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

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Running title: Probe Selection on CYP Reaction Phenotyping Using RAF

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This manuscript contains:

21 text pages (without references);
3 tables;
4 figures;
23 references;
247 words in Abstract;
508 words in Introduction;
1432 words in Discussion.

Abbreviations:

CL_{int} - Intrinsic clearance
CL_{int,u} - Unbound CL_{int}
CYP - Cytochrome P450
DDI - Drug-drug interaction
f_m - Fractional contribution of the enzyme involved in overall metabolic clearance
f_u - Unbound fraction

HLM - Human liver microsomes

HPLC - High-performance liquid chromatography

ICR - Intersystem clearance ratio

IS - Internal standard

K_m - Substrate concentration at which the reaction rate is half of maximal velocity

K_m,u - Unbound K_m

LC-MS/MS - Liquid chromatography coupled with tandem mass spectrometry

LC-TOFMS - Liquid chromatography coupled with time-of-flight mass spectrometry

m/z - Mass-to-charge ratio

PBPK - Physiologically based pharmacokinetic

PPB - Potassium phosphate buffer

RAF - Relative activity factor

RAF-scaled CL_int - RAF-scaled CL_int from rCYP to HLM

rCYP - Recombinant human cytochrome P450
Abstract

Accurately assessing the contribution of cytochrome P450 (CYP) isoforms to overall metabolic clearance is important for prediction of clinical drug-drug interaction (DDI). The relative activity factor (RAF) approach in CYP reaction phenotyping assumes that the interaction between CYP-selective probes and testing systems is the same as to drug candidate with those systems. To test this assumption, 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 (HLM). RAF value for CYP3A4 or CYP2C9 derived from a particular CYP-selective probe reaction was applied to calculate RAF-scaled CLint for other probe reactions of the same CYP 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. Additionally, 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 DDI.

Our study demonstrated a significant impact of probe substrate selection on CYP phenotyping using the RAF approach and ICR may provide a potential solution.
Introduction

Cytochrome P450 (CYP) enzymes are well known for their importance in metabolism of the majority of drugs (Coon, 2005; Guengerich, 2006). In the drug development process, quantification of contributions of CYP 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 interaction (DDI). A commonly used method for such assessment is the relative activity factor (RAF) approach using recombinant human CYP (rCYP) and human liver microsomes (HLM). In this approach, the intrinsic clearance (CL\textsubscript{int}) of a CYP-selective probe reaction is assessed in both systems (rCYP and HLM) to establish the RAF for each CYP isoform. The determination of RAF is highly depended on the HLM batch, rCYP expression quality, and the incubation conditions. Therefore, the RAF values are highly diverse among labs and specific to the experimental conditions applied in each lab as illustrated by several investigators (Venkatakrishnan et al., 2001; Soars et al., 2003; Uttamsingh et al., 2005; Emoto and Iwasaki, 2007). However, once the RAF values are established, the RAF of each CYP isoform can be subsequently applied to the CL\textsubscript{int} of a potential drug candidate measured in the rCYP system to assess each isoform’s relative contribution to metabolism in HLM, 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\textsubscript{int} from rCYP to HLM is consistent between the CYP-selective probe reaction and the metabolism of the drug candidate by that CYP isoform. However, to our knowledge, there has been no study to test this assumption. Furthermore, multiple binding sites with diverse substrate selectivity have been recognized for several CYP 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 CYP, 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 the present 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 $\text{CL}_{\text{int}}$ from rCYP to HLM (RAF-scaled $\text{CL}_{\text{int}}$) for the other probe reactions of the same CYP isoform in a crossover manner. The RAF-scaled $\text{CL}_{\text{int}}$ values from rCYP were then compared with the measured $\text{CL}_{\text{int}}$ in HLM (HLM $\text{CL}_{\text{int}}$), and an Intersystem Clearance Ratio (ICR) was created to gauge the difference between these two values. Additionally, RAF values were applied to three model drugs to determine the relative contributions of CYP3A4 versus CYP2C9 to their overall metabolic clearance. The objective of the current study was 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 CYP isoform. The potential application of ICR as a tool to identify the appropriate probe for RAF approach in CYP 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 Biotech (Santa Cruz, CA). Mixed gender pooled HLM was purchased from XenoTech (Lenexa, KS). rCYP 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

Analyses of probe reactions were performed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The mass spectrometer, AB Sciex 4000 QTrap triple quadrupole mass spectrometer (Framingham, MA), was interfaced with Shimadzu HPLC systems including LC-10AD binary pumps and SIL-HTC autosampler (Shimadzu Corporation, Kyoto, Japan). The HPLC column used was Zorbax XDB-C18 5µ 2.1 x 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 min, %B = 1; t = 3 min, %B = 80; t = 4 min, %B = 99; t = 4.1 min, %B = 1, and a total run time of 7.5 min. 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 Turbo Spray ionization source. The ionization spray voltage was set at 5000 V and the source temperature was maintained at 650 ºC. The CYP-selective probe reactions were assessed in the multiple reaction
monitoring mode with the transition of 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 the AB Sciex Analyst™ 1.6 software (Framingham, MA).

Parent depletion of losartan, meloxicam, and sildenafil was analyzed using liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOFMS) consisting of a SIL-30AC autosampler, a DGU-20A5R degasser, two LC-30AD pumps, a CTO-30A column oven (Shimadzu Corporation, Kyoto, Japan), and a TripleTOF 5600 mass spectrometer (AB Sciex, Framingham, MA). The HPLC column used was Sunfire C18 5µ 2.1 x 150 mm (Waters, Milford, MA). The mass spectrometer was calibrated using the APCI Positive Calibration Solution (AB Sciex, Framingham, MA). 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 min, %B = 1; t = 3.5 min, %B = 1; t = 4 min, %B = 99; t = 4.1 min, %B = 1, and a total run time of 6 min. 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 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, Framingham, MA).

**Protein Binding**

Final concentrations of probe substrates were set to the apparent substrate concentration at which the reaction rate is half of maximal velocity (K_m), and those of model drugs were 0.5 µmol/l. Probe substrate or model drugs were spiked with 0.5 mg/ml HLM or 50 pmol/ml rCYP isoforms. Three hundred microliters of each sample was added to the dialysis membrane side of the Rapid Equilibrium Devices® in duplicate; 500 µl of PPB (100 mmol/l, pH 7.4) was added to the outer well of the Rapid Equilibrium Devices®. The plate was then covered with a Breathe-Easy® sealing membrane and placed in a CO_2 incubator at 37°C while shaking at approximately 200 rpm for 4 hours. After the incubation, 50 µl was removed from each side of the membrane and matrix-matched. The resulting samples were extracted and analyzed by LC-TOFMS. The percentage of protein binding in 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 HLM or 50 pmol/ml rCYP isoforms, probe substrates, and PPB (100 mmol/l, pH 7.4) at a final volume of 200 µl. The concentration ranges for the probe substrates were as follows: phenacetin (5–200 µmol/l), diclofenac (1–40 µmol/l), tolbutamide (7.81–1000 µmol/l), S-warfarin (0.78–100 µmol/l), S-mephenytoin (5–200 µmol/l), bufuralol (2–70 µmol/l), midazolam (5–200 µmol/l), nifedipine (0.78–100 µmol/l), and testosterone (0.78–100 µmol/l). After pre-incubating in a
water bath at 37°C with gentle shaking for 2 min, the reactions were initiated by adding an NADPH solution (2 mg/ml final concentration), and continued for 1, 2, 5, 10, and 15 min for nifedipine and testosterone and 2, 5, 10, 20, and 30 min 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 IS for quantification of acetaminophen (CYP1A2), 4’-hydroxydiclofenac (CYP2C9), (S)-7-hydroxywarfarin (CYP2C9), (S)-4’-hydroxymephenytoin (CYP2C19), and 1’-hydroxymidazolam (CYP3A4). 3H9-Hydroxytolbutamide, 2H9-1’-hydroxybufuralol, dehydronifedipine-d6, and 6β-hydroxytestosterone-d7 were used as IS for quantification of 4-hydroxytolbutamide (CYP2C9), 1’-hydroxybufuralol (CYP2D6), dehydronifedipine (CYP3A4), and 6β-hydroxytestosterone (CYP3A4), respectively. After mixing and centrifugation at 1500 x g for 10 min, the supernatant was analyzed by LC-MS/MS for the formation of respective CYP-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 HLM or 50 pmol/ml rCYP isoforms, 0.5 µmol/l losartan, meloxicam, sildenafil, or positive controls, and PPB (100 mmol/l, pH 7.4) at a final volume of 200 µl. rCYP isoforms tested were CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The positive controls were 1 µmol/l phenacetin, 1 µmol/l diclofenac, 1 µmol/l S-mephenytoin, 1 µmol/l bufuralol, 1 µmol/l midazolam, and 0.5 µmol/l loperamide for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and HLM.
respectively. After pre-incubating in a water bath at 37°C with gentle shaking for 2 min, the reactions were initiated by adding an NADPH solution (final concentration 2 mg/ml) and incubated up to 30 min. At the designated incubation times of 2, 5, 15, and 30 min, the reaction samples were quenched by adding 200 µl of acetonitrile:methanol (1:1, by volume) containing IS of 0.05 µmol/l (R)-propranolol. After mixing and centrifugation at 1500 x g for 10 min at 4°C, the supernatant was analyzed by LC-TOFMS. The experiment was conducted in duplicate (N = 2).

**Data Analysis and Calculations**

For each CYP-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 rCYP and HLM systems. Based on these measured values, the unbound $CL_{int}$ ($CL_{int,u}$) for each probe was calculated.

The RAF for each CYP isoform was established based on the $CL_{int}$ obtained in rCYP and HLM.

$$RAF = \frac{V_{max, HLM} / K_{m, u, HLM}}{V_{max, rCYP} / K_{m, u, rCYP}}$$

Equation 1

The ICR was defined as the following equation.

$$ICR = \frac{RAF-scaled CL_{int, u}}{Measured HLM CL_{int, u}} = \frac{(V_{max, rCYP} / K_{m, u, rCYP}) * RAF}{V_{max, HLM} / K_{m, u, HLM}}$$

Equation 2

The relative contribution of a CYP isoform to total CYP-mediated clearance was calculated as reported in the literature (Bohnert et al., 2016 Supplementary).
% Contribution of a CYP to HLM CL_{int,u} = \frac{(V_{max, CYP} / K_{m, u, CYP}) \times RAF}{\sum ((V_{max, CYP} / K_{m, u, CYP}) \times RAF)} \times 100 \quad \text{Equation 3}

Results

Establishment of RAF

All CYP-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 rCYP and HLM using Equation 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 rCYP. When the RAF generated from a probe reaction (e.g. tolbutamide for CYP2C9 or midazolam for CYP3A4) was applied to the probe itself, the ICR between unbound RAF-scaled CL_{int} and HLM CL_{int}, as calculated using Equation 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 CYP 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. 1D and 1E).

**Impact of Selection of Probe Substrate Combination on CYP Phenotyping Assessments**

RAF values were applied to three model drugs (losartan, meloxicam, and sildenafil) to determine the relative contributions of CYP isoforms to their overall metabolic clearance. For each of the model drugs, the $\text{CL}_{\text{int,u}}$ and $f_u$ values were measured in HLM and in each rCYP isoform; the results are shown in Table 2. The $\text{CL}_{\text{int,u}}$ of each rCYP was then adjusted with the established RAF and summed up to calculate unbound RAF-scaled $\text{CL}_{\text{int}}$, which was compared with the unbound HLM $\text{CL}_{\text{int}}$ 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 $\text{CL}_{\text{int}}$ and ICR for each model drug (Table 3). Applying the values obtained from each combination to Equation 3, the $f_m$ of each CYP isoform was determined for losartan, meloxicam, and sildenafil (Fig. 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 CYP-selective probes. Generally, the most significant impact on $f_m$ occurred when various CYP3A4 probes were applied, while 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 CYP 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%; and 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. 2A and 2B; 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 of CYP2C9 was assessed as 69%, 47%, and 61%; and the f_m 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 CYP3A4 contribution ranging from 83% to 92% (Fig. 3A and 3B; Supplemental Table 2). Among all combinations of probe substrates, the ICR values closest to the unity 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 DDI are mandated by regulatory agencies before the initiation of advanced clinical trials (FDA, 2012; EMA, 2012). One of the crucial aspects of DDI is the potential for a drug to become the victim of a co-administered drug capable of modulating its metabolic clearance. Inhibition 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 DDI requires an accurate measurement of the $f_m$ 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 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, CYP 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 the application of RAF approach with rCYP and HLM systems.

In this study, we challenged the assumption that RAF value generated using a particular CYP-selective probe reaction can always accurately scale the $CL_{int}$ from rCYP to HLM. RAF value derived from a particular probe reaction was applied to other probe reactions of the same CYP isoform. The RAF-scaled $CL_{int}$ was then compared to 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 CYP probe from which the RAF was derived. However, when applying the RAF to other probes of
the same CYP, there were significant deviations of the ICR from the unity value (Fig. 1), suggesting the inadequacy of a specific RAF for scaling \( \text{CL}_{\text{int}} \) 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 beyond the scope of the current study, it is clear that protein binding in rCYP or HLM systems played an insignificant role, as the \( \text{CL}_{\text{int}} \) values used in the current 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 CYP isoform. Since the \( \text{CL}_{\text{int}} \) scaled from rCYP has been widely used for the assessment of \( f_m \) of the CYP to the overall clearance in HLM, our results further highlight the impact of probe substrate selection on the outcome of CYP reaction phenotyping using the RAF approach.

To further illustrate the impact on CYP phenotyping results, we selected losartan, meloxicam, and sildenafil as model drugs. CYP3A4 and CYP2C9 have been reported as the enzymes involved in oxidative metabolism of these drugs (Sica et al., 2005; Gates et al., 2005; Hyland et al., 2001). In these case studies, ICR was also generated by comparing the sum of RAF-scaled \( \text{CL}_{\text{int}} \) values obtained from rCYP and from direct measurement in HLM (HLM \( \text{CL}_{\text{int}} \)). Ideally, if the interaction between the CYP-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 ICR from unity has frequently been observed in CYP phenotyping using RAF or similar approaches. ICR that is less than unity (ICR < 1) is often attributed to potential involvement of drug metabolism enzymes other than the CYP isoforms included in the study. On the other hand, ICR that is greater than unity (ICR > 1) is...
also frequently observed. Our results imply that the selection of probe substrates for establishing 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 (Chesné et al., 1998; Türck et al., 1996) based on in vitro CYP-selective inhibitor approaches and clinical observations. In the current study, ICR values were observed to be closer to the unity value for these drugs when testosterone for CYP3A4 and diclofenac or tolbutamide for CYP2C9 was selected 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 (Fig. 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 (Fig. 2A, 2B, 3A, and 3B). The significant impact of probe substrate selection for 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 in comparison to CYP2C9 (Hyland et al., 2001; Muirhead et al., 2000). 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 CYP probe substrate selection on the result of CYP
reaction phenotyping using RAF approach. As demonstrated in these case studies, appropriate selection of a CYP probe substrate can be critical in accurate assessment of relative contribution of a CYP isoform to the overall metabolism of the drug of interest. In practice, there is minimum impact on overall resources once all the RAF values are established with various probes for a given CYP isoform. Subsequently, they can be applied to a drug candidate to generate the CYP $f_m$ values that associate with a set of ICR values. The closeness of 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 pharmacokinetic and DDI 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 CYP contribution to metabolic clearance is essential to cultivating confidence in PBPK modeling and simulation prior to clinical trials. The current study demonstrated a significant impact of probe substrate selection on CYP phenotyping studies using the RAF approach to generate $f_m$ values.
Our results highlight the necessity of assessing RAF from diverse probe substrates for CYP 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 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 CYP phenotyping, RAF values of a CYP isoform should be established with multiple probe substrates and applied respectively to the \( f_m \) assessment for the drug candidate. The selection of suitable probe substrate may be determined by the closeness of the overall ICR to the unity value. Although the current study focused on CYP3A4 and CYP2C9, the next steps would be to investigate the diverse probe substrates of other CYP isoforms and their potential impacts on the outcomes of CYP phenotyping using the RAF approach.
Acknowledgments

The authors would like to thank Mr. Andrew Hart, Drs. Kazutomi Kusano, Raku Shinkyo, and Takafumi Komori for their helps in the preparation of this manuscript and Dr. Scott Obach for advice on tabulation of data.
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
References


Footnotes

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Figure Legends

**Fig. 1.** Crossover analysis for Intersystem Clearance Ratio (ICR) of typical probe substrates of cytochrome P450 (CYP) 2C9 and CYP3A4 based on relative activity factor (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.

**Fig. 2.** Comparison of the relative percentage of contributions of cytochrome P450 (CYP) isoforms to the overall metabolic clearance of losartan using relative activity factor (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 cytochrome P450 (CYP) 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).

**Fig. 4.** Comparison of the relative percentage of contributions of CYP 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).
Table 1  CYP-selective probe reactions and their parameters that derive the RAF values for various CYP isoforms.

<table>
<thead>
<tr>
<th>CYP isoform</th>
<th>Probe Reaction</th>
<th>HLM</th>
<th>Recombinant CYP</th>
<th>RAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_{m,u}$ (µmol/L)</td>
<td>$V_{max}$ (µmol/L/min)</td>
<td>$f_u$</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>66.3</td>
<td>0.514</td>
<td>1.00</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4'-hydroxylation</td>
<td>22.4</td>
<td>0.040</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Tolbutamide 4-hydroxylation</td>
<td>236.5</td>
<td>0.058</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>S-warfarin 7-hydroxylation</td>
<td>10.2</td>
<td>0.002</td>
<td>0.83</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin 4'-hydroxylation</td>
<td>56.8</td>
<td>0.039</td>
<td>0.91</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol 1'-hydroxylation</td>
<td>5.4</td>
<td>0.060</td>
<td>0.57</td>
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<tr>
<td>CYP3A4</td>
<td>Midazolam 1'-hydroxylation</td>
<td>4.8</td>
<td>3.878</td>
<td>0.60</td>
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<tr>
<td></td>
<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>
</tr>
</tbody>
</table>
Table 2  Intrinsic clearance and protein binding values of losartan, meloxicam, and sildenafil measured in rCYP and HLM systems.

<table>
<thead>
<tr>
<th>Testing System</th>
<th>Losartan</th>
<th>Meloxicam</th>
<th>Sildenafil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL\text{int,u} (min\textsuperscript{-1})</td>
<td>f\textsubscript{u}</td>
<td>CL\text{int,u} (min\textsuperscript{-1})</td>
</tr>
<tr>
<td>rCYP1A2</td>
<td>0.0007</td>
<td>0.80</td>
<td>0.0029</td>
</tr>
<tr>
<td>rCYP2C9</td>
<td>0.0068</td>
<td>0.90</td>
<td>0.0039</td>
</tr>
<tr>
<td>rCYP2C19</td>
<td>0.0006</td>
<td>0.91</td>
<td>0.0073</td>
</tr>
<tr>
<td>rCYP2D6</td>
<td>0</td>
<td>0.83</td>
<td>0.0010</td>
</tr>
<tr>
<td>rCYP3A4</td>
<td>0.0134</td>
<td>0.82</td>
<td>0.0133</td>
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<tr>
<td>HLM</td>
<td>0.0042</td>
<td>0.88</td>
<td>0.0004</td>
</tr>
</tbody>
</table>
Table 3  RAF-scaled $\text{CL}_{\text{int}}$ and ICR values of losartan, meloxicam, and sildenafil using RAF values derived from various combinations of probe substrate for CYP2C9 and CYP3A4.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CYP3A4 Probe Substrate</th>
<th>CYP2C9 Probe Substrate</th>
<th>CYP3A4 Probe Substrate</th>
<th>CYP2C9 Probe Substrate</th>
<th>CYP3A4 Probe Substrate</th>
<th>CYP2C9 Probe Substrate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diclofenac</td>
<td>Tolbutamide</td>
<td>$S$-Warfarin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RAF-scaled $\text{CL}_{\text{int}}$ (min$^{-1}$)</td>
<td>Measured $\text{CL}_{\text{int}}$ (min$^{-1}$)</td>
<td>ICR $c$</td>
<td>RAF-scaled $\text{CL}_{\text{int}}$ (min$^{-1}$)</td>
<td>Measured $\text{CL}_{\text{int}}$ (min$^{-1}$)</td>
<td>ICR $c$</td>
</tr>
<tr>
<td>Losartan</td>
<td>Midazolam</td>
<td>0.0149</td>
<td>0.0042</td>
<td>3.5</td>
<td>0.0132</td>
<td>0.0042</td>
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<tr>
<td></td>
<td>Nifedipine</td>
<td>0.0127</td>
<td>0.0042</td>
<td>3.0</td>
<td>0.0110</td>
<td>0.0042</td>
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<tr>
<td></td>
<td>Testosterone</td>
<td>0.0032</td>
<td>0.0042</td>
<td>0.8</td>
<td>0.0015</td>
<td>0.0042</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>Midazolam</td>
<td>0.0140</td>
<td>0.0004</td>
<td>39.1</td>
<td>0.0130</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td>0.0117</td>
<td>0.0004</td>
<td>32.8</td>
<td>0.0107</td>
<td>0.0004</td>
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<tr>
<td></td>
<td>Testosterone</td>
<td>0.0024</td>
<td>0.0004</td>
<td>6.6</td>
<td>0.0014</td>
<td>0.0004</td>
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<tr>
<td>Sildenafil</td>
<td>Midazolam</td>
<td>1.1028</td>
<td>0.2663</td>
<td>4.1</td>
<td>1.0923</td>
<td>0.2663</td>
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<tr>
<td></td>
<td>Nifedipine</td>
<td>0.8988</td>
<td>0.2663</td>
<td>3.4</td>
<td>0.8883</td>
<td>0.2663</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>0.0530</td>
<td>0.2663</td>
<td>0.2</td>
<td>0.0425</td>
<td>0.2663</td>
</tr>
</tbody>
</table>

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

$^b$ Measured $\text{CL}_{\text{int}}$ based on $\text{CL}_{\text{int,u}}$ values of HLM in Table 2.

$^c$ ICR: Intersystem clearance ratio = RAF-scaled $\text{CL}_{\text{int}}$ / Measured $\text{CL}_{\text{int}}$. 

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Figure 1
Figure 2:

A) Percentage of CYP isoform for CL int of Losartan in Midazolam & Diclofenac, Midazolam & Tolbutamide, and Midazolam & S-Warfarin.

B) Percentage of CYP isoform for CL int of Losartan in Nifedipine & Diclofenac, Nifedipine & Tolbutamide, and Nifedipine & S-Warfarin.

C) Percentage of CYP isoform for CL int of Losartan in Testosterone & Diclofenac, Testosterone & Tolbutamide, and Testosterone & S-Warfarin.
Figure 3
Figure 4