies using the RAF approach to generate f_m values. Our results highlight the need to assess the RAF from diverse probe substrates for P450 isoforms, specifically CYP3A4 and CYP2C9. Considering the importance of f_m in risk assessment of a drug as a potential DDI victim, the appropriate selection of probe substrates for the RAF could have a significant impact on the accuracy of f_m assessment and on the accuracy of DDI assessment using PBPK modeling and simulation. As a general guidance to P450 phenotyping, RAF values of a P450 isoform should be established with multiple probe substrates and applied respectively to the f_m assessment for the drug candidate. Selection of a suitable probe substrate may be determined by the closeness of the overall ICR to the unity value. Although our study focused on CYP3A4 and CYP2C9, the next steps would be to investigate the diverse probe substrates of other P450 isoforms and their potential impacts on the outcomes of P450 phenotyping using the RAF approach.

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Authorship Contributions

Participated in research design: Siu, Lai.
Conducted experiments: Siu.
Performed data analysis: Siu, Lai.
Wrote or contributed to the writing of the manuscript: Siu, Lai.

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