In Vitro Hepatic Uptake in Human and Monkey Hepatocytes in the Presence and Absence of Serum Protein and Its In Vitro to In Vivo Extrapolation

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ABSTRACT

It is well documented that human hepatic clearance based on in vitro metabolism or transporter assays systematically resulted in under-prediction; therefore, large empirical scalars are often needed in either static or physiologically based pharmacokinetic (PBPK) models to accurately predict human pharmacokinetics (PK). In our current investigation, we assessed hepatic uptake in hepatocyte suspension in Krebs-Henseleit buffer in the presence and absence of serum. The results showed that the unbound intrinsic active clearance (CLu,int,active) values obtained by normalizing the unbound fraction in the buffer containing 10% serum were generally higher in monkey hepatocytes than that in human, and the species differences appeared to be compound dependent. Scaling up in vitro uptake values derived in assays containing species-specific serum can compensate for the species-specific variabilities when using cynomolgus monkey as a probe animal model. Incorporating SFs calibrated in monkey and together with scaled in vitro data can be a reliable approach for the prospective human PK prediction in early drug discovery.

SIGNIFICANCE STATEMENT

We investigated the protein effect on hepatic uptake in human and monkey hepatocytes and improved the in vitro to in vivo extrapolation using parameters obtained from the incubation in the present of serum protein. In addition, significantly higher active uptake clearances were observed in monkey hepatocytes than in human, and the species differences appeared to be compound dependent. The physiologically based pharmacokinetic model that incorporates scaling factors calibrated in monkey and together with scaled in vitro human data can be a reliable approach for the prospective human pharmacokinetics prediction.

Introduction

Accurately predicting hepatic clearance is essential for ranking and optimizing new chemical entities in the current drug discovery and development practices; furthermore, it is critically needed for understanding potential oral bioavailability, evaluating drug-drug interactions (DDIs), and determining doses in first-in-man trials. As systemic clearance (CL) is a fundamental pharmacokinetic (PK) parameter for human dose projection, discovery of bioavailable and metabolically stable small molecule drug candidates are ideal goals in early PK optimization. Prediction of systemic clearance for drug candidates by major elimination organ liver is more involved. Over the past two decades, many empirical and physiologically based approaches have been developed for human CL prediction (Ito and Houston, 2005; Chiba et al., 2009). For example, in vitro metabolic stability assays using liver derived systems such as liver microsomes, cytosols, and hepatocytes are routinely used for assessing enzyme stability in the early discovery stage as a high-throughput tool to select metabolically stable molecules in pharmaceutical companies (Obach et al., 1997). The rationale of these approaches is that the liver preparations prepared from human or preclinical species can reserve the enzyme activities and should reasonably represent in vivo clearance.

Recently, increasing recognition was given to transporter-mediated clearance in the role of affecting drug bioavailability (first-pass hepatic extraction) and elimination. Many pharmacogenomics and DDI studies in organic anion transporting polypeptide (OATP) substrates showed transporter-mediated clearance affecting systemic drug exposure (Lai et al., 2010, 2012; El-Kattan et al., 2016; Yee et al., 2018). Incorporating transporter-mediated CL in the prediction of overall hepatic CL, also

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ABBREVIATIONS: CL, clearance; CLu,int,active, unbound intrinsic active clearance; CLu,int,passive, unbound intrinsic passive clearance; SF, scaling factor.
known as the extended clearance concept, was first introduced by Siriani and Pang and was extensively investigated by other research groups (Siriani and Pang, 1997; Kunze et al., 2015; Patleia-Vrana and Unadkat, 2016; Benet et al., 2018). Currently, a range of in vitro tools with increasing sophistication of transporter expressions are used to characterize transporter-mediated CL parameters for human PK prediction. Among the in vitro systems, hepatocytes with expression of transporter and enzyme proteins that mimic in vivo are often preferred to estimate in vitro hepatic uptake CL in suspended, plated, and sandwich-cultured hepatocytes formats. However, the discrepancies in in vitro to in vivo extrapolation (IVIVE), e.g., underpredicting in vivo hepatic uptake CL, are concerns that merited further investigations (Jones et al., 2012; Barton et al., 2013). Commonly large empirical scaling factors (SFs) were applied for transporter-mediated CL to fit in vivo human PK (Jones et al., 2012). The need for large empirical SFs for IVIVE also holds true in preclinical species (Watanabe et al., 2009; Morse et al., 2017; De Bruyn et al., 2018). The SFs appeared to be compound dependent, and the highly protein-bound compounds tended to have larger SFs (Jones et al., 2012; Morse et al., 2017; De Bruyn et al., 2018). Over the past years, various efforts have been made to reduce the IVIVE SFs through optimizing in vitro methodologies, for example, to measure the difference of transporter expressions between in vitro and in vivo (Li et al., 2010), to incorporate human serum protein (Bowman et al., 2019; Kim et al., 2019), and to establish a “universal” SF from an internal/local in vitro system for laboratory specific parameters (De Bruyn et al., 2018). In our current investigation, hepatic uptake assays were conducted in suspension human and monkey hepatocytes in the presence or absence of their respective serum to elucidate the impact of protein on the active uptake for known OATP substrates. Species differences of intrinsic total uptake clearance (CL\textsubscript{int,uptake}) between human and monkey hepatocytes were also evaluated in the presence of serum protein. Additionally, a physiologically based pharmacokinetic (PBPK) model was developed to obtain SFs from the IVIVE in monkey and human. Our comprehensive investigation on species differences in hepatic uptake for 15 OATP substrates provided insightful information for the future usage of cynomolgus monkey as a probe animal model for SFs to predict human PK. Furthermore, the extensive analysis of IVIVE using compounds with a broader range of protein binding demonstrated the need for incorporating the protein-facilitated uptake for the human PK prediction.

Materials and Methods

Materials. Pitavastatin calcium was purchased from Fisher Scientific Company, LLC (Pittsburgh, PA). Bosentan hydrate, danoprevir, labetolol, repaglinide, valsartan, maraviroc, telmisartan, cerivastatin sodium, fluvasatin sodium, pravastatin sodium, atorvastatin calcium, rosuvastatin calcium, buphenine, warfarin, silicone oil, and mineral oil were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Gazoprevir was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Sorafenib was purchased from Selleck Chemicals (Houston, TX). Asunaprevir were synthesized in house. Cryopreserved human (lots XPM, YTW, and PZA) (Supplemental Table 1), cynomolgus monkey (lots PNC, VNV, and UHK) hepatocytes (Supplemental Table 2), in VitroGro-HT medium. The hepatocytes were centrifuged at 50g for 4 minutes at 4°C. After centrifuging, the cells were gently resuspended in ice-cold KHB buffer. Cell viability was determined by trypan blue staining. The cell viability of hepatocyte lots used in this study exceeded 80%. The hepatocytes were diluted to 2 million cells/ml in KHB with 10% (v/v) human and cynomolgus monkey serum, respectively. The compounds (1000× concentration in DMSO) were diluted in uptake buffer (KHB with or without 10% human or cynomolgus monkey serum). Prior to uptake experiments, cell suspension and uptake buffer containing 2× substrate concentration was incubated at warm or ice-cold water bath for 10 minutes to reach the uptake temperature at 37°C or 4°C. Uptake assays were initiated by adding the uptake buffer to cell suspension (1:1 in v/v), which resulted in 1× final substrate concentration in a cell density of 1 million cells/ml. For the uptake studies, all compounds were performed in 1 μM final concentration, except repaglinide, which had a final concentration of 0.1 μM. The incubations were terminated at 0.25, 0.5, 1, 1.5, and 5 minutes by collecting 100 μl of incubation mixture onto a microcentrifuge tube containing two layers preloaded. The bottom layer contained 100 μl of 3 M ammonium acetate, and the upper layer contained 100 μl oil mixture of silicone oil and mineral oil (density = 1.015). The microcentrifuge tubes were immediately centrifuged at 14,000 rpm for 14 seconds in Eppendorf benchtop centrifuge. The oil layer separated the cells from the uptake buffer. Microcentrifuge tubes were immediately placed on dry ice and transferred to −80°C freezer until analysis. The active transporter-mediated uptake was assessed at 37°C, and passive diffusion was assessed at 4°C, assuming minimal transporter activities at 4°C. For each batch of uptake experiment, rosuvastatin was included to monitor variations from batch to batch. The uptake assay was conducted in triplicates at each time point for all compounds. Human hepatocyte lot XPM and monkey hepatocyte lot UHK were used in the uptake study to compare the in vitro hepatic uptake clearance in the presence or absence of serum protein. Moreover, three donors of human (XPM, PZA, and YTW) and monkey (UHK, PNC, and VNV) hepatocytes were used to further assess the donor variability.

Liquid Chromatography–Tandem Mass Spectroscopy Analysis. Tips of microcentrifuge tubes containing hepatocyte pellets were cut and placed upside down in deep 96 well plates. One hundred microliters of deionized water was added to each well, and the cells were sonicated for 10 minutes. Two hundred microliters of 100% acetonitrile with internal standard, labetolol, was added to the wells for compound extraction. The samples were sonicated for 10 minutes, followed by shaking on a shaker for 20 minutes. After additional 5 minutes sonication, the samples were centrifuged at 4000 rpm for 20 minutes at 4°C. Standard curves for quantitation were prepared in blank hepatocyte pellets that were treated similarly to hepatocyte samples. One hundred fifty microliters aliquot of the spiked serum was transferred into 200 μl buffer containing 20% acetonitrile and 80% water with 0.1% formic acid. The samples were vortexed and centrifuged at 3500 rpm at 4°C for 20 minutes before liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. All the samples were analyzed on a Sciex Qtrap 6500 LC-MS/MS (Redwood City, CA) coupled with a Shimadzu Nexera-X2 ultra-high-performance liquid chromatograph (Shimadzu Corporation, Kyoto, Japan). Ten microliters of the sample were injected onto a Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) (Milford, MA) and eluted by gradient mobile phases of 0.1% formic acid in water (A) and acetonitrile (B). The LC-MS/MS conditions for each compound are summarized in Supplemental Table 3.

Unbound Fraction in Serum Protein. Serum protein binding of 15 compounds was determined in 100% and 10% human or monkey serum by equilibrium dialysis with a Rapid Equilibrium Dialysis Device (Thermo Fisher Scientific, Rockford, IL.). The DMSO stock solution of the test article was spiked into 100% or 10% (diluted in KHB buffer) human or monkey serum to a final concentration of 2 μM. One hundred microliters aliquot of the spiked serum was transferred to a 96-well deep-well plate as the T0 sample. Blank KHB buffer (pH 7.4, 100 μl) was added to the plate to make the matrix of 50:50 (v/v) serum:buffer. The T0 samples were incubated at 37°C for 4 hours. The spiked samples were placed into the sample chamber (300 μl), and the KHB buffer was placed into the adjacent buffer chamber (500 μl). The plate was sealed with a self-adhesive lid and incubated at 37°C on an orbital shaker (250 rpm) for 4 hours. The assay was
carried in duplicates. At the end of the incubations, aliquots (100 µl) were taken from both the serum and buffer chambers. Blank KHB buffer (100 µl) was added to the serum samples, and blank serum or 10% serum (100 µl) were added to the buffer samples. Finally, 300 µl of quench solution (50% acetonitrile and 50% methanol with 0.05% formic acid) containing internal standards (bucetin and warfarin) was added to each sample. The quenched samples were vortexed vigorously for 20 minutes and centrifuged at 4000 rpm at 10°C. The supernatants were transferred to a 96-well plate and analyzed by LC-MS/MS. The percentage free and percentage recovery of the test compound were calculated (Supplemental Table 4).

**In Vitro Uptake Data Analysis.** The CL<sub>u,intr,active</sub> and intrinsic passive uptake clearance (CL<sub>u,intr,passive</sub>) were obtained from the initial uptake rates at 37°C or 4°C, respectively. The initial uptake rates were estimated from the slopes of linear uptake phase using linear regression analysis. The intrinsic uptake clearance values were calculated by dividing the initial uptake velocity by the nominal substrate concentration. The intrinsic active uptake clearance (CL<sub>int,active</sub>) was calculated by subtracting the CL<sub>u,passive</sub> from CL<sub>int,uptake</sub>. The unbound intrinsic uptake clearance [unbound intrinsic active clearance (CL<sub>u,int,active</sub>) and unbound intrinsic passive clearance (CL<sub>u,int,passive</sub>)] was calculated by dividing intrinsic clearance by the measured unbound fraction in buffer containing 10% serum or 100% for study in serum free buffer.

The in vitro intrinsic CL values were expressed as microliters per minute per million cells. The scaled in vivo intrinsic clearances were calculated by multiplying hepatocellularity (125 million cells/g liver in human and 122 million cells/g liver in cynomolgus monkey) and liver weight and were adapted from SimCYP (version 17; Certara Ltd.).

**Hepatic Uptake in Human and Monkey Hepatocytes and Its IVIVE.** PK studies were performed in cynomolgus monkey to understand IVIVE of in vitro hepatic uptake parameters. The PK studies were performed in WuXi AppTec (Suzhou, China). All procedures were approved by an Institutional Animal Care and Use Committee. In brief, each cynomolgus monkey (n = 4 male, 3–5 kg) was dosed at 0.5 mg/kg in 5% DMSO/95% saline solution. Individual doses were calculated based on body weights recorded on the day of dose administration. The intravenous dose was administered as an approximately 30-minute infusion using a calibrated Harvard Apparatus PHD 2000 infusion pump via cephalic vein. Serial blood samples were collected via the femoral vein before dosing and at predefined time points. Blood samples were maintained on ice prior to centrifugation to obtain plasma (K<sub>2</sub>EDTA). Centrifugation began within 1 hour of collection. Plasma samples (approximately 500 µl) were placed into a 96-well tube containing 4 µl of formic acid (the final concentration of formic acid in plasma was approximately 2%), and samples were vortex mixed. The plasma samples were analyzed using LC-MS/MS.

Physiologically Based Pharmacokinetic Model Analysis for In Vivo Hepatic Uptake Parameters. A five-compartment liver model was adapted from previously published PBPK model for OATP substrates (Jones et al., 2012; Morse et al., 2015, 2017). The mass balance differential equations described previously (Jones et al., 2012; Morse et al., 2017) were employed in SAAM II (Epsilon Group, Charlotteville, VA). The tissue partition coefficient (Kp) for each nonliver tissue was obtained from SimCYP (version 17; Certara Ltd.). A fitting procedure for pitavastatin plasma PK curves was performed to determine in vivo hepatic clearance parameters, using a similar procedure previously published for other OATP substrates (Jones et al., 2012; Morse et al., 2017). In brief, the scaled unbound intrinsic CL parameters calculated from the in vitro uptake values obtained from uptake assay using the protein free buffer or buffer containing 10% serum were used as the initial estimates. The fitted values of CL<sub>u,active</sub>, CL<sub>u,passive</sub>, and unbound intrinsic biliary clearance (CL<sub>u,int,bil</sub>) were estimated by fitting the plasma PK curve. The pitavastatin monkey plasma PK data were obtained from in-house data, and the human plasma PK data were digitalized (GnData Graph Digitizer V 2.26.0) from previously published New Drug Application (NDA22363). The empirical SFs were calculated for each of CL<sub>u,int,active</sub> and CL<sub>u,int,passive</sub> by dividing the in vitro scaled value by the fitted value. The median of SFs across the drugs in the data set was calculated.

**Results**

**In Vitro Hepatic Uptake in Human and Monkey Hepatocytes in Presence or Absence of Serum Protein.** The impact of serum protein on the hepatic uptake was first assessed in in suspension human and monkey hepatocytes. The hepatocytes were incubated with known OATP substrates in KHB buffer with or without 10% serum. The unbound serum protein fraction of OATP substrates in both 100% and 10% serum buffer was measured, and data are summarized in Supplemental Table 4. All compounds had good recovery numbers (>80%). Because sorafenib is highly bound to serum protein, the percentage free in 100% human or monkey serum could not be determined. Among these OATP substrates, the serum protein binding values were highly correlated between human and monkey, as R² values were higher than 0.9 in both assays measured in 100% serum and 10% serum containing buffer (Fig. 1). The in vitro values of unbound intrinsic uptake clearance (CL<sub>u,int,active</sub>) and CL<sub>u,int,passive</sub> for 15 OATP substrates obtained from incubation with and without serum protein are summarized in Table 1. As shown in Table 1, the values of CL<sub>u,int,active</sub> obtained in the buffer containing 10% serum protein were generally higher than values obtained directly from the uptake in the protein free buffer in both human and monkey hepatic uptake studies. The shift ranged from 1.5- to 924.6-fold higher in human or 1- to 878.5-fold higher in monkey, respectively. The higher fold differences were observed in higher serum protein bound compounds. In addition, a similar trend of shift was observed in CL<sub>u,int,passive</sub> for highly protein bound compounds. Similar observations were reported in recent publications from other research groups (Bowman et al., 2019, 2020; Kim et al., 2019). In the nonparametric Spearman correlation test, the fold differences of CL<sub>u,int,passive</sub> and CL<sub>u,int,active</sub> in both human (Fig. 2, A and B) and monkey hepatocytes (Fig. 2, C and D) were negatively correlated with the serum protein binding values.

**In Vitro to In Vivo Extrapolation of Hepatic Uptake Clearances in PBPK Modeling.** Recent publications suggested that performing in vitro hepatic uptake studies in the presence of protein could improve the prediction of transporter-mediated hepatic clearance (Bowman et al., 2019; Kim et al., 2019). In our current study, the impact of addition of serum protein in the uptake assay on the improvement of IVIVE was further investigated in PBPK modeling. The in vivo intrinsic CL fitted parameters were estimated by human or monkey PBPK models reported previously (Jones et al., 2012; Morse et al., 2015, 2017), except for pitavastatin. The IVIVE of pitavastatin in monkey and human was performed by a curve fit of plasma PK using in-house PBPK model adapted from previously published models (Jones et al., 2012; Morse et al., 2017) (Fig. 3). As shown in Table 2, the SFs for CL<sub>u,int,active</sub> ranged from 0.1 to 7.7 with the median of 0.8 in human or the median of 0.9 in monkey when using the scaled in vitro parameters obtained from the incubation without serum protein, whereas the SFs ranged from 0.1 to 10.3 with the median of 0.5 in human or the median of 0.6 in monkey when applying the in vitro parameters from the incubation with 10% serum. On the other hand, the SFs for CL<sub>u,int,active</sub> ranged from 7.3 to 106 with the median of 24.2 in human or 22.7 in monkey when in vitro parameters were acquired from the incubation in serum free buffer. As expected, the SFs for CL<sub>u,int,active</sub> ranged from 2.3 to 23.7 with the median of 4.6 in human or 7.1 in monkey when the model incorporated the scaled in vitro parameters from the incubation in buffer containing 10% serum. The results indicated that the empirical SFs were significantly larger when using in vitro parameters obtained in the serum free buffer, as compared with the parameters obtained from the incubation with serum protein added.

**Differences of Hepatic Uptake Clearance in Human and Monkey Hepatocytes for Known OATP Substrates.** To assess the species differences and donor variability in transporter-mediated uptake in hepatocytes between human and cynomolgus monkey, hepatic uptake assays were conducted in two additional donors for each species. The
in vitro uptake assays were performed in the buffer with 10% respective human and monkey serum. The values of in vitro hepatic uptake clearance were then adjusted by the fraction of unbound in 10% serum buffer. The values of \( CL_{\text{u,int,passive}} \), \( CL_{\text{u,int,active}} \), and \( CL_{\text{u,int,passive}} \) for each donor in human and monkey hepatocytes are summarized in the Table 3. In general, the values of uptake clearances obtained from different lots were within 2-fold range, and the values of \( CL_{\text{u,int,passive}} \) between human and monkey hepatocytes were comparable (two-tailed \( P = 0.4 \) in Wilcoxon paired non-parametric test). One the other side, the \( CL_{\text{u,int,active}} \) in monkey hepatocytes were about 2-fold higher than in human hepatocytes (two-tailed \( P = 0.0001 \) in Wilcoxon paired non-parametric test).

Discussion

As a general practice, in vitro metabolic stability is routinely examined using hepatic preparations, and hepatic uptake studies in suspended, plated, and sandwich-cultured hepatocytes are used to estimate hepatic transporter-mediated uptake clearance in early drug discovery. In vitro parameters obtained are then scaled to in vivo

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Serum free buffer</th>
<th>Buffer with 10% serum</th>
<th>Fold difference b</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( CL_{\text{u,int,passive}} )</td>
<td>( CL_{\text{u,int,active}} )</td>
<td></td>
</tr>
<tr>
<td>Human hepatic uptake: lot XPM</td>
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<tr>
<td>Asunaprevir</td>
<td>119.7 (52.3)</td>
<td>19.4 (2.9)</td>
<td>6.24</td>
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<td>Atorvastatin</td>
<td>47.9 (2.0)</td>
<td>5 (1.9)</td>
<td>9.56</td>
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<td>Bosentan</td>
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<td>4.9 (1.2)</td>
<td>5.84</td>
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<td>6.2 (0.2)</td>
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<td>4.8 (2.9)</td>
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<td>12.4 (2.9)</td>
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<td>Human hepatic uptake: lot UHK</td>
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<td>21.6 (1.9)</td>
<td>1.9 (0.3)</td>
<td>11.00</td>
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**a**The in vitro uptake clearance was assessed in single donor of human (lot XPM) and monkey (lot UHK) hepatocytes. The initial uptake rates were estimated from the slopes of linear uptake phase using linear regression fitting. The experiments were conducted in triplicates for each time point. The mean values were averaged from the triplicates, and the S.D.s are presented in parentheses. The intrinsic uptake clearance values were calculated by dividing the initial uptake velocity by the nominal substrate concentration. The \( CL_{\text{int,active}} \) was calculated by subtracting the \( CL_{\text{int,passive}} \) from \( CL_{\text{int,uptake}} \). In vitro uptake clearance is reported as microliters per minute per million cells. In vitro uptake clearance measured in uptake buffer containing 10% human or monkey serum was obtained by normalizing the free fraction of protein binding in 10% serum buffer.

**b**Fold difference = \( CL_{\text{u,int,passive}} \) in 10% serum buffer/\( CL_{\text{u,int,passive}} \) in serum free buffer.
clearances by multiplying the hepatocellularlarity and liver weight (Li et al., 2009; Bi et al., 2012). Although this physiologically based IVIVE appears to be the best approach, systematic underprediction is a well known issue (Yamagata et al., 2017; Bowman and Benet, 2019). To bridge the gap of transporter-mediated clearance IVIVE, empirically derived SFs were widely used to parametrize the transporter-associated clearance such as \( \text{CL}_{u,\text{int,active}} \), \( \text{CL}_{u,\text{int,passive}} \), and unbound intrinsic biliary clearance (\( \text{CL}_{u,\text{int,bile}} \)). In addition, confidence in the prediction is low for the compounds that are less permeable and subject to transporter substrates with high protein binding (Jones et al., 2012, 2015; Yamagata et al., 2017; De Bruyn et al., 2018). Recently, Kim et al. showed that the \( \text{CL}_{u,\text{int,active}} \) increases in the presence of human serum albumin for 11 highly protein bound drugs (Kim et al., 2019). As a result, an improved IVIVE for 11 OATP substrates was achieved using uptake parameters obtained from the incubation in the presence of human serum protein (Kim et al., 2019). Moreover, Bowman et al. also showed similar results by doing uptake studies in the presence of 100% plasma for high protein binding substrates such as atorvastatin and pitavastatin in fresh isolated rat hepatocytes (Bowman et al., 2019). Collectively, a theory of “protein-facilitated” or “transporter-induced protein binding shift” uptake was proposed for the improved IVIVE (Baik and Huang, 2015; Zhang et al., 2015; Miyauchi et al., 2018; Bowman et al., 2019). In the present investigation, we measured the in vitro \( \text{CL}_{\text{int,uptake}} \) in suspension hepatocytes in the buffer with or without 10% human or cynomolgus monkey serum. As expected, in vitro \( \text{CL}_{u,\text{int,active}} \) values obtained from the incubation in the buffer containing 10% serum protein were greatly increased compared with the \( \text{CL}_{u,\text{int,active}} \) values obtained from the incubation in protein free buffer (Table 1). The fold differences highly correlated with the protein binding (Fig. 2), with particularly larger differences being observed for the drugs that were highly protein bound, such as sorafenib (925-fold), asunaprevir (23-fold), and valsartan (15-fold) in human hepatocytes. Similar results were observed in monkey hepatocytes. Therefore, the SFs for \( \text{CL}_{u,\text{int,active}} \) were significantly lower for each compound, especially for highly protein bound drugs, when using scaled \( \text{CL}_{u,\text{int,active}} \) from the incubation in the buffer containing 10% serum protein (Table 2). As expected, less fold shift was observed for lower protein binding compounds such as rosuvastatin and pravastatin. Interestingly, the larger empirical SFs were needed for pravastatin IVIVE in both human and in monkey, which is consistent with the recent report by De Bruyn et al. (2018). These data suggested that uptake clearance of pravastatin was significantly underestimated in vitro, even in the incubation with serum protein. Pravastatin has been reported to be a substrate for many other uptake and efflux transporters (Table 4), and the protein binding is low (57% free in human serum)
higher values of CLu,int,passive observed, especially for highly protein
increased with addition of protein in the uptake experiments as a result of
as well as our present investigation showed that passive diffusion also
appeared to be comparable with the literature SFs using uptake values
protein, and IVIVE involved various elimination pathways are war-
Further investigation is needed in this field.

volume and surface area may affect passive diffusion. Collectively, the
can be defined as the permeability-surface area, the change of cell
mechanism is changing the interaction between the binding to the serum
affected both active uptake and passive diffusion. One possible
(Supplemental Table 4). In addition, renal elimination also significantly
contributes to overall systemic clearance (pravastatin drug label). In
addition, previous publication suggested addition of protein had effect on
both Vmax and Km of transport kinetics (Bowman et al., 2019, 2020; Kim et al., 2019). In the present study, only a single concentration was
used in the uptake assay. Thus, further studies on the contribution of
each transporter, the preservation of transporter function in cryopre-
used in the uptake assay. Thus, further studies on the contribution of

Moreover, recent published studies (Bowman et al., 2019, 2020) and
as well as our present investigation showed that passive diffusion also
increased with addition of protein in the uptake experiments as a result of
higher values of CLu,int,passive observed, especially for highly protein
bound compounds (Fig. 2; Table 1). The data suggested that protein
affected both active uptake and passive diffusion. One possible
mechanism is changing the interaction between the binding to the serum
protein and nonspecific binding to the cells membrane when adding protein to uptake buffer. For example, the serum protein (with
compound highly bound) may bind to cell membrane during the
incubation, and the binding of serum protein on the cell membrane may be not fully washed with buffer or separated by the oil layer. When
we lysed cells, the cell membrane fraction was included in the analysis.
Such contamination may confound the results. Another possible reason
is that the solutes in plasma/serum may change the osmotic pressure of
cell membrane, which may be different in protein free buffer. As in
previous reports, even in an isotonic environment, animal cells face
a problem in maintaining cell volume (Lodish et al., 2000). The solutes
and other molecules in the plasma/serum may affect the function of
ATP-Na/K pump and ion movements and sequentially change the cell
volume and surface area. As the intrinsic clearance of passive diffusion
can be defined as the permeability-surface area, the change of cell
volume and surface area may affect passive diffusion. Collectively, the
mechanisms of protein affecting passive diffusion remain unknown.
Further investigation is needed in this field.

Many studies showed that a “middle-out” approach can reasonably
capture clinical PK profiles and predict clinical DDIs when using PBPK
models (Varma et al., 2012; Barton et al., 2013; Duan et al., 2017).
Unfortunately, human PK data are unlikely to be available during drug
discovery and early development phase, which limits the application of
the “middle-out” modeling. As such, to incorporate transporter-mediated
clearance for human PK prediction during compound selection and
engage PBPK modeling along lead optimization, one of critical options
is to derive SFs from preclinical species and apply the SFs for
prospective human PK prediction. However, concerns remain when
translating preclinical animal data to human due to poor protein
homology for drug transporters. Being a species with a high degree of
homology to human OATP isoforms (Shen et al., 2013; Kimoto et al.,
2017; De Bruyn et al., 2018), cynomolgus monkey is a promising
preclinical species that can be used in transporter characterization to
bridge the gaps of human IVIVE. As such, we further characterized
hepatic uptake in human and monkey hepatocytes under the condition in
the buffer containing 10% serum. Three hepatocyte lots were included in
the uptake studies for each species to evaluate variation among
difference lots or donors. As shown in Table 3, among 15 known
OATP and other transporter substrates (Table 4), the uptake values
in different lots were generally within 2-fold. Although the binding to
hepatocytes cannot be ignored in the incubation, the binding to the
hepatocytes should be similar in both human and monkey. After the
correction of serum binding, the values of in vitro CLu,int,passive were
generally comparable between human and monkey. On the other hand,
in vitro CLu,int,active in monkey was signifyingly higher than in human
(>2-fold) and appeared to be compound dependent (Table 3). It is worth
noting that the SFs of CLu,int,passive for bosentan and rosuvastatin in
monkey were higher than in human, although the SFs of CLu,int,active
were comparable in human and monkey for both bosentan and
rosuvastatin, therefore, the large difference of SFs in CLu,int,passive
between human and monkey was less likely due to in vitro experiments.
For bosentan and rosuvastatin, the PBPK modeling of human and
monkey were performed by two different groups. In addition, the curve
fitting in human PBPK solely relied on plasma PK data (Jones et al.,
<table>
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<tr>
<th>Compound</th>
<th>Lot</th>
<th>Uptake in human hepatocytes (µl/min per million cells)a</th>
<th>Lot</th>
<th>Uptake in monkey hepatocytes (µl/min per million cells)a</th>
<th>Mean Fold Differencec</th>
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<td>C_{u,int}^{passive}b</td>
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*TABLE 3*

Summary of in vitro hepatic uptake intrinsic clearance for 15 OATP substrates.
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<tr>
<th>Compound</th>
<th>Uptake in human hepatocytes (µl/min per million cells)$^a$</th>
<th>Uptake in monkey hepatocytes (µl/min per million cells)$^a$</th>
<th>Mean Fold Difference$^c$</th>
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<td>$\text{CL}_{\text{int,passive}}^b$</td>
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$^a$The in vitro uptake clearance was assessed in three donors of human (lots PZA, XPM, and YTW) and monkey (lots PNC, UHK, and VNV) hepatocytes. The initial uptake rates were estimated from the slopes of linear uptake phase using linear regression fitting. The intrinsic uptake clearance values were calculated by dividing the initial uptake velocity by the nominal substrate concentration. For each donor, the experiments were conducted in triplicates for each time point. The mean values were averaged from the triplicates, and the S.D. are presented in parentheses. Additionally, the donor mean value and the donor S.D. value for each compound were obtained from the three donors using the mean value of each donor. The $\text{CL}_{\text{int,active}}$ was calculated by subtracting the $\text{CL}_{\text{int,passive}}$ from $\text{CL}_{\text{int,uptake}}$. In vitro uptake clearance is reported as microliters per minute per million cells.

$^b$In vitro uptake clearance measured in uptake buffer containing 10% human or monkey serum was obtained by normalizing the free fraction of protein binding in 10% serum buffer.

$^c$Fold difference = mean value of data from three monkey hepatocyte lots/mean value of data from three human hepatocyte lots.
mediated uptake parameters for prospective human PK prediction. Human hepatocytes for 15 known OATP substrates, and the difference of uptake, such as difference of transporter expression, the relative binding as well as the difference in transport kinetics. Nevertheless, in human, it is recommended to consider the species difference in protein binding. Protein binding data were correlated well among these 15 compounds comparable between human and monkey (Table 2). Although the serum protein binding data were correlated well among these 15 compounds between human and monkey (Fig. 1), another study observed poor correlation of protein binding between preclinical species and human (Lombardo et al., 2013). In this regard, when interpreting protein facilitated uptake clearance and translating from preclinical animal to human, it is recommended to consider the species difference in protein binding as well as the difference in transport kinetics. Nevertheless, since overall difference of hepatic uptake obtained from the incubation in the buffer containing serum proteins incorporates multiple species-specific variables to compensate the complexities affecting the hepatic uptake, such as difference of transporter expression, the relative contribution of each transporter, substrate affinity of each transporter, and protein binding. SFs derived from the IVIVE in monkey can be reliably used in PBPK models for prospective human PK prediction.

In summary, the IVIVE of transporter-mediated clearance was significantly improved when using hepatic uptake parameters obtained from the incubation with serum protein in the uptake experiments. The species differences were found in hepatic uptakes between money and monkey for bosentan and rosuvastatin. In the case of pitavastatin, the uptake and efflux transporters of each selected compounds are summarized from Drug Interaction Solution Database, University of Washington (https://didb.druginteractionsolutions.org).

### TABLE 4
Summary of transporters involved in transporting selected compounds in human

<table>
<thead>
<tr>
<th>Compound</th>
<th>OATPs</th>
<th>OATs</th>
<th>Other transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosuvastatin</td>
<td>1A2, 1B1, 1B3, 2B1</td>
<td>OAT3</td>
<td>NTPC, BCRP, MRP1-5, P-gp</td>
</tr>
<tr>
<td>Cervastatin</td>
<td>1B1, 1B3, 2B1</td>
<td>-</td>
<td>P-gp, BCRP, MRP</td>
</tr>
<tr>
<td>Flavastatin</td>
<td>1B1, 1B3, 2B1</td>
<td>-</td>
<td>NTPC, BCRP, MRP2</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>1A2, 1B1, 1B3, 2B1</td>
<td>-</td>
<td>NTPC, BCRP, MRP3, MRP4, P-gp</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>1A2, 1B1, 1B3, 2B1</td>
<td>-</td>
<td>NTPC, BCRP, MRP1, MRP2, MRP4, P-gp</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>1B1, 1B3</td>
<td>-</td>
<td>OCT1, MRP2, P-gp</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>1A2, 1B1, 1B3, 2B1</td>
<td>OAT3, OAT4, OAT7</td>
<td>NTPC, BCRP, MRP1, MRP2, MRP4, P-gp</td>
</tr>
<tr>
<td>Danoprever</td>
<td>1B1, 1B3</td>
<td>-</td>
<td>MRP2, P-gp</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>1B1</td>
<td>-</td>
<td>P-gp</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1B1, 1B3, 2B1</td>
<td>-</td>
<td>BCRP, P-gp</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>1B1, 1B3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Grazoprever</td>
<td>1B1, 1B3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>1B3, 2B1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Asunaprever</td>
<td>1B1, 2B1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Valsartan</td>
<td>1B1, 1B3</td>
<td>OAT1, OAT3</td>
<td>MRP2, P-gp</td>
</tr>
</tbody>
</table>

2012), whereas both liver and plasma data were used in curve fitting in monkey model (Morse et al., 2017). Different models and fitting process may contribute to the different SFs of Cl\textsubscript{int,passive} obtained in human and monkey for bosentan and rosuvastatin. In the case of pitavastatin, both human and monkey PBPK models were developed in house for plasma PK curve fitting; the SFs of Cl\textsubscript{int,passive} and Cl\textsubscript{int,active} were comparable between human and monkey (Table 2). Although the serum protein binding data were correlated well among these 15 compounds between human and monkey (Fig. 1), another study observed poor correlation of protein binding between preclinical species and human (Lombardo et al., 2013). In this regard, when interpreting protein facilitated uptake clearance and translating from preclinical animal to human, it is recommended to consider the species difference in protein binding as well as the difference in transport kinetics. Nevertheless, since overall difference of hepatic uptake obtained from the incubation in the buffer containing serum proteins incorporates multiple species-specific variables to compensate the complexities affecting the hepatic uptake, such as difference of transporter expression, the relative contribution of each transporter, substrate affinity of each transporter, and protein binding. SFs derived from the IVIVE in monkey can be reliably used in PBPK models for prospective human PK prediction.

In summary, the IVIVE of transporter-mediated clearance was significantly improved when using hepatic uptake parameters obtained from the incubation with serum protein in the uptake experiments. The species differences were found in hepatic uptakes between money and monkey for bosentan and rosuvastatin. In the case of pitavastatin, the uptake and efflux transporters of each selected compounds are summarized from Drug Interaction Solution Database, University of Washington (https://didb.druginteractionsolutions.org).

### Acknowledgments
We thank Drs. Bernard Murray and Yang Song for helpful scientific discussions.

### Authorship Contributions
Participated in research design: Liang, Smith, Lai.
Conducted experiments: Liang, Park, DeForest, Hao, Zhao, Niu, Wang.
Performed data analysis: Liang, Park, Hao, Zhao, Lai.
Wrote or contributed to the writing of the manuscript: Liang, Park, Lai.

### References


Supplemental materials

Title: In Vitro Hepatic Uptake in Human and Monkey Hepatocytes in the Presence and Absence of Serum Protein and Its In Vitro to In Vivo Extrapolation

Authors: Xiaomin Liang, Yeojin Park, Natalie DeForest, Jia Hao, Xiaofeng Zhao, Congrong Niu, Kelly Wang, Bill Smith and Yurong Lai

Journal: Drug Metabolism and Disposition

DMD-AR-2020-000163
**Supplemental Table S1.** Human primary hepatocyte donor demographics

<table>
<thead>
<tr>
<th>Human hepatocytes donor</th>
<th>Donor</th>
<th>Age</th>
<th>Gender</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>YTW</td>
<td>Single</td>
<td>19</td>
<td>M</td>
<td>WH</td>
</tr>
<tr>
<td>XPM</td>
<td>Single</td>
<td>57</td>
<td>M</td>
<td>WH</td>
</tr>
<tr>
<td>PZA</td>
<td>Single</td>
<td>76</td>
<td>M</td>
<td>WH</td>
</tr>
</tbody>
</table>

**Supplemental Table S2.** Monkey primary hepatocyte donor demographics

<table>
<thead>
<tr>
<th>Monkey hepatocytes donor</th>
<th>Donor</th>
<th>Gender</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHK</td>
<td>Single</td>
<td>M</td>
<td>Cynomolgus</td>
</tr>
<tr>
<td>VNV</td>
<td>Single</td>
<td>M</td>
<td>Cynomolgus</td>
</tr>
<tr>
<td>PNC</td>
<td>Single</td>
<td>M</td>
<td>Cynomolgus</td>
</tr>
</tbody>
</table>
Supplemental Table S3. LC-MS/MS conditions

LC methods

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>0.6 ml/min</td>
</tr>
<tr>
<td>Gradient (B concentration %)</td>
<td>0.2 min 10% B; 1.2 min 99% B; 1.5 min 99% B; 1.55 min 10% B</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: 1% acetonitrile in water with 0.2% formic acid&lt;br&gt;B: 1% water in acetonitrile with 0.2% formic acid</td>
</tr>
</tbody>
</table>

Mass transitions for 15 compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>ESI mode</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asunaprevir</td>
<td>positive</td>
<td>748.3</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>negative</td>
<td>559.3</td>
</tr>
<tr>
<td>Bosentan</td>
<td>positive</td>
<td>552.2</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>negative</td>
<td>460.3</td>
</tr>
<tr>
<td>Danoprevir</td>
<td>negative</td>
<td>730.3</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>negative</td>
<td>412.3</td>
</tr>
<tr>
<td>Grazoprevir</td>
<td>positive</td>
<td>767.342</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>positive</td>
<td>514.323</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>negative</td>
<td>422.2</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>negative</td>
<td>423.3</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>positive</td>
<td>453.28</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>positive</td>
<td>482.1</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>positive</td>
<td>465.1</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>positive</td>
<td>515.293</td>
</tr>
<tr>
<td>Valsartan</td>
<td>positive</td>
<td>436.387</td>
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</table>
### Supplemental Table S4. Protein binding in human and monkey serum

<table>
<thead>
<tr>
<th>Compound</th>
<th>100% Human Serum</th>
<th>10% Human Serum</th>
<th>100% monkey Serum</th>
<th>10% monkey Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Free</td>
<td>% Recovery</td>
<td>% Free</td>
<td>% Recovery</td>
</tr>
<tr>
<td>Asunaprevir</td>
<td>0.68</td>
<td>86</td>
<td>4.91</td>
<td>97</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>3.15</td>
<td>101</td>
<td>27.55</td>
<td>101</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1.89</td>
<td>97</td>
<td>18.37</td>
<td>102</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>0.93</td>
<td>97</td>
<td>13.88</td>
<td>103</td>
</tr>
<tr>
<td>Danoprevir</td>
<td>2.80</td>
<td>92</td>
<td>25.78</td>
<td>103</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>1.02</td>
<td>89</td>
<td>11.58</td>
<td>94</td>
</tr>
<tr>
<td>Grazoprevir</td>
<td>1.14</td>
<td>91</td>
<td>8.95</td>
<td>97</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>21.48</td>
<td>98</td>
<td>75.85</td>
<td>119</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.39</td>
<td>104</td>
<td>5.37</td>
<td>102</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>56.69</td>
<td>116</td>
<td>84.17</td>
<td>103</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.86</td>
<td>99</td>
<td>12.10</td>
<td>106</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>16.98</td>
<td>98</td>
<td>67.32</td>
<td>110</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>N.A.</td>
<td>89</td>
<td>0.03</td>
<td>98</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>0.65</td>
<td>98</td>
<td>11.18</td>
<td>97</td>
</tr>
<tr>
<td>Valsartan</td>
<td>0.11</td>
<td>102</td>
<td>1.89</td>
<td>105</td>
</tr>
</tbody>
</table>

(1). The % free of test compound was calculated by the following equation:
\[
% \text{ Free} = \left( \frac{\text{Peak area ratio of analyte over internal standard in the buffer chamber}}{\text{Peak area ratio of analyte over internal standard in the serum chamber}} \right) \times 100\%
\]
(2). The % recovery of test compound was calculated by the following equation:
\[
% \text{ Recovery} = \left( \frac{\text{Ratio of the peak areas of analyte over internal standard in the buffer chamber} \times \text{buffer volume}}{\text{Ratio of the peak areas of analyte over internal standard in the serum}} \right) \times 100\%
\]
chamber*serum volume)/ (Ratio of the peak areas of analyte over internal standard in the T0 sample*serum volume) × 100%

(3). N.A.: not applicable. The binding of sorafenib in 100 % serum cannot be experimentally determined due to highly protein bound.

(4) Data was presented as mean of duplicates.
Supplemental Figures
Supplemental Figure S1. The time-course profile for the hepatic uptake. The error bars were represented standard deviation of triplicates.

A. Hepatic uptake of human PZA lot hepatocytes in 10% serum buffer

B. Hepatic uptake of human XPM lot hepatocytes in 10% serum buffer
C. Hepatic uptake of human YTW lot hepatocytes in 10% serum buffer

D. Hepatic uptake of monkey PNC lot hepatocytes in 10% serum buffer
E. Hepatic uptake of monkey UHK lot hepatocytes in 10% serum buffer

F. Hepatic uptake of monkey VNV lot hepatocytes in 10% serum buffer
G. Hepatic uptake of human XPM lot hepatocytes in serum free buffer

H. Hepatic uptake of monkey UHK lot hepatocytes in serum free buffer