Novel Method to Predict *In Vivo* Liver-to-Plasma K_{puu} for OATP Substrates Using Suspension Hepatocytes

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Abbreviations

BSA, bovine serum albumin; CI, confidence interval; DDI, drug-drug interactions; DHP, dehydropravastatin; HPLC, high performance liquid chromatography; $IC_{50,}$ half maximal inhibitory concentration; IS, internal standard; I.V., intravenous; $f_{u,}$ fraction unbound; $f_{u,cell}$, fraction unbound of cells; $f_{u,liver}$, fraction unbound of liver; $f_{u,media}$, fraction unbound of media; $f_{u,p}$, fraction unbound of plasma; K_p, partition coefficient; $K_{puu,}$ unbound partition coefficient; NASH, non-alcoholic steatohepatitis; IVIVE, *In vitro – in vivo* extrapolation/correlation; LC, liquid chromatography; LC-MS/MS, liquid chromatography– tandem mass spectrometry; MDCK-LE, Madin-Darby Canine Kidney-low efflux cell line; MWCO, molecular weight cutoff; MVA, mevalonic acid; NHP, non-human primate; OATP, organic anion-transporting polypeptide; P_{app}, apparent permeability; PD, pharmacodynamics; PET, positron emission tomography; PK, pharmacokinetics; PK/PD, pharmacokinetics/pharmacodynamics; PBS, phosphate buffered saline; RH, relative humidity; RPM, revolutions per minute; RT, room temperature.

Abstract

The ability to predict human liver-to-plasma unbound partition coefficient (K_{puu}) is of great importance to estimate unbound liver concentration, develop PK/PD relationships, predict efficacy and toxicity in the liver, and model drug-drug interaction (DDI) potential for drugs that are asymmetrically distributed into the liver. A novel *in vitro* method has been developed to predict *in vivo* K_{puu} with good accuracy using cryopreserved suspension hepatocytes in InVitroGRO HI media with 4% BSA. Validation was performed using six OATP substrates with rat *in vivo* K_{puu} data from IV infusion studies where steady state was achieved. Good i*n vitro-in vivo* correlation (IVIVE) was observed as the *in vitro* K_{puu} values were mostly within two fold of *in vivo* K_{puu}. Good K_{puu} IVIVE in human was also observed with *in vivo* K_{puu} data of dehydropravastatin from positron emission tomography and *in vivo* K_{puu} data from PK/PD modeling for pravastatin and rosuvastatin. Under the specific K_{puu} assay conditions, the drug metabolizing enzymes and influx/efflux transporters appear to function at physiological levels. No scaling factors are necessary to predict *in vivo* K_{puu} from *in vitro* data. The novel *in vitro* K_{puu} method provides a useful tool in drug discovery to project *in vivo* K_{puu}.

Introduction

Liver is an important organ for many disease targets, such as dyslipidemia, diabetes, obesity, and NASH. It is critical to understand the unbound drug concentration in the liver, as it impacts pharmacological activity, metabolic and biliary clearance, and DDI (Smith et al., 2010). For compounds that are not actively transported and are not influenced by membrane potential or pH gradient (Scott et al., 2016), the unbound drug concentration in the liver will be the same as that in the plasma. In this case, the unbound partition coefficient (K_{puu}) between liver and plasma is close to 1. When compounds are uptake transporter substrates (e.g., OATPs), K_{puu} values can be greater than 1 due to active influx. K_{puu} represents the distribution of unbound drugs between liver and plasma *in vivo* or between hepatocytes and media *in vitro* when multiple processes, including metabolism, uptake, efflux, and passive diffusion, have achieved steady state. K_{puu} can be described using the extended clearance equation incorporating the multiple mechanisms (Shitara et al., 2006; Watanabe et al., 2010; Yabe et al., 2011). It is important to be able to estimate *in vivo* K_{puu}, since it is the link between unbound plasma concentration and unbound liver concentration. Because it is challenging to measure unbound liver concentration directly in higher species (e.g., NHP) and humans, the ability to predict K_{puu} will enable direct estimation of unbound liver concentrations from unbound plasma concentrations.

Currently, several *in vitro* methods (Riccardi et al., 2016) are available to estimate K_{puu}, including the binding method (Mateus et al., 2013), the kinetic method (Yabe et al., 2011) and the temperature method (Shitara et al., 2013). However, validation of these

methods with *in vivo* exposure/pharmacology or *in vitro* activity data is fairly limited (Shitara et al., 2013; Riccardi et al., 2016). IVIVE for K_{puu} using hepatocyte systems for OATP substrates has not been established. Several studies have shown internalization or down-regulation of transporters in the hepatocyte systems (Roelofsen et al., 1995; Bow et al., 2008; Kimoto et al., 2012; Kunze et al., 2014; Bridget et al., 2015; Vildhede et al., 2015), although others have shown no significant difference in transporter abundance between hepatocytes and liver tissues (Prasad et al., 2014; Badee et al., 2015). It is uncertain whether a direct translation is possible without scaling factors from *in vitro* hepatocytes to *in vivo* K_{puu}. In this study, we explored the IVIVE of K_{puu} using cryopreserved suspension rat and human hepatocytes for OATP substrates. The ability to predict *in vivo* liver-to-plasma K_{puu} from *in vitro* systems will provide a useful tool in drug discovery to predict unbound liver concentration as well as clearance and dose, design drugs for liver targeting, develop PK/PD (pharmacokinetic/pharmacodynamics) relationships for disease targets residing in the liver, and model DDI due to inhibition /induction of liver enzymes when transporters are involved in the distribution processes.

Materials and Methods

Materials

Test compounds were obtained from obtained from Sigma-Aldrich (St. Louis, MO) or Pfizer (Groton, CT). PF-04991532 (Compound 19 in the reference) (Pfefferkorn et al., 2012) and PF-05187965 (Compound 7 in the reference) (Stevens et al., 2013) were synthesized according to the methods reported in the referenced publications. Rat (14 male and 14 female, pooled) and human (6 male, pooled) plasma, cryopreserved human hepatocytes (Lot DCM, custom-pooled of both male and female, 10 donors) and Wistar-Han rat hepatocytes (Lot VSU, 35 male pooled donors) were purchased from BioreclamationIVT, LLC (Hicksville, NY). Human liver (1 male donor) was from Analytical Biological Services Inc. (Wilmington, DE). Wistar-Han rat liver (4 male donors) was obtained internally at Pfizer Global Research and Development (Groton, CT). Williams' medium E (WEM Gibco-BRL, catalog #C1984, custom formula number 91-5233EC) contained 26 mM sodium bicarbonate and 50 mM HEPES, InVitroGRO HI media, and MPER buffer were purchased from Thermo Fisher Scientific (Waltham, MA). BSA (free of fatty acid, catalog # A4612) and other reagents were from Sigma-Aldrich unless specified. The equilibrium dialysis device (96-well format) and cellulose membranes (MWCO 12-14K) were obtained from HTDialysis, LLC (Gales Ferry, CT). Breathe Easy™ sealing membranes were obtained from Sigma-Aldrich.

Determination of Fraction Unbound

In preparation of *in vitro* f_u measurement, rat and human liver tissues were homogenized in phosphate buffered saline (1:5 tissue : PBS dilution) at RT with an Omni TH tissue

homogenizer (Omni International, Kennesaw, GA). A probe (7 mm x 110 mm) was used for 30 second pulses at high speed. InVitroGRO HI media containing 4% BSA and plasma were used directly without any dilution for binding determination. Before an experiment, the dialysis membranes were soaked in water for 15 minutes, 30% ethanol / water for 15 minutes, and PBS for 15 minutes or overnight. The dialysis device was put together following the instructions from the manufacturer (http://htdialysis.com/page/1puq4/Operating_Instructions.html). Compound stock solutions were prepared at 200 μM in DMSO, added to matrices (1:100 dilution), and mixed well with a multichannel pipettor (Eppendorf®, VWR, Radnor, PA). The final test compound concentration for equilibrium dialysis was 2 μM with 1% DMSO. A aliquot (150 μL) of matrix (plasma, liver homogenate or assay media) containing 2 μM test compound was added to the donor side of the membrane and PBS (150 μL) was added to the receiver side of the membrane. The dialysis device was sealed with Breathe Easy™ membranes. Quadruplicates were used for each compound in binding experiments. The dialysis device was incubated in a humidified incubator (75% relative humidity (RH), 5% CO_2/95% air) at 37°C for 6 hours at 200 RPM with an orbital shaker (VWR, Radnor, PA). Alternative binding methods (pre-saturation or dilution) were also used for highly bound compounds to ensure equilibrium had been achieved (Riccardi et al., 2015). At the incubation was completed, matrix samples (15 μL) from the donor wells were added to 45 μL of PBS in a 96-well plate. Dialyzed PBS (45 μL) from the receiver wells were added to the blank matrix (15 μL). Matrix material (15 μL) containing with 2 μM compound from both before and after incubation was taken and added to 45 μL PBS in a 96-well plate. They were used assess recovery and stability of the samples. Cold

acetonitrile (200 µL) containing internal standard (IS, a cocktail of 5 ng/mL terfenadine and 0.5 ng/mL tolbutamide) was added to all the samples for protein precipitation. The samples were vortexed for 3 minutes (VWR, Radnor, PA) and centrifuged at 3000 RPM for 5 minutes (Allegra 6R, Beckman Coulter, Fullerton, CA) at RT. The supernatant was transferred, dried down, reconstituted with solvents and injected to LC-MS/MS for analysis. Sertraline was used on every incubation plate for quality control. Calculation of fraction unbound (f_u) has been discussed previously (Riccardi et al., 2015; Riccardi et al., 2016).

In Vitro K_{puu} Measurement

The cryopreserved hepatocytes were thawed and re-suspended in WEM. The number of cells and viability were determined using the Trypan Blue exclusion method. Cell suspensions were centrifuged (Allegra 6R, Beckman Coulter, Fullerton, CA) at 50 g, RT for 3 minutes. Media were removed and cells were re-suspended in InVitroGRO HI media supplemented with 4% BSA. Test compounds (1 mM) were prepared in DMSO and 1 µL was added to the suspended hepatocytes at 0.5 million cells/mL in 1 mL. The final compound concentration is 1 µM with 0.1% DMSO. Two to four replicates were used for each compound. The suspension was incubated at 37 °C in a humidified incubator (75% RH, 5% CO_2/95% air) with for 4 hours to ensure steady state was reached. At the end of the incubation, the cell suspension was centrifuged for 3 minutes at 500 RPM and supernatant was sampled to determine the medium concentration. The remaining medium was removed from the hepatocytes. The cells were washed with cold PBS 3 times (1 mL each time) and lysed with 75 µL MPER buffer. The solution of the

lysed cells was sampled for analysis. Cold acetonitrile with IS was added to both the supernatant and the cell lysed solutions and mixed. The solution was centrifuged at 3000 RPM for 5 min at RT and the supernatants were transferred for LC-MS/MS analysis using standard curves from both media and cells. Calculation of *in vitro* K_{puu} has been discussed previously (Riccardi et al., 2016). Here, $f_{u,cell}$ is replaced with $f_{u,liver}$ since the two values are quite comparable as liver comprised of 80% hepatocytes by volume (Bayliss and Skett, 1996).

In Vivo Rat Liver-to-Plasma K_{puu} Determination

The intravenous (I.V.) infusion experiments in rats were conducted at BioDuro (Shanghai, China). Wistar-Han rats (male, n=3, fed) were infused I.V. through a jugular vein cannula (JVC) at a rate of 4 - 9 μL/min with test compounds using a programmable pump (Harvard 2000, Harvard Apparatus, Holliston, MA). The doses were selected based on the I.V. bolus data and the detection limit. Infusion time was determined using a duration greater than 5 times the terminal half-life that was predetermined from IV bolus data. Under this condition, K_{puu} should be close to steady state, because greater than 97% steady state is achieved at 5 times half-life (Ito, 2011; Hedaya, 2012). Dose and formulation of the compounds are summarized in Table 1. At the end of infusion, blood samples were obtained from the carotid artery catheter and livers were also collected. Liver samples were rinsed with saline and patted dry with a paper towel and the blood vessels attached were also removed all to minimize the potential contamination from blood and bile. Since the total volume of the biliary tree is quite small compared to the liver for both rat (0.5%) and human (0.3%) (Casali et al., 1994; Masyuk et al., 2001),

the impact of bile contamination on liver concentration is likely to be small. Concentrations were determined using LC-MS/MS. Both pravastatin and its isomer (van Haandel et al., 2016), 3'α-hydroxy-pravastatin, were included in the calculation of K_{puu}. Free concentrations were calculated by multiplying the total plasma or total liver concentration by the f_u values of plasma or liver. The i*n vivo* unbound liver-to-plasma ratio, K_{puu}, was obtained by dividing steady state unbound liver concentration by unbound plasma concentration.

LC-MS/MS Quantification

A generic LC-MS/MS method is discussed here and equivalent methods were also used based on compound properties. Two LC mobile phases were used: (A) 95% 2 mM ammonium acetate in water and 5% 50/50 methanol/acetonitrile, (B) 90% 50/50 methanol/acetonitrile and 10% 2 mM ammonium acetate in water, or (A) water with 0.1% formic acid, (B) acetonitrile with 0.1% formic acid. A flow rate of 0.5 mL/min was used with solvent gradient from 5% (B) to 95% (B) over 1.1 minutes to elute the compounds from the UPLC column (BEH C18, 1.7 µm, 50x2.1 mm, Waters, Milford, MA). The injection volume was 10 µL and the cycle time was 2.5 minutes / injection. A CTC PAL autosampler (LEAP Technologies, Carrboro, NC), an Agilent 1290 binary pump (Santa Clara, CA) and an AB Sciex (Foster City, CA) API 6500 triple quadrupole mass spectrometer with a TurboIonSpray source in MRM mode were used for sample analysis. Data collection processing and analysis were conducted with Analyst™ 1.6.1 software (Applied Biosystems, Foster City, CA).

Human in Vivo K_{puu} Estimation Based on PK/PD Modeling

The *in vivo* human K_{puu} of rosuvastatin and paravastatin were calculated as the ratio between *in vitro* and *in vivo* IC_{50} values for HMG-CoA reductase inhibition. The *in vitro* IC_{50} values were obtained from the literature (McTaggart et al., 2001; Holdgate et al., 2003; Gazzerro et al., 2012). The *in vivo* IC_{50} was estimated using PK/PD modeling (See supplementary material: Equation S1). The rosuvastatin model published previously has adapted to estimate the *in vivo* IC_{50} in humans (Aoyama et al., 2010). This is a two compartment PK model developed with an indirect PD response incorporating circadian rhythm of mevalonic acid (MVA) production (Aoyama et al., 2010). Pravastatin plasma PK (Pan et al., 1990; van Luin et al., 2010) and plasma MVA concentrations in response to pravastatin (Nozaki et al., 1996) were used in the PK/PD modeling with the same method as rosuvastatin. All modeling was performed in NONMEM 7.2 (ICON Plc, Dublin, Ireland).

Calculation Methods

F_u was calculated with Equation (1) based on compound concentrations or area ratios between test compound and IS. For samples with diluted matrices, f_u was obtained with Equations (2) and (3), where D is the dilution factor. The calculations of recovery and stability are shown in Equation (4) and Equation (5), respectively. *In vitro* K_{puu}, unbound cell concentration and unbound medium concentration were obtained using Equations (6) – (8).

$$f_u = \frac{\text{Receiver Concentration}}{\text{Donor Concentration}} \qquad \text{Eq (1)}$$

$$\text{Diluted } f_{u,d} = \frac{\text{Receiver Concentration}}{\text{Donor Concentration}} \qquad \text{Eq (2)}$$

$$\text{Undiluted } f_u = \frac{1/D}{((1/f_{u,d})-1)+1/D} \qquad \text{Eq (3)}$$

$$\%\ \text{Recovery} = \frac{\text{Donor Concentration} + \text{Receiver Concentration}}{\text{Donor Concentration at Time Zero}} \text{ x } 100\% \qquad \text{Eq (4)}$$

$$\text{Stability as } \%\ \text{Remaining} = \frac{\text{Concentration at Last Time Point}}{\text{Concentration at Zero Time Point}} \text{ x } 100\% \qquad \text{Eq (5)}$$

$$K_{puu} = \frac{C_{u,cell}}{C_{u,\,medium}} \qquad \text{Eq (6)}$$

$$C_{u,cell} = C_{total,cell} \text{ x } f_{u,cell} \qquad \text{Eq (7)}$$

$$C_{u,medium} = C_{total,medium} \text{ x } f_{u,medium} \qquad \text{Eq (8)}$$

Results

Development of IVIVE requires high quality *in vivo* K_{puu} data in order to verify the *in vitro* results. As *in vivo* rat K_{puu} data can be obtained relatively easily by using IV infusion to ensure steady state has been achieved, K_{puu} IVIVE was first developed using the rat animal model. The method was then extended to humans, where quality *in vivo* K_{puu} data are quite limited. Four statins (cerivastatin, fluvastatin, rosuvastatin and pravastatin) and two Pfizer internal liver-targeting compounds (PF-04991532 (Pfefferkorn et al., 2012) and PF-05187965 (Stevens et al., 2013)) were used for the study of rat IVIVE. All six compounds are OATP substrates consisting of a carboxylic acid functional group. Cerivastatin and pravastatin are OATP1B1 substrates, rosuvastatin and fluvastatin are substrates of OATP1B1, 1B3 and 2B1, and the two Pfizer compounds (PF-04991532 and PF-05187965) are substrates of OATP1B1 and OATP1B3 (University of Washington Drug-Drug Interaction Database, UCSF-FDA TransPortal, (Pfefferkorn et al., 2012; Stevens et al., 2013)) The plasma and liver concentrations, measured at steady state, from *in vivo* rat IV infusion studies are shown in Table 2. K_{puu} was calculated based on *in vivo* liver-to-plasma K_p and *in vitro* f_{up} and $f_{u,liver}$ from the equilibrium dialysis assay. The *in vivo* K_{puu} values of the compounds range from 2.2 to 57 covering a wide range of liver distribution properties. The *in vitro* K_{puu} data using cryopreserved suspension rat hepatocytes are summarized in Table 3. *In vitro* K_{puu} was calculated by multiplying *in vitro* K_p with $f_{u,cell}$ and dividing by $f_{u,media}$. Since $f_{u,cell}$ is similar to $f_{u,liver,}$ as 80% of liver cells are hepatocytes (Bayliss and Skett, 1996), rat $f_{u,liver}$ data from Table 2 were used as $f_{u,cell}$ for the *in vitro* K_{puu} calculation. From this and previous studies (Riccardi et al., 2016), a 4-hour incubation is a conservative time point to achieved steady

state in cells for most compounds. For example, the K_{puu} values for cerivastatin in rat hepatocytes were 39±2.0, 34±3.2, 24±1.4, 31±0.42 and 23±2.3 at 1, 2, 3, 4 and 5 hours with 0.1 μM incubation, suggesting steady state has been achieved even at a 1 hour incubation. The comparison between *in vitro* K_{puu} from suspension rat hepatocytes and *in vivo* rat K_{puu} is shown in Table 4. A good correlation between *in vitro* and *in vivo* has been observed and the K_{puu} values are mostly within two-fold of each other. The lower K_{puu} value of pravastatin compared to the other three statins could potentially be due to higher biliary clearance, higher basolateral efflux, and lower active uptake. It appears that the drug metabolizing enzymes and influx/efflux transporters are functioning at the physiological levels in the suspension rat hepatocytes under the specific assay conditions. No scaling factors are necessary to predict *in vivo* K_{puu} from *in vitro* data.

High quality *in vivo* K_{puu} data from human are very scarce. Liver biospy is invasive and positron emission tomography (PET) imaging has a number of limitations (e.g., interference from metabolites, nonspecific binding to tissues at low doses). Nevertheless, a few *in vivo* human K_{puu} values are available to evaluate the *in vitro* K_{puu} method. Human liver PET data has been reported for [^{11}C]dehydropravastatin (DHP) (Shingaki et al., 2014). The K_{puu} was estimated to be 2.0 using terminal phase (post 15 minutes) DHP data and in-house measured pravastatin human f_{up} (0.64) and $f_{u,liver}$ (0.17) values. It has been shown that the transporter and dispositional properties of DHP and pravastatin are very similar using *in vitro* sandwich cultured human hepatocyte assay and *in vivo* rat studies (personal communication with Prof. Yuichi Sugiyama). Therefore, the K_{puu} value of DHP can be used as a surrogate for K_{puu} of pravastatin.

Because high quality directly measured human *in vivo* K_{puu} data are fairly limited, human K_{puu} values of rosuvastatin and pravastatin were obtained indirectly using PK/PD modeling. Other statins were not included in the modeling due to interference of active metabolites or insufficient literature data. The human *in vivo* liver-to-plasma K_{puu} of rosuvastatin was estimated to be 10. The average of literature reported value for *in vitro* IC_{50} was 7 nM, ranging from 2 to 10 (McTaggart et al., 2001; Holdgate et al., 2003; Gazzerro et al., 2012). The *in vivo* IC_{50} of rosuvastatin was fitted to be 2.1 ng/mL with a 95% confidence interval (CI) of 1.87 – 2.32 ng/mL. The corresponding unbound IC_{50} was 0.7 nM (95% CI 0.62 – 0.78 nM) using in house measured f_{up} of 0.16. The PK/PD model fits the data well (supplementary material, Figure S1) and the parameter estimates agree with those published previously (supplementary material, Table S1) (Aoyama et al., 2010). The human *in vivo* liver-to-plasma K_{puu} of pravastatin was estimated to be 5.3. The average of the reported *in vitro* IC_{50} values was 48 nM ranging from 29 to 70 nM (Gazzerro et al., 2012). The *in vivo* IC_{50} of pravastatin was modeled to be 6.0 ng/mL with a 95% CI of 3.6 – 12.7 ng/mL, converting to unbound *in vivo* IC_{50} of 9.0 nM (95% CI of 5.4 – 19.3 nM) based on in-house measured f_{up} of 0.64. The modeling results are shown in the supplementary material, Table S2 and Figure S2.

The comparison of human *in vivo* K_{puu} from PET or PK/PD modeling and *in vitro* K_{puu} from suspension human hepatocytes is shown in Table 5. The *in vitro* assay predicted *in vivo* DHP K_{puu} from PET data well (2.3 vs. 2.0). The K_{puu} data from PK/PD modeling carry some uncertainties as it is an indirect *in vivo* measure of K_{puu} from many *in vitro* and *in vivo* parameters. Certain assumptions were made to obtain the K_{puu} values from

PK/PD modeling. It is assumed that the *in vitro* assays fully capture the *in vivo* conditions, and that the measured *in vitro* IC_{50} can be directly used to quantitatively explain the PD (e.g., MVA) response to unbound liver drug concentrations. In addition, there are only eight and five (respectively) time point measurements of plasma concentrations of MVA in each group of the rosuvastatin and paravastatin studies, and only one PD study of each statin was available to be included in the analysis. Giving the uncertainty of the *in vivo* K_{puu} values from PK/PD modeling, it is reasonable that the *in vitro* K_{puu} data are within 2- and 4-fold of the *in vivo* K_{puu} estimates from PK/PD. The human K_{puu} IVIVE is similar to that observed in rat based on limited data, suggesting the *in vitro* K_{puu} method with suspension hepatocytes is a suitable *in vitro* tool to predict *in vivo* K_{puu} under specific assay conditions. As more human *in vivo* K_{puu} data become available, the performance of *in vitro* K_{puu} method will continue to be verified.

Discussion

Determination of unbound liver concentration is critical to understand pharmacology of disease targets in the liver, develop PK/PD relationships, predict DDI potentials, and anticipate liver toxicity. The ability to predict *in vivo* liver-to-plasma K_{puu} from *in vitro* assays is highly desirable as there is no easy way to measure human unbound liver concentration. With K_{puu} values, unbound liver concentrations can be estimated from unbound plasma concentrations, which can be readily measured. As reported in the literature, a direct translation of *in vitro* K_{puu} to *in vivo* K_{puu} can be challenging that transporter protein levels and functions in the *in vitro* systems are quite different than *in vivo* (Roelofsen et al., 1995; Bow et al., 2008; Kimoto et al., 2012; Kunze et al., 2014; Bridget et al., 2015; Vildhede et al., 2015). Typically, empirical scaling factors are needed to predict *in vivo* drug disposition from *in vitro* data (Jones et al., 2012; Li et al., 2014). The scaling factors are system dependent and can vary with assay conditions, such as cell culture time/media, plated vs. suspension cells, BSA vs. no proteins, cell types (transfected cells vs. hepatocytes), and medium composition. However, under this specific assay condition with cryopreserved hepatocyte suspension in InVitroGRO HI media with 4% BSA, the transporters and enzymes appear to be functioning at the physiological level. Good K_{puu} IVIVE has been observed in both rat and human without any scaling factors. Both the specific assay media and the physiological amount of BSA (4%) in the assay are important to generate good K_{puu} IVIVE. This is the first time that an *in vitro* assay shows good prediction of *in vivo* K_{puu} for OATP substrates. Based on the extended clearance concept, K_{puu} is affected by intrinsic clearance of passive diffusion, active hepatic uptake, sinosuidal efflux, biliary excretion, as well as

metabolism. Based on some literature data, cryopreserved suspended hepatocytes did not retain proper functional activity of efflux transporters. This was likely due to the internalization or down-regulation of some transporters (Bow et al., 2008). However, the information is controversial in the literature, as efflux activity has been reported in suspension hepatocytes of multiple species including rat, human, dog and monkey (Li et al., 2008). The mechanistic understand of why this particular assay condition seems to perform better others requires further investigation.

The *in vivo* K_{puu} measures the unbound drug concentration between liver and arterial blood rather than liver blood. The liver blood has lower drug concentrations than arterial blood for high extraction drugs. Theoretically, for compounds with high liver extraction, the *in vivo* measured K_{puu} will be lower than the *in vitro* experimental K_{puu}. However, in practice, these differences have not been observed. The *in vitro* K_{puu} values are shown to be both higher or lower than the *in vivo* K_{puu}. This could potentially be due to experimental variability from both *in vitro* and *in vivo* assays, making it difficult to detect the differences. This theoretical difference between *in vivo* and *in vitro* K_{puu} appears to be inconsequential for K_{puu} IVIVE.

In this study, we observed good correlation between *in vivo* K_{puu} from PK/PD modeling and *in vitro* K_{puu}. The *in vivo* K_{puu} was calculated as the IC_{50} ratio of HMG-CoA inhibition between *in vitro* and *in vivo*. The *in vitro* IC_{50} was measured using human liver microsomes. Assuming *in vitro* IC_{50} fully captures *in vivo* conditions (e.g. enzyme activity, substrate concentration, pH, and temperature), has no impact of transporters and

represents the intrinsic inhibitory activity, this measured *in vitro* IC_{50} should explain the *in vivo* effect directly if intracellular unbound drug concentration in the liver is known. However, in the absence of liver drug concentration, the human *in vivo* IC_{50} was estimated using PK/PD modeling based on unbound plasma concentration. The unbound statin concentration in the liver can be higher than unbound plasma concentrations due to OATP active uptake. Therefore, the IC_{50} ratio between *in vitro* and *in vivo* reflects the unbound drug concentration difference between liver and plasma and can be used as a surrogate for K_{puu}. This novel *in vitro* K_{puu} method provides a new tool to assess *in vivo* K_{puu} in drug discovery. The information is useful to estimate human unbound liver drug concentrations, predict efficacy and model DDI risks for drugs that are active influx/efflux in the liver by transporters.

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Authorship Contributions

Participated in research design: Riccardi, Lin, Li, Niosi, Ryu, Hua, Atkinson, Kosa, Litchfield, Di.

Conducted experiments: Riccardi, Ryu, Hua.

Performed data analysis: Riccardi, Lin, Li, Niosi, Ryu, Hua, Atkinson, Kosa, Litchfield, Di.

Wrote or contributed to the writing of the manuscript: Li, Atkinson, Kosa, Di.

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Table 1. Rat IV Infusion Experimental Conditions

Compounds	**Dose (mg/kg/h)**	**Dosing Vehicle**
Cerivastatin	0.10	Saline
Fluvastatin	0.10	12% (w/v) SBECD (sulfobutylether-β-cyclodextrin) in water
Rosuvastatin	0.10	12% (w/v) SBECD in water
Pravastatin	0.10	12% (w/v) SBECD in water
PF-04991532	0.12	Phosphate buffered saline (PBS), pH 7.4
PF-05187965	1.2	12%(w/v) SBECD in water with 3 molar equivalent 1N HCl

Table 2. *In Vivo* Rat IV Infusion Study for Determination of Liver-to-Plasma K_{puu}

Compounds	Plasma Concentration at Terminal Time Point (ng/mL)	Liver Concentration at Terminal Time Point (ng/mL)	Total Liver-to-Plasma Ratio (K_p)	Rat Plasma f_{up}	Rat Liver $f_{u,liver}$	In Vivo Rat Liver-to-Plasma K_{puu}
Cerivastatin	261 ± 70	6717 ± 960	27 ± 8.0	0.016 ± 0.001	0.017 ± 0.001	29 ± 8.5
Fluvastatin	245 ± 34	8990 ± 1490	37 ± 7.4	0.011 ± 0.001	0.013 ± 0.001	44 ± 8.7
Rosuvastatin	14 ± 3.7	178 ± 50	13 ± 2.2	0.044 ± 0.009	0.19 ± 0.02	57 ± 9.5
Pravastatin	33 ± 19	171 ± 15	6.7 ± 4.5	0.54 ± 0.02	0.18 ± 0.02	2.2 ± 1.5
PF-04991532	172 ± 67	1170 ± 364	7.1 ± 2.0	0.12 ± 0.01	0.096 ± 0.02	5.7 ± 1.6
PF-05187965	76 ± 21	323 ± 13	4.4 ± 1.0	0.27 ± 0.01	0.15 ± 0.02	2.4 ± 0.57

Table 3. *In Vitro* K_p and K_{puu} between Cells and Media in Rat Suspension Hepatocytes

Compounds	$f_{u,media}$	$f_{u,cell}$*	K_p	K_{puu}
Cerivastatin	0.022 ± 0.003	0.017 ± 0.001	27± 2.6	21 ± 2.0
Fluvastatin	0.016 ± 0.001	0.013 ± 0.001	27± 1.8	22 ± 1.5
Rosuvastatin	0.19 ± 0.02	0.19 ± 0.02	35 ± 0.6	35 ± 0.6
Pravastatin	0.49 ± 0.06	0.18 ± 0.02	8.3 ± 0.8	3.0 ± 0.3
PF-04991532	0.12 ± 0.01	0.096 ± 0.02	8.9 ± 0.1	7.1 ± 0.1
PF-05187965	0.36 ± 0.01	0.15 ± 0.02	10 ± 0.4	4.2 ± 0.2

* $F_{u,cell}$ is assumed to be the same as $f_{u,liver}$ in Table 2, as 80% of the liver is made of hepatocytes by volume (Bayliss and Skett, 1996).

Table 4. Correlation between In Vivo Rat Liver-to-Plasma K_{puu} and In Vitro Suspension Rat Hepatocyte K_{puu}

Compounds	P_{app} MDCK-LE (10^{-6} cm/s) *	In Vivo Rat Liver-to-Plasma K_{puu}	In Vitro Suspension Rat Hepatocyte K_{puu}	Fold Difference in Vivo K_{puu} /in Vitro K_{puu}
Cerivastatin	10.3	29 ± 8.5	21 ± 2.0	1.4
Fluvastatin	7.8	44 ± 8.7	21 ± 1.5	2.1
Rosuvastatin	0.9	57 ± 9.5	35 ± 0.6	1.6
Pravastatin	0.4	2.2 ± 1.5	2.9 ± 0.3	0.8
PF-04991532	1.0	5.7 ± 1.6	7.1 ± 0.1	0.8
PF-05187965	0.5	2.4 ± 0.6	4.2 ± 0.2	0.6

* P_{app} was measured using MDCK-LE method (Di et al., 2011) and the values were obtained from the following references (Pfefferkorn et al., 2012; Stevens et al., 2013; Varma et al., 2015).

Table 5. Correlation between *in Vitro* and *in Vivo* K_{puu} for Humans

Compounds	*In Vivo* Liver-to-Plasma K_{puu}	*In Vitro* Suspension Hepatocyte K_{puu}
Pravastatin	2.0 (DHP, PET), 5.3 (PK/PD)	2.3 ± 0.2
Rosuvastatin	10 (PK/PD)	39 ± 5.0

Pravastatin human f_{up} = 0.64 ± 0.16, $f_{u,liver}$ = 0.17 ± 0.02, $f_{u,media}$ = 0.49 ± 0.06

Rosuvastatin human f_{up} = 0.16 ± 0.05, $f_{u,liver}$ = 0.31 ± 0.02, $f_{u,media}$ = 0.19 ± 0.02