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

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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 (CLint) and measured CLint 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 CLint for other probe reactions of the same P450 isoform in a crossover manner and compared with the measured HLM CLint. 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 (CLint) 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 CLint 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 CLint 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 CLint from rP450 to HLMs (RAF-scaled CLint) for the other probe reactions of the same P450 isoform in a crossover manner. The RAF-scaled CLint values from rP450 were then compared with the measured CLint in HLMs (HLM CLint), 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 (fm) of P450 isoforms. The potential application of the ICR as a tool to identify the

ABBREVIATIONS: CLint, intrinsic clearance; CLint,u, unbound intrinsic clearance; DDI, drug-drug interaction; fm, fractional contribution of the enzyme involved in overall metabolic clearance; fu, 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 were purchased from XenoTech (Lenexa, KS). Reduced 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 5 μm 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 = 3.5 minutes, %B = 1; 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 turbo spray ionization source. The ionization spray voltage was set at 5000 V and the source temperature was 650°C. The parent depletion of losartan, meloxicam, and sildenafil was analyzed using liquid chromatography–time-of-flight mass spectrometry (TOFMS) consisting of an SIL-30AC autosampler, a DGU-20A5R degasser, a DGU-20A5R degasser, and positive control were processed by MultiQuant 2.0.2 (AB Sciex). Peak area ratios of analyte to internal standard (IS) were calculated based on each peak area obtained at ASPET Journals on December 5, 2021 dmd.aspetjournals.org Downloaded from

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, 0.5 μM losartan, meloxicam, sildenafil, or positive controls, 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 (7.8–1000 μM), S-warfarin (0.78–100 μM), S-mephentoin (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/ml (R)-propranolol was used as the IS for quantification of acetaminophen (CYP2A12), 4’-hydroxydiclofenac (CYP2C9), (S)-7-hydroxywarfarin (CYP2C9), (S)-4’-hydroxyphenytoin (CYP2C19), and 1’-hydroxymidazolam (CYP3A4). 2H9-Hydroxytolbutamide, 2H9-1’-hydroxybufuralol, dehydroindeneidipine, and 6f6-hydroxytestosterone-6f were used as the IS for quantification of 4-hydroxytolbutamide (CYP2C19), 1’-hydroxybufuralol (CYP2D6), dehydroindeneidipine, and 6f6-hydroxytestosterone-3f (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-mephentoin, 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 Km and Vmax values were measured following typical Michaelis–Menten kinetics. The CL_{int} for each system was calculated from the measured Vmax and Km of the rP450 value was corrected using the unbound fraction (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,u} obtained in rP450 and HLMs (eq: 1):

\[
RAF = \frac{V_{\text{max}, \text{HLM}}/K_{\text{m}, \text{u}, \text{HLM}}}{V_{\text{max}, \text{P450}}/K_{\text{m}, \text{u}, \text{P450}}} \quad (1)
\]

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

\[
ICR = \frac{\text{RAF}-\text{scaled \, CL}_{\text{int}, \text{u}, \text{HLM}}}{\text{Measured \, HLM \, CL}_{\text{int}, \text{u}, \text{HLM}}} (\frac{V_{\text{max}, \text{P450}}/K_{\text{m}, \text{u}, \text{P450}} \times \text{RAF}}{V_{\text{max}, \text{HLM}}/K_{\text{m}, \text{u}, \text{HLM}}}) \quad (2)
\]

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):

\[
\text{Contribution of a P450 to HLM} \, CL_{\text{int}, \text{u}, \text{HLM}} = \frac{(V_{\text{max}, \text{P450}}/K_{\text{m}, \text{u}, \text{P450}} \times \text{RAF})}{\sum_{i=1}^{n} \frac{(V_{\text{max}, \text{P450}}/K_{\text{m}, \text{u}, \text{P450}} \times \text{RAF})}{100}} \quad (3)
\]

Results

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

![Fig. 1](https://example.com/f1.png)

**TABLE 1**

<table>
<thead>
<tr>
<th>P450 Isoform</th>
<th>Probe Reaction</th>
<th>HLMs</th>
<th>rP450</th>
<th>RAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K_{\text{max}}</td>
<td>V_{\text{max}}</td>
<td>f_u</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>66.3</td>
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<td>1.00</td>
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<td>Diclofenac 4'-hydroxylation</td>
<td>22.4</td>
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<td>Tolbutamide 4-hydroxylation</td>
<td>236.5</td>
<td>0.058</td>
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<td>S-Warfarin 7-hydroxylation</td>
<td>10.2</td>
<td>0.002</td>
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<td>S-Mephenytoin 4'-hydroxylation</td>
<td>56.8</td>
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<tr>
<td>CYP2D6</td>
<td>Bufuralol 1′-hydroxylation</td>
<td>5.4</td>
<td>0.060</td>
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<tr>
<td>CYP3A4</td>
<td>Midazolam 1'-hydroxylation</td>
<td>4.8</td>
<td>3.878</td>
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<td>Nifedipine dehydrogenation</td>
<td>8.4</td>
<td>0.754</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Testosterone 6β-hydroxylation</td>
<td>10.2</td>
<td>0.668</td>
<td>0.78</td>
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</table>
applied to the probe itself, the ICR between unbound RAF-scaled $CL_{int,u}$ and HLM $CL_{int,u}$ 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$_{int,u}$, which was compared with the unbound HLM CL$_{int,u}$ 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$_{int,u}$ and ICR for each model drug (Table 3). Applying the values obtained from each combination to eq. 3, the $fm$ of each probe substance was determined for CYP3A4 probes, while less impact was demonstrated for CYP2C9 probes. Generally, the most significant impact on $fm$ 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 $fm$ 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 $f_u$ values ranging from 8% to 22%, and a predominant CYP3A4 contribution, with $f_u$ 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 CYP2C9, combined with the RAF for CYP2C9 derived from diclofenac, tolbutamide, and S-warfarin, the $f_u$ value of CYP2C9 was assessed as 69%, 47%, and 61% and the $f_u$ 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 $f_u$ 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**


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**TABLE 2**

<table>
<thead>
<tr>
<th>Testing System</th>
<th>Losartan</th>
<th>Meloxicam</th>
<th>Sildenafil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL$_{int,u}$</td>
<td>$f_u$</td>
<td>CL$_{int,u}$</td>
</tr>
<tr>
<td>rCYP3A4</td>
<td>0.0007</td>
<td>0.80</td>
<td>0.0029</td>
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<tr>
<td>rCYP3A4</td>
<td>0.0141</td>
<td>0.88</td>
<td>0.0004</td>
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**TABLE 3**

<table>
<thead>
<tr>
<th>Drug</th>
<th>CYP3A4 Probe Substrate</th>
<th>Diclofenac</th>
<th>Tolbutamide</th>
<th>S-Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAF-Scaled CL$_{int,u}$</td>
<td>Measured CL$_{int,u}$</td>
<td>ICR</td>
<td>RAF-Scaled CL$_{int,u}$</td>
</tr>
<tr>
<td></td>
<td>min$^{-1}$</td>
<td>min$^{-1}$</td>
<td></td>
<td>min$^{-1}$</td>
</tr>
<tr>
<td>Losartan</td>
<td>Midazolam</td>
<td>0.0149</td>
<td>0.0042</td>
<td>3.5</td>
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<tr>
<td></td>
<td>Nifedipine</td>
<td>0.0117</td>
<td>0.0042</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Tolbutamide</td>
<td>0.0032</td>
<td>0.0042</td>
<td>0.8</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>Midazolam</td>
<td>0.0140</td>
<td>0.0004</td>
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<tr>
<td></td>
<td>Nifedipine</td>
<td>0.0117</td>
<td>0.0004</td>
<td>32.8</td>
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<td>Tolbutamide</td>
<td>0.0024</td>
<td>0.0004</td>
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<td>Sildenafil</td>
<td>Midazolam</td>
<td>1.1028</td>
<td>0.2663</td>
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<td></td>
<td>Nifedipine</td>
<td>0.8988</td>
<td>0.2663</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>0.0530</td>
<td>0.2663</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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$a$ RAF-scaled CL$_{int,u}$ = $\sum (CL_{int,u} \times$ RAF); based on CL$_{int,u}$ values of rP450 in Table 2 and RAF values in Table 1.

$b$ Measured CL$_{int,u}$ based on CL$_{int,u}$ values of HLMs in Table 2.

$c$ ICR = RAF-scaled CL$_{int,u}$/measured CL$_{int,u}$.
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 $C_{L_{int}}$ 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 $C_{L_{int}}$ was then compared with the measured HLM $C_{L_{int}}$ 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 $C_{L_{int}}$ 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 $C_{L_{int}}$ values used in our study were
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 fm 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 fm 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, fm 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 fm 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 fm 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 fm 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 fm 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 fm 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 fm 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

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