**Special Section on Prediction of Human Pharmacokinetic Parameters from In Vitro Systems**

**In Vitro–In Vivo Correlation for Low-Clearance Compounds Using Hepatocyte Relay Method**

Li Di, Karen Atkinson, Christine C. Orozco, Carrie Funk, Hui Zhang, Thomas S. McDonald, Beijing Tan, Jian Lin, Cheng Chang, and R. Scott Obach

Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc., Groton, Connecticut

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**ABSTRACT**

In vitro–in vivo correlation (IVIVC) of intrinsic clearance in preclinical species of rat and dog was established using the hepatocyte relay method to support high-confidence prediction of human pharmacokinetics for low-clearance compounds. Good IVIVC of intrinsic clearance was observed for most of the compounds, with predicted values within 2-fold of the observed values. The exceptions involved transporter-mediated uptake clearance or metabolizing enzymes with extensive extrahepatic contribution. This is the first assay available to address low clearance challenges in preclinical species for IVIVC in drug discovery. It extends the utility of the hepatocyte relay method in addressing low clearance issues.

**Introduction**

An important goal of drug metabolism and pharmacokinetics scientists in drug discovery is to be able to predict human pharmacokinetics (PK) and dosing regimen using in vitro data from human-derived reagents such as hepatocytes and liver microsomes. To increase high confidence in prediction, it is desirable to accurately predict PK parameters for the same compound(s) in at least two preclinical species, typically rats and dogs, using corresponding in vitro reagents from three species (Wilby et al., 2011). Therefore, developing in vitro–in vivo correlation (IVIVC) in preclinical species is highly valuable to gain confidence in human translation.

Clearance is one of the most challenging PK parameters to accurately predict due to species differences in drug-metabolizing enzymes, potential for multiple metabolic pathways, and contribution of drug transport. Chemical series that demonstrate strong IVIVC in preclinical species (e.g., rats, dogs) are more likely to give a reliable prediction of human clearance from in vitro data (Wilby et al., 2011). However, for low-clearance compounds (defined as intrinsic clearance not measurable using standard liver microsomal or hepatocyte assays), in vitro hepatic clearance values are not usually available due to a lack of appropriate tools in assessing poorly metabolized compounds in drug discovery. This leads to insufficient supporting evidence of adequate IVIVC from preclinical species, and therefore low confidence in human translation.

In drug discovery, low-intrinsic-clearance compounds have increased significantly over the years due to the effective use of high-throughput absorption, distribution, metabolism, excretion, and toxicity screening and early metabolite identification, which enable a rapid structure-activity relationship to address high-intrinsic clearance as a liability (Di et al., 2012). In addition, new chemical space of drug discovery is being explored, such as liver targeting (Oballa et al., 2011; Pfefferkorn et al., 2012) and once-weekly dosing (Rangan et al., 2007), and all of these require low clearance. Although low clearance can be a beneficial attribute in a new drug to increase exposure, prolong half-life, and reduce dose, it presents a challenge for drug metabolism and pharmacokinetics scientists, because tools available in drug discovery to accurately measure low clearance are limited. As a consequence, it is often inaccurately assumed that no metabolism is occurring when intrinsic clearance is below the limit of in vitro microsomal or hepatocyte stability assays. This frequently leads to inaccurate prediction of human clearance, half-life, and dose. To address low clearance challenges, the hepatocyte relay method was recently developed to predict human intrinsic clearance for low-intrinsic-clearance compounds (Di et al., 2012). In this study, we expanded the hepatocyte relay method to predict rat and dog intrinsic clearance to establish IVIVC in three preclinical species to support high-confidence translation of human IVIVC for low-clearance compounds. This is the first in vitro tool available to study low clearance in rats and dogs when limited to measuring intrinsic clearance by monitoring substrate depletion.

**Materials and Methods**

Test compounds were obtained from Pfizer Global Material Management (Groton, CT) or purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were obtained from Sigma-Aldrich unless otherwise specified. Ninety-six-well
polypropylene plates were from Thermo Fisher Scientific (Pittsburgh, PA) and 24-well polystyrene plates were obtained from Costar (Sigma-Aldrich).

**Rat and Dog Hepatocyte Relay Method.** The rat and dog hepatocyte relay methods are similar to the human hepatocyte relay assay previously reported (Di et al., 2012). Pooled cryopreserved male beagle dog hepatocytes from 5 dogs and male Wistar-Han rat hepatocytes from 12 rats were purchased from Celsis IVT (Baltimore, MD). Upon thawing, the hepatocytes were resuspended in Williams’ E medium (WEM Gibco-BRL, Invitrogen, Grand Island, NY; catalogue number C1984, custom formula number 91-5233EC) supplemented with 50 mM HEPES and 26 mM NaHCO3. The cells were counted using the trypan blue exclusion method, and the 24-well hepatocyte plates containing 0.5 million cells/ml were spiked with a compound at a final concentration of 1 µM (final dimethylsulfoxide of 0.025% and MeOH of 0.125%), in a final incubation volume of 0.5 ml. The plates were covered with Breath-Easy gas-permeable membranes or their equivalent (Diversified Biotech, Dedham, MA) and incubated at 37°C with 95% O2/5% CO2, 75% relative humidity for 4 hours at 150 rpm on an orbital shaker in an incubator. At time 0 and 4 hours, 25 µl of hepatocyte suspension was removed from the incubation and added to 50 µl of cold acetonitrile containing internal standard to quench the reaction. The samples were centrifuged (Eppendorf, Hauppauge, NY) at 3000g for 10 minutes at room temperature, and 50 µl of supernatant was transferred to a clean plate, dried completely, and reconstituted in 50% methanol/50% water prior to liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis. The remaining hepatocyte suspension in the incubation plate was centrifuged (3000g, 10 minutes, room temperature), and 25 µl of supernatant was removed and added to 50 µl of cold acetonitrile containing internal standard to determine the concentration of the supernatant. A total of 300 µl of the remaining supernatant was transferred to a clean 24-well plate and stored at −80°C until the next relay experiment. For the second relay experiment, the supernatant plates were warmed to 37°C for 30 minutes, and hepatocytes were added to the samples to yield a final cell density of 0.5 million cells/ml. The plates were incubated at 37°C for 4 hours, sampled, and processed as described earlier. Five relays were performed to give a total incubation time of 20 hours. A buffer control plate (without hepatocytes) was run under the same conditions to monitor any nonmetabolic decline. Standard curves were prepared using the same matrix material.

**LC/MS/MS Quantification.** The LC mobile phases were as follows: (A) high-performance liquid chromatography–grade water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid, or (A) 95% 2 mM ammonium acetate in water and 5% 50/50 methanol/acetonitrile and (B) 90% 50/50 methanol/acetonitrile and 10% 2 mM ammonium acetate in water. A solvent gradient from 5% (B) to 95% (B) over 2.0 minutes at a flow rate of 0.5 ml/min was used to elute the compounds from the column (Kinetex C18, 30 × 2 mm, 2.6µm; Phenomenex, Torrance, CA). The cycle time was 3 minutes/injection. An aliquot of 15 µl was injected for analysis using a CTC PAL autosampler (LEAP Technologies, Carborro, NC). The analysis was conducted with Shimadzu high-performance liquid chromatography AD20 pumps (Shimadzu, Columbia, MD) connected to an AB Sciex (Foster City, CA) API 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source using multiple reaction monitoring mode. Analyst 1.5.2 software (Applied Biosystems, Foster City, CA) was applied to data collection. MultiQuant software (version 2.1) from AB Sciex was used for data processing and analysis. Metabolite (200 ng/ml, positive mode), imidethacin (250 ng/ml, negative mode), and terfenadine (40 ng/ml, positive mode) were used as internal standards for LC/MS/MS quantification in positive and negative ion multiple reaction monitoring mode, respectively. Only one internal standard was used in the final calculation depending on the ionization mode. All test compounds had good linearity with R2 > 0.99, and the lower limit of quantitation was 1 nM for all compounds.

**Calculations.** The data transformation and calculation for the hepatocyte relay assay has been discussed previously (Di et al., 2012). The hepatocyte relay assay requires transfer of the supernatant between plates for the different relay incubations. Drug is lost due to hepatocyte uptake, nonspecific binding, or other reasons, and dilution of the drug occurs as new hepatocytes and medium are added post transfer. These losses necessitate a correction before comparison with time 0 concentrations. The correction equation is as follows (eq. 1):

\[
C_{\text{corrected}} = C_n \times \frac{\text{recovery}}{\text{transfer volume}} \times \frac{V_{\text{transfer}}}{V_{\text{total}}} \times \frac{C_{\text{total}}}{C_{\text{supernatant}}}
\]

Here, \(C_{\text{corrected}}\) is the total concentration of the well after transfer corrected for loss and dilution. Recovery accounts for the fraction of the compound that is irretrievable due to nonspecific binding to the well or other sources, and should be set to unity unless it is known that drug is lost after each transfer at a location not captured in the total-concentration measurement. Recovery can be estimated experimentally by measuring the change in concentration of a representative solution from before and after addition to a well in the absence of cells (buffer control). The \(n\) variable is the number of transfers. The ratio of the well volume to the supernatant-transfer volume corrects for the dilution which occurs after each transfer. Equation 1 assumes that the ratio is fixed, i.e., the total and transfer volumes remain constant throughout the relay assay. Deviations can be handled by eq. 2. The final term, the product of the ratios of the total and supernatant concentrations in the steps previous to the transfer, corrects for loss in the hepatocyte precipitate. The corrected values can be compared directly to values obtained at early time points prior to any transfer.

\[
C_{\text{corrected}} = C_n \times \frac{\text{recovery}}{\text{transfer volume}} \times \frac{V_{\text{transfer}}}{V_{\text{total}}} \times \frac{C_{\text{total}}}{C_{\text{supernatant}}}
\]

Half-life was calculated using eq. 3, and intrinsic clearance was obtained using eq. 4 for rats and eq. 5 for dogs with the corrected concentration \(C_{\text{corrected}}\) discussed earlier (Hosea et al., 2009).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>In Vivo Rat Intrinsic Clearance</th>
<th>In Vitro Intrinsic Clearance from Rat Hepatocyte Relay Method ± S.D.</th>
<th>Fold Difference In Vivo/Relay Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>4.3 (Nakazawa et al., 1985)</td>
<td>3.0 ± 0.76</td>
<td>1.4</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>9.9 (Ashforth et al., 1995; Mandula et al., 2006)</td>
<td>9.8 ± 0.71</td>
<td>0.61</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>6.5 (Belpaire et al., 1990)</td>
<td>2.4 ± 1.35</td>
<td>2.7</td>
</tr>
<tr>
<td>Famotidine</td>
<td>9.4 (Matsuzaki et al., 2008)</td>
<td>6.6 ± 2.31</td>
<td>1.4</td>
</tr>
<tr>
<td>(±)-Warfarin</td>
<td>19 (Yaoobi and Levy, 1977; Hirate et al., 1990)</td>
<td>12 ± 0.01</td>
<td>1.6</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>20 (Eldershaw et al., 1996)</td>
<td>18 ± 0.39</td>
<td>1.1</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>27 (McPherson and Lee, 1977; Kurata et al., 2010)</td>
<td>16 ± 1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Naproxen</td>
<td>38 (Huntjes et al., 2006)</td>
<td>37 ± 5.04</td>
<td>1.0</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>47 (Tahara et al., 2005)</td>
<td>21 ± 1.44</td>
<td>2.3</td>
</tr>
<tr>
<td>(±)-Acebutolol</td>
<td>88 (Puiguerre-Miller and Jamali, 1997)</td>
<td>7.9 ± 0.89</td>
<td>11</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>85-154 (Watanabe et al., 2010a)</td>
<td>54 ± 2.77</td>
<td>1.6-2.9</td>
</tr>
<tr>
<td>(±)-Ketoprofen</td>
<td>155 (Satterwhite and Boudinot, 1992)</td>
<td>70 ± 12</td>
<td>2.2</td>
</tr>
<tr>
<td>Prazosin</td>
<td>196 (Ohkura et al., 1999)</td>
<td>45 ± 0.76</td>
<td>4.3</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1916 (Huang et al., 2012)</td>
<td>79 ± 0.05</td>
<td>24</td>
</tr>
</tbody>
</table>
In vivo intrinsic clearance was calculated using the well-stirred model (Ashforth et al., 1995) (eq. 6) based on in vivo i.v. plasma clearance data from the literature, where \( Q_H \) is hepatic blood flow (70 and 40 ml/min/kg for rats and dogs, respectively), \( CL_b \) is hepatic blood clearance, \( f_{up} \) is fraction unbound in plasma, and \( R_B \) is the ratio of blood to plasma concentration.

\[
CL_{int} = \frac{CL_b f_{up}}{R_B} \left( 1 - \frac{CL_b}{Q_H} \right)
\]

ACD/Laboratories software version 12.0 (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) was used to calculate log D/P for all compounds.

**Results**

A set of low-clearance compounds in rats and dogs was selected for the study based on available literature data and low intrinsic hepatic metabolic clearance values. In vivo i.v. clearance values were obtained from the literature, and hepatic clearance values were calculated by subtracting renal and biliary clearance from plasma clearance. Variability of the in vivo data can lead to disconnect of IVIVC. Intravenous data from multiple strains were used assuming minimal strain difference in clearance (Richmond et al., 2010). No oral PK data were included to ensure data quality. In vivo intrinsic clearance was calculated from in vivo hepatic clearance using the well-stirred model (Hallifax et al., 2010) by incorporating literature or in-house plasma protein binding and blood-to-plasma ratio. Intrinsic clearance values from hepatocyte relay were obtained using five relays (20-hour accumulative incubation time) with a density of 0.5 million cell/ml and were reported as mean values of two or three replicates. All test compounds had significant turnover under the assay conditions and demonstrated good linearity. Hepatocyte binding was not incorporated for correction of unbound intrinsic clearance, since all compounds have \( f_{u,hep} \) (fraction unbound in the hepatocyte incubation) values close to 1 based on the calculated values (Kilford et al., 2008) estimated using log P/D from the ACD software.

**Rat IVIVC for Low-Clearance Compounds.** The rat intrinsic clearance values from both the in vivo and in vitro hepatocyte relay methods are summarized in Table 1. The in vivo rat intrinsic clearance values ranged from 4 to 2000 ml/min/kg. The in vivo literature clearance values were from a variety of rat strains (e.g., Sprague-Dawley, Wistar, Fischer) and both genders, since data from the Wistar-Han rat was not available for all compounds. Strain and gender difference in drug metabolism was assumed to be minimal (Richmond et al., 2010), although exceptions do exist for certain compounds. The hepatocytes used in the rat relay assay were Wistar-Han, which was a standard strain for the Pfizer internal rat hepatocyte stability assay. In general, most compounds showed good IVIVC, with the in vitro hepatocyte relay assay within 2- to 3-fold of in vivo intrinsic clearance values (Fig. 1). The exceptions had a high hepatic uptake component by transporters [e.g., fexofenadine (Poirier et al., 2009), fluvastatin (Watanabe et al., 2010a), ketoprofen (Morita et al., 2005), prazosin (Qin et al., 1994), and bosentan (Huang et al., 2012)] or potential contribution from extrahepatic metabolism [e.g., hydrolysis of acebutolol (Andresen and Davis, 2009)].
IVIVC of Low Clearance

<table>
<thead>
<tr>
<th>Compounds</th>
<th>In Vivo Dog Intrinsic Clearance</th>
<th>In Vitro Intrinsic Clearance from Dog Hepatocyte Relay Method ± S.D.</th>
<th>Fold Difference In Vivo/Relay Method</th>
</tr>
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<tr>
<td></td>
<td>ml/min/kg</td>
<td>ml/min/kg</td>
<td></td>
</tr>
<tr>
<td>Ranitidine</td>
<td>7.7 (Bayliss and Cross, 2000)</td>
<td>7.4 ± 0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>11.6 (Kimura et al., 1999)</td>
<td>9.0 ± 3.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>14 (Balani et al., 2002)</td>
<td>14 ± 1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Naproxen</td>
<td>22 (Suh et al., 1997)</td>
<td>20 ± 3.0</td>
<td>1.1</td>
</tr>
<tr>
<td>(+/-)-Ketoprofen</td>
<td>48 – 114 (Granero and Amidon, 2008; Neirinckx et al., 2011)</td>
<td>24 ± 1.2</td>
<td>2.0–4.7†</td>
</tr>
</tbody>
</table>

† with the exception of ketoprofen.

Discussion

Rats and dogs are the most common preclinical species used in drug metabolism, PK, pharmacodynamics, and safety evaluation studies of new chemical entities. Good IVIVC in rats and dogs usually translates to a high-confidence prediction of human PK and clinical dose. There were no adequate in vitro tools to determine low clearance in drug discovery. If a compound has no measurable turnover in liver microsomes or hepatocytes under the standard assay conditions, compounds would be reported as having no metabolism or being below a certain cutoff clearance value. The development of the hepatocyte relay method enables determination of clearance values for low-clearance compounds early in drug discovery to support structure–activity relationship, prioritization of chemical series, and for human PK and dose projection (Di et al., 2012). The hepatocyte relay method has been shown to correlate well with in vivo human intrinsic clearance. Here, the IVIVC in rats and dogs was evaluated for low-clearance compounds to assess application of the method in preclinical species.

Typically, the hepatocyte relay method used an incubation time of 20 hours, which allowed an estimation of half-life in hepatocytes of up to 40 hours (Di et al., 2004). In theory, the limit of the low clearance value that can be measured decreases with an increasing number of relays. However, based on the theoretical calculation, intrinsic clearance values drop most significantly for the first few relays. As incubation time goes beyond 20 hours, there is little gain in clearance values from each additional relay. Therefore, in practice, five relays are the standard assay conditions used to support drug discovery programs. However, the development of the hepatocyte relay method enables determination of clearance values for low-clearance compounds early in drug discovery to support structure–activity relationship, prioritization of chemical series, and for human PK and dose projection (Di et al., 2012). The hepatocyte relay method has been shown to correlate well with in vivo human intrinsic clearance. Here, the IVIVC in rats and dogs was evaluated for low-clearance compounds to assess application of the method in preclinical species.

As shown in Tables 1–3 and Fig. 1, the rat, dog, and human hepatocyte relay method gave good prediction of in vivo intrinsic clearance [additional human data were collected during this study and added to the previously published set (Di et al., 2012)]. For most compounds...
the in vivo and in vitro intrinsic clearance ratios were within 2-fold, suggesting good IVIVC. The exceptions involved significant active uptake from transporters or contribution of extrahepatic metabolism. Figure 2 shows the IVIVC of five compounds with rat, dog, and human data. Good human IVIVC was observed when both rats and dogs demonstrated strong IVIVC (i.e., ranitidine, tolbutamide, antipyrine, and naproxen). For ketoprofen, the rank order of the species is correct; the in vivo values are underpredicted. About 5-fold underprediction of human in vivo intrinsic clearance was observed for ketoprofen. Correction factor derived from rats and dogs can be applied to enhance human prediction accuracy of intrinsic clearance. Weak or no IVIVC in preclinical species often translated to low-confidence prediction of human in vivo clearance from in vitro data.

In rats, significant contribution of transporter-mediated clearance was observed for ketoprofen (Poirier et al., 2009), leading to underprediction of in vivo clearance based on metabolic clearance from hepatocytes since transporter uptake also contributes to the systemic clearance (Yamazaki et al., 1996; Shitara et al., 2006; Watanabe et al., 2010b). In addition, potential extrahepatic contribution of UGT metabolism in rats can lead to underprediction of in vivo clearance.
(Terrier et al., 1999). In dogs, the major elimination pathway for ketoprofen was through UGT metabolism, and the kidneys made a significant contribution to the metabolism (Granero and Amidon, 2008). In humans, ketoprofen was mainly metabolized by CYP2C9 and UGT2B7 (Kilford et al., 2009). The extraplatelet contribution of UGT metabolism could potentially contribute to the underprediction of intrinsic clearance using hepatocytes for ketoprofen.

This study indicated that the rat and dog hepatocyte relay method gave good prediction of in vivo hepatic clearance for low-clearance compounds. The hepatocyte relay method extended the low limit for intrinsic clearance measurement by about 10-fold, which enhanced our ability to address the challenges of low-intrinsic-clearance compounds. The good in vivo prediction of clearance by the rat and dog hepatocyte relay methods highlights the wide applications of the relay approach not only in human intrinsic clearance prediction (Di et al., 2012) but also in preclinical species.

Conclusions

The rat and dog hepatocyte relay method has been successfully developed and applied to predict in vivo clearance for low-clearance compounds. Good IVIVC was observed for most of the compounds, with the ratio of in vivo to in vitro intrinsic clearance 2-fold. The outliers were due to the contribution of transporter-mediated clearance or extraplatelet metabolism. This is the first method available to study low-intrinsic-clearance rat compounds in rats and dogs in drug discovery. It highlights the broad applications of the hepatocyte relay method in preclinical as well as humans for developing IVIVC.

Acknowledgments

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Authorship Contributions

Participated in research design: Di, Atkinson, Orozco, Funk, Zhang, McDonald, Tan, Obach.
Conducted experiments: Atkinson, Orozco, Funk, McDonald, Tan.
Performed data analysis: Di, Atkinson, Orozco, Funk, Zhang, McDonald, Tan, Obach, Chang, Obach.

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