

# Novel Method to Predict In Vivo Liver-to-Plasma $K_{puu}$ for OATP Substrates Using Suspension Hepatocytes<sup>§</sup>

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## 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 the drug-drug interaction 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 i.v. infusion studies where a steady state was achieved. Good in vitro-in vivo

correlation (IVIVE) was observed as the in vitro  $K_{puu}$  values were mostly within 2-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 physiologic 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 nonalcoholic steatohepatitis. It is critical to understand the unbound drug concentration in the liver, because 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 a 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 the unbound liver concentration directly in higher species (e.g., nonhuman primate) 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 are 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 downregulation of transporters in the hepatocyte systems (Roelofsen et al., 1995; Bow et al., 2008; Kimoto et al., 2012; Kunze et al., 2014; Morse 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, to design drugs for liver targeting, to develop pharmacokinetic (PK)/pharmacodynamics (PD) 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 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

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**ABBREVIATIONS:** BSA, bovine serum albumin; CI, confidence interval; DDI, drug-drug interaction; DHP, dehydropravastatin; DMSO, dimethylsulfoxide;  $f_u$ , 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; IS, internal standard; IVIVE, In vitro-in vivo extrapolation/correlation;  $K_p$ , partition coefficient;  $K_{puu}$ , unbound partition coefficient; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MVA, mevalonic acid; OATP, organic anion-transporting polypeptide; PBS, phosphate-buffered saline; PD, pharmacodynamics; PET, positron emission tomography; PK, pharmacokinetics; RT, room temperature.

reference) (Stevens et al., 2013) were synthesized according to the methods reported in the referenced publications. Rat plasma (from 14 males and 14 females, pooled) and human plasma (from 6 males, pooled), 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 Biologic Services Inc. (Wilmington, DE). Wistar-Han rat liver (four male donors) was obtained internally at Pfizer Global Research and Development (Groton, CT). Williams' Medium E (catalog #C1984, custom formula number 91-5233EC; ThermoFisher Scientific, Waltham, MA) contained 26 mM sodium bicarbonate and 50 mM HEPES, InVivoGRO HI media, and M-PER buffer were purchased from ThermoFisher Scientific. 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 (molecular weight cutoff, 12,000–14,000) 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 fraction unbound ( $f_u$ ) measurement, rat and human liver tissues were homogenized in PBS (1:5 tissue: PBS dilution) at RT with an Omni TH tissue homogenizer (Omni International, Kennesaw, GA). A probe ( $7 \times 110$  mm) was used for 30-second pulses at high speed. InVivoGRO 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://www.htdialysis.com>). Compound stock solutions were prepared at 200  $\mu$ M in dimethylsulfoxide (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  $\mu$ M with 1% DMSO. An aliquot (150  $\mu$ L) of matrix (plasma, liver homogenate, or assay media) containing 2  $\mu$ M test compound was added to the donor side of the membrane, and PBS (150  $\mu$ L) was added to the receiver side of the membrane. The dialysis device was sealed with Breathe-Easy Membranes (Sigma-Aldrich). Quadruplicates were used for each compound in binding experiments. The dialysis device was incubated in a humidified incubator (75% relative humidity, 5%  $CO_2/95\%$  air) at 37°C for 6 hours at 200 rpm with an orbital shaker (VWR). Alternative binding methods (before saturation or dilution) were also used for highly bound compounds to ensure that equilibrium had been achieved (Riccardi et al., 2015). After the incubation was completed, matrix samples (15  $\mu$ L) from the donor wells were added to 45  $\mu$ L of PBS in a 96-well plate. Dialyzed PBS (45  $\mu$ L) from the receiver wells was added to the blank matrix (15  $\mu$ L). Matrix material (15  $\mu$ L) containing 2  $\mu$ M compound from both before and after incubation was taken and added to 45  $\mu$ L PBS in a 96-well plate. They were used to assess the recovery and stability of the samples. Cold acetonitrile (200  $\mu$ L) 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) 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 analyzed by LC-MS/MS. Sertraline was used on every incubation plate for quality control. Calculation of  $f_u$  has been discussed previously (Riccardi et al., 2015, 2016).

**In Vitro  $K_{puu}$  Measurement.** The cryopreserved hepatocytes were thawed and resuspended in William's Medium E. The number of cells and viability were determined using the Trypan Blue exclusion method. Cell suspensions were centrifuged (Allegra 6R; Beckman Coulter) at 50g at RT for 3 minutes. Media were removed and cells were resuspended in InVivoGRO HI media supplemented with 4% BSA. Test compounds (1 mM) were prepared in DMSO, and 1  $\mu$ L was added to the suspended hepatocytes at 0.5 million cells/ml in 1 ml. The final compound concentration is 1  $\mu$ M 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% relative humidity, 5%  $CO_2/95\%$  air) with for 4 hours to ensure that the 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 three times (1 ml each time) and lysed with 75  $\mu$ L M-PER 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 minutes 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 i.v. infusion experiments in rats were conducted at BioDuro (Shanghai, People's Republic of China). Wistar-Han rats (male,  $n = 3$ , fed) were infused i.v. through a jugular vein cannula at a rate of 4–9  $\mu$ l/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 five times the terminal half-life that was predetermined from i.v. bolus data. Under this condition,  $K_{puu}$  should be close to steady state, because a greater than 97% steady state is achieved at five times the 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 all the blood vessels attached were also removed to minimize the potential contamination from blood and bile. Since the total volume of the biliary tree is quite small compared with 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'- $\alpha$ -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 in vivo unbound liver-to-plasma ratio,  $K_{puu}$ , was obtained by dividing the steady-state unbound liver concentration by the 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 and (B) 90% 50/50 methanol/acetonitrile and 10% 2 mM ammonium acetate in water, or (A) water with 0.1% formic acid and (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 ultra-performance LC column (BEH C18, 1.7  $\mu$ m, 50  $\times$  2.1 mm; Waters, Milford, MA). The injection volume was 10  $\mu$ L, and the cycle time was 2.5 minutes/injection. A CTC PAL autosampler (LEAP Technologies, Carboro, NC), an model 1290 binary pump (Agilent, Santa Clara, CA) and an API 6500 triple quadrupole mass spectrometer with a TurboIonSpray source (AB Sciex, Foster City, CA) 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  $K_{puu}$  values for rosuvastatin and pravastatin were calculated as the ratio between in vitro and in vivo  $IC_{50}$  values for 3-hydroxy-3-methylglutaryl-CoA reductase inhibition. The in vitro  $IC_{50}$  values were obtained from the literature (McTaggart et al., 2001; Holdgate et al., 2003; Gazzero et al., 2012). The in vivo  $IC_{50}$  values were estimated using PK/PD modeling (Supplemental eq. 1). 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

TABLE 1  
Rat i.v. infusion experimental conditions

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

SBECD, sulfobutylether- $\beta$ -cyclodextrin.

TABLE 2  
In vivo rat i.v. 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 $f_{u,p}$	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

method as rosuvastatin. All modeling was performed in NONMEM 7.2 (ICON Plc, Dublin, Ireland).

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

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

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

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

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

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

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

$$C_{u,cell} = C_{total,cell} \times f_{u,cell} \quad \text{Eq. (7)}$$

$$C_{u,medium} = C_{total,medium} \times f_{u,medium} \quad \text{Eq. (8)}$$

## Results

Development of IVIVE requires high-quality in vivo  $K_{puu}$  data to verify the in vitro results. As in vivo rat  $K_{puu}$  data can be obtained relatively easily by using i.v. 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, OATP1B3, and OATP2B1; 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 i.v. infusion studies are shown in Table 2.  $K_{puu}$  was calculated based on in vivo liver-to-plasma  $K_p$  and in vitro  $f_{u,p}$  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 time is a conservative time point to achieve a steady state in cells for most compounds. For example, the  $K_{puu}$  values for cerivastatin in rat hepatocytes were  $39 \pm 2.0$ ,  $34 \pm 3.2$ ,  $24 \pm 1.4$ ,  $31 \pm 0.42$ , and  $23 \pm 2.3$  at 1, 2, 3, 4 and 5 hours with 0.1  $\mu\text{M}$  incubation, suggesting that a steady state has been achieved even after 1 hour of 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 2-fold of each other. The lower  $K_{puu}$  value of pravastatin compared with 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 physiologic levels in the rat hepatocytes in suspension under the specific assay conditions. No scaling factors are necessary to predict in vivo  $K_{puu}$  data from in vitro data.

High-quality in vivo  $K_{puu}$  data from humans are very scarce. Liver biopsy 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 [ $^{11}\text{C}$ ]dehydropravastatin (DHP) (Shingaki et al., 2014). The  $K_{puu}$  was estimated to be 2.0 using terminal-phase (after 15 minutes) DHP data and in-house-measured pravastatin human  $f_{u,p}$  (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 an in vitro sandwich-cultured human hepatocyte assay and in vivo rat studies (Y. Sugiyama, personal communication).

TABLE 3  
In vitro  $K_p$  and  $K_{puu}$  between cells and media in rat suspension hepatocytes

Compounds	$f_{u,media}$	$f_{u,cell}^a$	$K_p$	$K_{puu}$
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

<sup>a</sup> $f_{u,cell}$  is assumed to be the same as  $f_{u,liver}$  in Table 2, because 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) <sup>a</sup>	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

MDCK-LE, Madin-Darby Canine Kidney-low efflux cell line;  $P_{app}$ , apparent permeability.

<sup>a</sup> $P_{app}$  was measured using the MDCK-LE method (Di et al., 2011), and the values were obtained from the following references: Pfefferkorn et al. (2012), Stevens et al. (2013), and Varma et al. (2015).

Therefore, the  $K_{puu}$  value of DHP can be used as a surrogate for the  $K_{puu}$  value 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 the 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 value reported in the literature for in vitro  $IC_{50}$  was 7 nM, ranging from 2 to 10 nM (McTaggart et al., 2001; Holdgate et al., 2003; Gazzero 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 an in-house-measured  $f_{u,p}$  of 0.16. The PK/PD model fits the data well (Supplemental Fig. 1), and the parameter estimates agree with those published previously (Supplemental Table 1) (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 an unbound in vivo  $IC_{50}$  of 9.0 nM (95% CI, 5.4–19.3 nM) based on an in-house-measured  $f_{u,p}$  of 0.64. The modeling results are shown in Supplemental Table 2 and Supplemental Figure 2.

The comparison of human in vivo  $K_{puu}$  from PET or PK/PD modeling and in vitro  $K_{puu}$  from human hepatocytes in suspension is shown in Table 5. The in vitro assay predicted in vivo DHP  $K_{puu}$  values from PET data well (2.3 versus 2.0). The  $K_{puu}$  data from PK/PD modeling carry some uncertainties because 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  $IC_{50}$  value 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 time point measurements of plasma concentrations of MVA, respectively, in each group of the rosuvastatin and pravastatin 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-fold and 4-fold of the in vivo  $K_{puu}$  estimates from PK/PD modeling. The human  $K_{puu}$  IVIVE is similar to that observed in the rat based on limited data, suggesting that 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 the in vitro  $K_{puu}$  method will continue to be verified.

## Discussion

Determination of the unbound liver concentration is critical to understand the 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  $K_{puu}$  values from in vitro assays is highly desirable as there is no easy way to measure the 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 because transporter protein levels and functions in the in vitro systems are quite different than those in the in vivo systems (Roelofsen et al., 1995; Bow et al., 2008; Kimoto et al., 2012; Kunze et al., 2014; Morse 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 versus suspension cells, BSA versus no proteins, cell types (transfected cells versus 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 physiologic level. Good  $K_{puu}$  IVIVE has been observed in both rat and human without any scaling factors. Both the specific assay media and the physiologic 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, sinusoidal 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 downregulation 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 understanding 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

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 <sup>a</sup>	2.0 (DHP, PET), 5.3 (PK/PD)	2.3 ± 0.2
Rosuvastatin <sup>b</sup>	10 (PK/PD)	39 ± 5.0

<sup>a</sup>Pravastatin human  $f_{u,p}$  = 0.64 ± 0.16,  $f_{u,liver}$  = 0.17 ± 0.02,  $f_{u,media}$  = 0.49 ± 0.06.

<sup>b</sup>Rosuvastatin human  $f_{u,p}$  = 0.16 ± 0.05,  $f_{u,liver}$  = 0.31 ± 0.02,  $f_{u,media}$  = 0.19 ± 0.02.

measured  $K_{puu}$  value will be lower than the in vitro experimental  $K_{puu}$  value. However, in practice, these differences have not been observed. The in vitro  $K_{puu}$  values are shown to be both higher and lower than the in vivo  $K_{puu}$  values. 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  $K_{puu}$  values appears to be inconsequential for  $K_{puu}$  IVIVE.

In this study, we observed good correlation between in vivo  $K_{puu}$  values from PK/PD modeling and in vitro  $K_{puu}$ . The in vivo  $K_{puu}$  was calculated as the  $IC_{50}$  ratio of 3-hydroxy-3-methylglutaryl-CoA inhibition between in vitro and in vivo values. The in vitro  $IC_{50}$  was measured using human liver microsomes. Assuming that the 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 the 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 values 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 have active influx/efflux in the liver by transporters.

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#### Authorship Contributions

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