Rosuvastatin Liver Partitioning in Cynomolgus Monkeys: Measurement In Vivo and Prediction Using In Vitro Monkey Hepatocyte Uptake

Bridget L. Morse, Hong Cai,1 Jamus G. MacGuire, Maxine Fox, Lisa Zhang, Yueping Zhang, Xiaomei Gu, Hong Shen, Elizabeth A. Dierks, Hong Su, Chiwu E. Luk, Punit Marathe, Yue-Zhong Shu, W. Griffith Humphreys, and Yurong Lai


Received June 11, 2015; accepted September 3, 2015

ABSTRACT

Unbound plasma concentrations may not reflect those in target tissues, and there is a need for methods to predict tissue partitioning. Here, we investigate the unbound liver partitioning (Kp_u,u) of rosuvastatin, a substrate of hepatic organic anion transporting polypeptides, in cynomolgus monkeys and compare it with that determined using hepatocytes in vitro. Rosuvastatin (3 mg/kg) was administered orally to monkeys and plasma and liver (by ultrasound-guided biopsy) collected over time. Uptake into monkey hepatocytes was evaluated up to steady state. Binding in monkey plasma, liver, and hepatocytes was determined using equilibrium dialysis. Mean in vivo Kp_u,u was 118 after correcting total liver partitioning by plasma and liver binding. In vitro uptake data were analyzed by compartmental modeling to determine active uptake clearance, passive diffusion, the intracellular unbound fraction, and Kp_u,u. In vitro Kp_u,u underpredicted that in vivo, resulting in the need for an empirical in vitro to in vivo scaling factor of 10. Adjusting model parameters using hypothetical scaling factors for transporter expression and surface area or assuming no effect of protein binding on active transport increased partitioning values by 1.1-, 6-, and 9-fold, respectively. In conclusion, in vivo rosuvastatin unbound liver partitioning in monkeys was underpredicted using hepatocytes in vitro. Modeling approaches that allow integrating corrections from passive diffusion or protein binding on active uptake could improve the estimation of in vivo intracellular partitioning of this organic anion transporting substrate. A similar assessment of other active hepatic transport mechanisms could confirm and determine the extent to which limited accumulation in isolated hepatocytes needs to be considered in drug development.

Introduction

Recent progress toward understanding drug transport has called into question previous assumptions of drug disposition and tissue accumulation. Specifically, the underlying principles of active transport allow unbound concentrations across a membrane to differ, undermining the notion that unbound plasma concentrations equal those in tissue and may therefore serve as a surrogate for unbound tissue concentrations. As tissue concentrations are directly responsible for many effects pertaining to drug safety and efficacy, of late, there has been increasing emphasis placed on methods for measuring or predicting tissue drug concentrations, particularly for compounds whose disposition is governed by drug transporters (Chu et al., 2013b).

Although noninvasive imaging for monitoring target tissue concentrations is now feasible for some therapeutic agents in the clinic (Bauer et al., 2012; van Velden et al., 2015), methods to predict intracellular tissue concentrations are needed in drug discovery and development. Although total tissue partitioning can be readily measured in rodents, there are noted interspecies differences in hepatic transporter homology and expression between rodents and humans (Chu et al., 2013a); therefore, it cannot be assumed that tissue partitioning in humans can be reliably predicted directly from that in rodents. Additionally, methods for measuring tissue concentrations in vivo do not enable measurement of unbound concentrations, which are responsible for drug effects. As such, in vitro methods for predicting intracellular unbound concentrations and unbound tissue partitioning are sought after.

As the liver is often the site of drug action and toxicity, attention has focused on the use of hepatocytes in vitro for predicting unbound liver partitioning in vivo. A number of approaches have been described using rat or human hepatocytes for the uptake of substrates for the hepatic

ABBREVIATIONS: KHB, Krebs-Henseleit buffer; Kp, total tissue partition coefficient; Kp_u,u, unbound tissue partition coefficient; LC-MS/MS, liquid chromatography–mass spectrometry; OATP, organic anion transporting polypeptide.
predicted using monkeys (Shen et al., 2013, 2015). In this research, we demonstrated interactions for rosuvastatin in humans were quantitatively mediated for rosuvastatin in humans were quantitatively cryopreserved hepatocytes and a similar in vivo disposition and OATP-OATPs, similar transport kinetics were observed in monkey and human uptake/inhibition activity for probe compounds (Shen et al., 2013). Furthermore, for rosuvastatin, a broad substrate of monkey and human OATPs, similar transport kinetics were observed in monkey and human cryopreserved hepatocytes and a similar in vivo disposition and OATP-mediated interactions for rosuvastatin in humans were quantitatively reproduced using monkeys (Shen et al., 2013, 2015). In this research, we demonstrate the use of a novel method for liver sampling in cynomolgus monkeys and evaluate the monkey as a model for in vivo to in vivo extrapolation of rosuvastatin unbound liver partitioning.

Materials and Methods

Rosuvastatin was purchased from Toronto Research Chemicals (North York, ON, Canada). Carbamazepine and rifampicin SV were purchased from Sigma-Aldrich (St. Louis, MO). InVitroGRO HT medium and Krebs-Henseleit buffer (KHB) were purchased from Bioreclamation IVT (Baltimore, MD). All other chemicals and reagents were of analytical grade.

In Vivo Rosuvastatin Pharmacokinetic Study in Monkeys. Animal studies were approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee and performed under the standards recommended by the Guide for the Care and Use of Laboratory Animals. Male cynomolgus monkeys were obtained from BioCulture (Mauritius) Ltd. (Riviere de Anguilles, Mauritius). Monkeys (n = 3; 6–8 kg) were fasted overnight starting at 3 p.m. Then, the following morning, they were dosed with rosuvastatin (3 mg/kg) in solution by oral gavage. Blood and liver samples were taken at 1, 6, and 24 hours after rosuvastatin administration. Blood samples (3–5 ml/time point) were collected from a central venous port into EDTA tubes and immediately spun to collect plasma. Liver samples were collected by ultrasound-guided biopsy. Monkeys were administered ketamine (5–10 mg/kg) and dexmedetomidine (0.02 mg/kg) intramuscularly prior to the biopsy procedure. Throughout the procedure, the anesthetic depth was monitored by toe pinch reflex, gross purposeful movement, and palpebral reflex. For local analgesia, 0.1–0.2 ml of 2% lidocaine was subcutaneously injected at the biopsy site. With the monkey in a dorsal recumbent position, hair was clipped from the right cranial abdomen and skin disinfection was conducted. A routine transabdominal ultrasound was conducted to identify an appropriate liver biopsy site free of great vessels, the gallbladder, and adjacent organs. Samples were taken from the right medial or right lateral lobe. A sterile 18-gauge biopsy needle was slowly advanced under the skin until it was visualized at the appropriate biopsy site and then the spring-loaded biopsy apparatus was discharged to obtain a core of the liver tissue (~4–8 mg). At completion of the procedure, ultrasound visualization of the biopsy site was maintained to check for signs of hemorrhaging and dexmedetomidine was reversed using atipamezole (0.15 mg/kg). One liver sample was taken from one monkey at each time point (n = 1/time point). Three weeks later, the procedure was repeated in the same animals. Blood samples were again collected 1, 6, and 24 hours postdose in all animals and one liver sample was again collected from one animal at 1, 6, and 24 hours (n = 1/time point). The liver sampling was staggered so that the same time point was not collected from the same animal on the two study days. On each study day, the animals were fed at approximately 8 hours postdose. Liver samples were snap frozen on dry ice, and all samples were stored at −80°C until rosuvastatin concentrations were determined by liquid chromatography–mass spectrometry (LC-MS/MS).

In Vitro Uptake of Rosuvastatin in Monkey Hepatocytes

Uptake of rosuvastatin was evaluated in monkey hepatocytes in suspension using the oil-spin method. Female cynomolgus monkey cryopreserved hepatocytes (10 female-pooled lot #0012-1407) were purchased from Invitro ADMET Laboratories, LLC (Columbia, MD). Hepatocytes were thawed at 37°C and then placed in InVitroGRO HT medium. Cells were spun down and reconstituted in KHB to a density of 2 × 10⁶ viable cells/ml. Cell viability was >90%, as determined by Trypan blue staining. Rosuvastatin uptake was evaluated from 0.25 to 15 minutes at 0.2 and 1 μM at 37°C and at 1 μM at 4°C. As uptake at 4°C may underestimate passive diffusion (Poirier et al., 2008), uptake was also assessed at 37°C in the absence and presence of 100 μM rifampicin SV from 0.25 to 1.5 minutes. All time points were evaluated in triplicate. For evaluation at 37°C, cells were prewarmed for 3 minutes prior to the initiation of rosuvastatin uptake. For evaluation at 4°C, cells were kept on ice after reconstitution. To initiate uptake, an equal volume of KHB containing 2 × the rosuvastatin concentration was added. Aliquots were taken at specified time points and added to centrifuge tubes containing 100 μl of a 5:1 silicone to mineral oil ratio (density = 1.015) and 50 μl of 2 M ammonium acetate and then spun down using table-top centrifuges at 10,000 rpm for 10 seconds to stop transport. Tubes were immediately placed on dry ice and stored at −80°C until rosuvastatin concentrations were determined by LC-MS/MS.

In Vitro Binding

Binding of rosuvastatin in monkey plasma, liver, and hepatocyte lysate was determined using equilibrium dialysis. All samples were isolated from in-house animals. The hepatocytes used were cryopreserved cells that were isolated from liver samples using the two-step collagenase perfusion method (Berry and Friend, 1969). All matrices were evaluated at 1 μM rosuvastatin in quadruplicate. Binding in plasma was evaluated with no dilution. Liver binding was evaluated in 2-, 4-, and 8-fold diluted hemogenate. Cryopreserved hepatocytes were thawed at 37°C in InVitroGRO HT and reconstituted to 15 × 10⁶ cells/ml in KHB. The cells were killed by several freeze/thaw cycles, followed by hydrolytic lysis. They were then reconstituted and diluted to 1, 5, and 15 × 10⁶ cells/ml for binding experiments. All dilutions were made in 133 mM sodium phosphate buffer. Equilibrium dialysis was carried out in a 96-well microequilibrium dialysis device (HTDialysis, LLC, Gales Ferry, CT). Prior to equilibration, membranes of 12–14 kDa cutoff were placed in distilled water for 30 minutes, followed by sodium phosphate buffer for 30 minutes. After aligning the membranes in the apparatus, 150 μl of each matrix sample was pipetted opposite 150 μl of sodium phosphate buffer and incubated at 37°C for 4 hours. As rosuvastatin may undergo metabolism/interconversion to rosuvastatin lactone, the stability of rosuvastatin acid over 4 hours was evaluated by incubating rosuvastatin in separate samples in all matrices at each dilution and similarly incubating at 37°C. Recovery of rosuvastatin acid from these samples was 89–103% in the various matrices, indicating little interconversion in vitro. After 4 hours, 20 μl of the matrix sample and 50 μl of buffer were taken. The matrix sample was added to 50 μl of blank buffer, and buffer was added to 20 μl of blank matrix to maintain a similar composition between the buffer and samples for the determination of rosuvastatin concentrations by LC-MS/MS. From diluted matrices, undiluted fraction unbound was determined as previously described (Kalvass and Maurer, 2002). For hepatocytes, the dilution factor was determined by using a value of 1 mg protein/10⁶ cells, which was compared with the protein concentration measured in whole monkey liver via spectrophotometric assay. All samples were stored at −80°C until rosuvastatin concentrations were determined by LC-MS/MS.

LC-MS/MS Measurement of Rosuvastatin

In Vivo Samples. Liver samples were homogenized 1:5 in distilled water. One hundred microliters of the homogenized sample was mixed with 200 μl of
acetonitrile containing the internal standard (100 nM rosuvastatin-d6). Samples were then vortexed and centrifuged for 10 minutes at 3500 rpm. The supernatant was then dried under nitrogen gas and reconstituted in 50 μl of mobile phase A. Plasma and liver standard curves were prepared by adding 10 μl of 10× standard into 90 μl of blank plasma or liver homogenate, and then precipitated, dried, and reconstituted similarly to the samples. In vivo samples were run on a high-performance liquid chromatography system consisting of a Shimadzu LC-10AD VP pump (Shimadzu Corp., Kyoto, Japan) and HTC Pal autosampler (CTC Analytics AG, Zwingen, Switzerland). The HPLC system was connected to an AB Sciex 4000 QTRAP mass spectrometer (MDS Sciex, Concord, ON, Canada). Five microliters of the sample were injected onto an Atlantis dC18 column mobile phase A to B ratio. In vitro samples were run on a Shimadzu UPLC system from 3 to 3.1 minutes, and 15% B maintained from 3.1 to 5 minutes. Multiple with the following gradient: 15% B to 60% B over 2 minutes, 60% B to 95% B acetonitrile (B). Elution of rosuvastatin and the internal standard was achieved rosuvastatin and carbamazepine, respectively, in the positive mode. The calibration curves for all matrices ranged from 1 to 1000 nM (standard, 200 μl of acetonitrile-containing internal standard (100 nM carbamazepine) in 200 μl of acetonitrile was then added, and samples were vortexed for 30 minutes. Standards were prepared by adding stock solution to blank hepatocyte pellets, and then were treated similarly to hepatocyte samples. A 225 μl aliquot of the standard and samples was filtered through a 0.45-μm hydrophilic low-binding polytetrafluoroethylene filter plate, and then the filtrates were dried under nitrogen gas and reconstituted in 150 μl of a 4:1 mobile phase A to B ratio. Plasma, liver, and hepatocyte lysate samples from in vitro binding experiments were quantitated with a standard curve made in a similar matrix (5:2, blank buffer to matrix ratio). To the 70 μl of each sample or standard, 200 μl of acetonitrile-containing internal standard (100 nM carbamazepine) was added and samples were vortexed. A 200-μl aliquot of each was then filtered through a 0.45-μm hydrophilic low-binding PTFE filter plate, and then the filtrates were dried under nitrogen gas and reconstituted in 150 μl of the 4:1 mobile phase A to B ratio. In vitro samples were run on a Shimadzu UPLC system (Shimadzu Corp.) connected to an AB Sciex 6500 QTRAP mass spectrometer (MDS Sciex). Five microliters of the sample were injected onto an Atlantis dC18 column (5 μm, 2.1 × 10 mm) (Waters Corp., Milford, MA). Mobile phases were 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Elution of rosuvastatin and rosuvastatin-d6 was achieved with the following gradient at a flow rate of 0.4 ml/min: 10% B to 60% B over 3 minutes, 60% B maintained from 3 to 3.5 minutes, 60% B to 90% B from 3.5 to 3.6 minutes, 90% B maintained from 3.6 to 4 minutes, return to 10% B from 4 to 4.1 minutes, and 10% B maintained from 4.1 to 4.5 minutes. Multiple reaction monitoring mass transitions were 482.2→270.2 and 488.2→276.2 for rosuvastatin and rosuvastatin-d6, respectively, in the positive mode. The calibration curves for all matrices ranged from 1 to 1000 nM (r² = 0.999 and 0.997 for plasma and liver, respectively).

**In Vitro Samples.** Hepatocyte pellets were cut from microcentrifuge tubes and sonicated following the addition of 100 μl of distilled water for 15 minutes. The internal standard (100 nM carbamazepine) in 200 μl of acetonitrile was then added, and samples were vortexed for 30 minutes. Standards were prepared by adding stock solution to blank hepatocyte pellets, and then were treated similarly to hepatocyte samples. A 225 μl aliquot of the standard and samples was filtered through a 0.45-μm hydrophilic low-binding polytetrafluoroethylene filter plate, and then the filtrates were dried under nitrogen gas and reconstituted in 150 μl of a 4:1 mobile phase A to B ratio. Plasma, liver, and hepatocyte lysate samples from in vitro binding experiments were quantitated with a standard curve made in a similar matrix (5:2, blank buffer to matrix ratio). To the 70 μl of each sample or standard, 200 μl of acetonitrile-containing internal standard (100 nM carbamazepine) was added and samples were vortexed. A 200-μl aliquot of each was then filtered through a 0.45-μm hydrophilic low-binding PTFE filter plate, and then the filtrates were dried under nitrogen gas and reconstituted in 150 μl of the 4:1 mobile phase A to B ratio. In vitro samples were run on a Shimadzu UPLC system (Shimadzu Corp.) connected to an AB Sciex 6500 QTRAP mass spectrometer (MDS Sciex). Five microliters of the sample were injected onto an Atlantis dC18 column (5 μm, 2.1 × 10 mm). Mobile phases were 0.1% formic acid in water (A) and acetonitrile (B). Elution of rosuvastatin and the internal standard was achieved with the following gradient: 15% B to 60% B over 2 minutes, 60% B to 95% B from 2 to 2.5 minutes, 95% B maintained from 2.5 to 3 minutes, return to 15% B from 3 to 3.1 minutes, and 15% B maintained from 3.1 to 5 minutes. Multiple reaction monitoring mass transitions were 482.2→258.2 and 237.1→194.0 for rosuvastatin and carbamazepine, respectively, in the positive mode. The calibration curves for all matrices ranged from 1 to 1000 nM (r² = 0.993–1.0).

**Data Analysis**

Total in vivo liver partitioning (Kp) for rosuvastatin was determined for each liver and corresponding plasma sample and over time with eqs. 1 and 2, respectively

$$K_p = \frac{C_{liver}}{C_{plasma}}$$  

$$K_p = \frac{AUC_{liver}}{AUC_{plasma}}$$

where $C_{liver}$ and $C_{plasma}$ represent the total rosuvastatin concentration in the liver and plasma. The area under the concentration time value curves of rosuvastatin were calculated from 0 to 24 hours using the Bailer method in Phoenix WinNonLin 6.3 (Certara L.P., Princeton, NJ). Unbound liver partitioning ($K_{pl}$) was determined with eq. 3

$$K_{pl} = K_p \cdot \frac{f_{liver}}{f_{plasma}}$$

where $f_{liver}$ and $f_{plasma}$ represent the unbound fraction in monkey liver and plasma, respectively, as determined by equilibrium dialysis.

Mathematical modeling of the in vitro rosuvastatin uptake was performed using a two-compartment model, similar to those previously described (Poirier et al., 2008; Menochet et al., 2012a). The total intracellular amount in each hepatocyte sample was determined by multiplying the measured rosuvastatin concentration by the reconstitution volume. Total intracellular concentrations were then calculated from total intracellular amounts, assuming a cell volume of 4 μl/10⁶ cells (Reinoeso et al., 2001). As changes in rosuvastatin medium concentrations over time were not expected in these experiments, the medium concentration was not measured and only the intracellular rosuvastatin concentrations were included in the model fitting. All modeling was performed using Phoenix WinNonLin 6.3 (Certara L.P.). The model structure is shown in Fig. 1. Model equations are as below for uptake at 37°C:

$$\frac{dC_{medium}}{dt} = \frac{-C_{medium} \cdot f_{medium} \cdot (C_{up} + PS_{diff}) + C_{cell} \cdot f_{cell} \cdot PS_{diff}}{V_{medium}}$$

and at 4 and 37°C with rifamycin SV

$$\frac{dC_{cell}}{dt} = \frac{-C_{medium} \cdot f_{medium} \cdot PS_{diff} + C_{cell} \cdot f_{cell} \cdot PS_{diff}}{V_{cell}}$$

where $C_{medium}$ and $C_{cell}$ represent the total rosuvastatin concentration in the medium and intracellular compartments, respectively; $V_{medium}$ and $V_{cell}$ represent the volume of the medium and intracellular compartments, respectively; $f_{medium}$ and $f_{cell}$ represent the unbound fraction in the medium and intracellular compartments, respectively; $C_{up}$ represents the unbound active uptake clearance; and $PS_{diff}$ represents the unbound passive diffusion clearance. $V_{cell}$ and $V_{medium}$ were fixed to 4 and 1000 μl/10⁶ cells, respectively. Rosuvastatin was presumed to be completely unbound in the medium, and $f_{medium}$ was fixed to 1. Fitted parameters included $PS_{diff}$, $C_{up}$, and $f_{cell}$. We further evaluated the effect of varying $f_{cell}$ values on the fitted clearance parameters by fixing $f_{cell}$ from 0.05 to 1 and fitting $C_{up}$ and $PS_{diff}$, which could be estimated using only data at 37°C when $f_{cell}$ was fixed. Initial conditions for the intracellular compartment were fixed at the intercept at time = 0, which was determined by linear regression using the initial linear uptake rate, and initial conditions for the medium compartment were fixed to the nominal rosuvastatin concentration added prior to initiation of uptake. Rosuvastatin medium concentrations over time were simulated using the obtained fitted parameters to validate the assumption of negligible change in this compartment. Any simulated changes in $C_{medium}$ were included in the calculation of in vitro $Kp$ and $Kp_{s,w}$, which were determined as below, using $C_{cell}$ and $C_{medium}$ at 15 minutes after steady state was obtained.

![Fig. 1. In vitro model structure. Model parameters (C_{up}, PS_{diff} and f_{cell}) were fit to measured intracellular rosuvastatin concentrations using hepatocyte uptake data at 37 and 4°C, simultaneously. *C_{up} included only for data at 37°C, as indicated in model equations under Materials and Methods. C_{medium}, f_{medium} and V_{medium} represent the rosuvastatin concentration, fraction unbound, and volume in the medium compartment, respectively. C_{cell}, f_{cell} and V_{cell} represent the rosuvastatin concentration, fraction unbound, and volume in the intracellular compartment, respectively. C_{up} and PS_{diff} represent unbound active and unbound diffusion clarity, respectively.](image-url)
Prediction of Rosuvastatin Liver Partitioning in Monkeys

\[ K_p = \frac{C_{\text{cell}}}{C_{\text{medium}}} \]  

(8)

\[ K_{p,u,u} = K_p \cdot f_{\text{cell}} \]  

(9)

Following the estimation of in vitro parameters, simulations using the two-compartment model were performed to test various hypotheses to explain observed in vitro to in vivo discrepancy in \( K_{p,u,u} \). In these simulations, the volumes of the extracellular and intracellular compartments were fixed to those for plasma and liver, respectively, of a 5-kg monkey (Bischoff et al., 1971). The in vitro clearance parameters determined using the two-compartment model were scaled up to that of the whole liver by similarly using the physiologic values for a 5-kg cynomolgus monkey and published scaling factor for hepatocellularity (Bischoff et al., 1971; Houston, 1994). The fraction unbound in the extracellular and intracellular compartments was fixed to those currently measured in the plasma and liver. The initial concentration in the extracellular compartment was arbitrarily fixed at 1 μM and that of the intracellular compartment was fixed at 0 μM. To evaluate the role of uptake transporter expression, recent data comparing OATP expression in unplated monkey hepatocytes to that in liver tissue were used to scale \( C_{\text{lp}} \) from that measured in hepatocytes to that in the liver (Wang et al., 2014). Given similar reported \( K_m \) values for rosuvastatin uptake by cynomolgus monkey OATP1B1 and OATP1B3 and a similar expression in monkey liver (Shen et al., 2013; Wang et al., 2014), contribution of these two transporters was considered equal. OATP2B1 activity was not considered as the same recent report determined its expression was negligible in monkeys compared with OATP1B1 and OATP1B3. Equation 10 was used to calculate liver active uptake clearance

\[
C_{\text{lp,iv}} = \frac{C_{\text{lp,hepatocyte (in vivo) \times OATP1B1/OATP1B3 expression in liver}}}{\text{OATP1B1/OATP1B3 expression in hepatocytes}}
\]  

(10)

where \( C_{\text{lp,hepatocyte (in vivo)}} \) represents in vivo unbound \( C_{\text{lp}} \) scaled directly from in vitro hepatocyte uptake, and \( C_{\text{lp,iv}} \), represents the scaled in vivo unbound \( C_{\text{lp}} \) after accounting for hepatocyte-to-liver differences in transporter expression. Simulations were then carried out by replacing the value for \( C_{\text{lp}} \) with \( C_{\text{lp,iv}} \) in the model. A scaling factor for \( P_{\text{Sdiff}} \) was also considered. Using rat hepatocytes, \( P_{\text{Sdiff}} \) for seven compounds was reported higher in suspension than in short-term culture (Yabe et al., 2011; Menochet et al., 2012a), with a mean difference of 6-fold. Simulations were then carried out assuming a 6-fold lower value on \( P_{\text{Sdiff}} \). Finally, many publications have called into question the restriction of drug-transporter interaction by extracellular protein binding (Burczynski et al., 2001; Blanchard et al., 2006; Poulin et al., 2012). Therefore, simulations were also carried out assuming active uptake transport was not limited by extracellular protein binding by removing this parameter from \( C_{\text{lp}} \) in model equations.

**Results**

**In Vivo Rosuvastatin Liver \( K_p \), Binding, and \( K_{p,u,u} \) in Monkeys.** Individual plasma and liver rosuvastatin concentrations following oral administration in monkeys are displayed in Fig. 2. Liver concentrations greatly exceeded plasma concentrations, with individual \( K_p \) values for each set of livers and corresponding plasma samples ranging from 69 to 145. Partitioning was similar at each time point, indicating hepatic transport had reached equilibrium at the time points assessed. Using overall area under the concentration time curve values, the value for total \( K_p \) was 118. The calculated rosuvastatin fraction unbound in the plasma and liver were both 0.12, resulting in an in vivo \( K_{p,u,u} \) value of 118. Calculated fraction unbound in hepatocytes at 15 × 10⁶ cells/ml was similar to that in the liver (0.13). Undiluted fraction unbound values at lower hepatocyte concentrations were considered invalid, as undiluted fraction unbound becomes imprecise when the measured fraction unbound approaches 80% (Pfeifer et al., 2013), and these values were 77 and 86% at 1 and 5 × 10⁴ cells/ml, respectively.

**Prediction of In Vivo Liver Partitioning from In Vitro Hepatocyte Uptake.** As shown in Fig. 3A, uptake at 4°C and that with rifamycin were overlapping, indicating passive diffusion of rosuvastatin is similar at 4 and 37°C. In vitro model fitting is shown in Fig. 3B, indicating a good fit of data at 37 and 4°C by the two-compartment model. Simulated medium concentrations are shown in Fig. 3C, verifying little change over time as presumed, assuming mass balance is maintained. The fitted in vitro values for \( f_{\text{cell}}, \) \( C_{\text{lp}} \), and \( P_{\text{Sdiff}} \) were 0.50 (7%), 54.5 (8%) \( \mu \text{L} \text{mL}^{-1} \text{min}^{-1} \), and 4.48 (13%) \( \mu \text{L} \text{mL}^{-1} \text{min}^{-1} \), respectively. The coefficient of variation for each parameter in parentheses indicated the model was able to estimate \( C_{\text{lp}}, \) \( P_{\text{Sdiff}}, \) and \( f_{\text{cell}} \) simultaneously with good precision. Using this fitted \( f_{\text{cell}} \) value, the calculated value for in vitro \( K_{p,u,u} \) was 12, underestimating in vivo \( K_{p,u,u} \) by ~10-fold.

The effect of varying \( f_{\text{cell}} \) values on fitted clearance parameters is displayed in Fig. 4. When \( f_{\text{cell}} \) was fixed, \( C_{\text{lp}} \) and \( P_{\text{Sdiff}} \) could be reliably estimated using only the 37°C data (coefficient of variation < 20% for both parameters). As shown, imprecision of \( f_{\text{cell}} \) when the value is high (≥0.5) has little effect on rosuvastatin \( C_{\text{lp}} \) or \( P_{\text{Sdiff}} \); however, imprecision in this parameter at low values appears to greatly affect both clearance parameter estimations. Fixing the rosuvastatin \( f_{\text{cell}} \) to a lower value than the true value in the in vitro system would underestimate \( C_{\text{lp}} \), overestimate \( P_{\text{Sdiff}} \), and therefore underestimate \( K_{p,u,u} \). Fixing \( f_{\text{cell}} \) to the highest value of 1 resulted in a \( K_{p,u,u} \) of 25, which is still underpredicting in vivo \( K_{p,u,u} \) by ~5-fold.

**Model Simulations to Hypotheses Underlying the Underprediction of In Vivo \( K_{p,u,u} \).** As shown in Table 1, when the clearance values, volumes, and binding parameters were fixed to those resembling plasma and liver, in contrast to in vitro studies, a decrease in extracellular (plasma) rosuvastatin concentrations could be observed at steady state compared with that initially, as would be with systemic rosuvastatin administration in vivo. Given maintenance of the ratio of active and passive clearances from those determined in vitro, the in vivo \( K_{p,u,u} \) was still similarly underestimated by ~10-fold. Correcting \( C_{\text{lp}} \) for the reported hepatocyte-to-liver OATP expression made no significant improvement on this underprediction. Corrections on \( P_{\text{Sdiff}} \) and assuming active transport is not limited by protein binding improved \( K_{p,u,u} \) estimation similarly to that observed in vivo. This suggests differences between membrane permeability in vitro and in vivo and that plasma protein binding may not limit active transport. Interestingly, with the volumes scaled to those of plasma and liver, the corrections that affected \( K_{p,u,u} \) significantly affected the extracellular (plasma) steady-state concentrations, with little effect on those in the intracellular (liver) compartment.
A fitted model parameters. Data were fit simultaneously. (C) Simulated medium concentrations from resulting observed (symbols, mean) and fitted (lines) rosvastatin uptake at 37 and 4 °C. All data were fit simultaneously. (C) Simulated medium concentrations from resulting fitted model parameters.

Discussion

Assessing drug concentrations and partitioning in target tissues is crucial to understanding pharmacokinetic/pharmacodynamic relationships for drug efficacy, drug-drug interactions, and off-target toxicity. Although the hepatocyte partitioning of multiple compounds has been evaluated in vitro and in situ in rodents, we present an initial evaluation of the translation of these in vitro methods to in vivo data in cynomolgus monkeys. In utilizing in vitro to in vivo extrapolation for the prediction of unbound hepatocyte partitioning, we present an initial evaluation in vitro and in situ in rodents, we present an initial evaluation of the in vitro to in vivo prediction of hepatic accumulation cannot be made, whereas the liver remains the site of action for many transporter substrates. In the present work, we present a method of liver sampling that allows for the collection of plasma and liver over time in monkeys and report an underprediction of unbound steady-state hepatic partitioning for rosvastatin using cryopreserved hepatocytes in a species with a similar OATP homology and activity as that in humans (Shen et al., 2013).

Although calculation of total drug partitioning in vitro and in vivo is relatively straightforward, the accurate determination of the intracellular unbound fraction is essential for indirect determination of $K_{pu,a}$ from $K_p$. In vivo tissue unbound fraction is traditionally determined by measurement using equilibrium dialysis in diluted homogenate or cell lysate of the tissue of interest (Mariappan et al., 2013), as performed in the current study. In silico methods have been also used for the prediction of in vivo tissue unbound fraction and tissue partitioning (Poulin and Theil, 2000). The use of the currently measured binding value of 0.12 for $f_{tu,iv}$ is supported by the similar fraction unbound determined in hepatocyte lysate (at a density of $15 \times 10^6$ cells/ml). Multiple approaches have been described for the determination of in vitro $f_{tu,iv}$ using hepatocytes (Yabe et al., 2011; Menochet et al., 2012a; Shibata et al., 2013). The in vitro value estimated currently with the two-compartment model of 0.50 agrees with those previously determined in rat hepatocytes of 0.48 and 0.51 (Yabe et al., 2011; Menochet et al., 2012a). An interesting observation is that the in vitro $f_{tu,iv}$ is higher than the measured $f_{tu,iv}$. Due to saturation of nonspecific binding sites in vitro, the in vitro $f_{tu,iv}$ may overestimate the true value in tissue (Zamek-Gliszczynski et al., 2013). The importance of understanding the in vitro $f_{tu,iv}$ parameter is emphasized in Fig. 4, in which applying too low of a value, such as that directly measured in tissue, could greatly affect both active and passive volumes and further underestimate the $K_{pu,a}$. In the current analysis, even assuming the largest in vitro $f_{tu,iv}$ value of 1 for rosvastatin, the in vitro data still underestimate the in vivo $K_{pu,a}$ by 5-fold, indicating that even in the uncertainty of in vitro $f_{tu,iv}$, there still exists an in vitro to in vivo discrepancy in the prediction of unbound hepatocyte partitioning.

The utility of modeling of the in vitro uptake has been recently emphasized as it allows simultaneous determination of multiple clearance parameters and intracellular unbound fraction (Zamek-Gliszczynski et al., 2013). A further benefit of employing mathematical modeling is the ability to perturb parameters and test hypotheses. As mentioned, previous efforts investigating the extrapolation of in vitro transport parameters to those in vivo using physiologically based modeling have concluded a need for scaling factors on in vitro $Cl_{up}$ to accurately predict in vivo plasma data.
including analyses specifically on rosuvastatin (Jones et al., 2012; Jamei et al., 2014). Several hypotheses for this have hence been generated, including low transporter expression/activity in vitro and the possibility of OATP expression at other sites in vivo (e.g., muscle) causing the high apparent in vivo plasma clearance. Although our data cannot rule out the involvement of other tissues, we can confirm with the current data that hepatic uptake is underestimated in vitro and that OATP expression elsewhere cannot be the sole cause for the necessity of an empirical scalar for rosuvastatin. To reconcile underprediction in vitro, we first attempted to integrate in vitro and in vivo OATP expression in monkey tissues, which unsurprisingly did not resolve the in vitro to in vivo discrepancy, given that reported OATP expression in isolated hepatocytes is almost 90% of that determined in the liver (Wang et al., 2014). The accurate estimation of passive diffusion is often neglected for clearance prediction of drugs in which active transport plays a large role; however, the value of PSdiff can be critical for the estimation of tissue partitioning. Given that the architecture of plated hepatocytes likely more closely represents that in vivo, we attempted scaling PSdiff using a previously reported difference for seven drugs in suspension versus short-term plated hepatocytes. With the application of this scaling factor, the in vitro to in vivo disconnect on KPu,u was almost eliminated. Another assumption in all clearance prediction is that only the unbound drug is available for permeation or metabolism. This has been challenged, however, as it has been demonstrated that plasma protein interactions at the cell surface may actually increase drug permeability (Poulin et al., 2012). Furthermore, for many transporter substrates, hepatic extraction is high despite their low unbound fraction. For example, for most statins, hepatic extraction is ≥70%, whereas the unbound fraction in the plasma is ≤10%, suggesting protein binding is not limiting for OATP-mediated hepatic extraction of these drugs (Igel et al., 2002). Removing this assumption on the protein binding effects was completely able to reconcile the underprediction of in vivo KPu,u from in vitro data; however, it is likely that in vivo plasma protein binding cannot be completely discounted and differences in affinity between transporter and plasma proteins may need to be considered. Given that KPu,u could hypothetically be reconciled through scaling of any of multiple parameters, it must be noted that the observed underprediction for rosuvastatin may not be quantitatively similar for other OATP substrates possessing different physicochemical properties and/or also undergoing metabolism. Prediction of plasma clearance for OATP substrates from in vitro data has demonstrated the need for drug-dependent scalars (Jones et al., 2012). Furthermore, use of different scalars or scaling of different parameters may be needed for substrates of other hepatic transporters, such as organic cation transporter 1, as hepatocyte isolation may have differing effects on the expression of various transporters (Soars et al., 2009; Lundquist et al., 2014). Overall, the simulation results warrant further investigations on the exact mechanisms contributing to the underestimation of unbound partitioning in vitro, including evaluation of a wider range of transporter substrates.

In summary, we present a novel methodology for the determination of in vivo drug liver partitioning in cynomolgus monkeys via ultrasound-guided biopsy. Using this technique, unbound liver partitioning of rosuvastatin in monkeys was extensive, further confirming the role of active hepatic uptake transporters in rosuvastatin hepatic accumulation in this species. Although in vitro hepatocyte uptake predicted the presence of active uptake and a KPu,u greater than unity, current in vitro methods were not able to quantitatively predict unbound partitioning in vivo, with a 10-fold underprediction. Multiple explanations for this in vitro underprediction exist, which need to be further explored. In vitro to in vivo extrapolation of other compounds needs to be evaluated using similar methods to determine if underprediction of KPu,u is similar in monkeys for substrates of other hepatic transporters or OATP substrates undergoing multiple processes, including enzyme metabolism.

### Authorship Contributions

**Participated in research design:** Lai, Cai, Shu, Morse, L. Zhang, Shen, Dierks, MacGuire, Marathe, Su.

**Conducted experiments:** MacGuire, Fox, Luk, Cai, Y. Zhang, L. Zhang, Morse, Su, Gu.

**Contributed new reagents or analytic tools:** MacGuire, Dierks, Lai, Cai.

**Performed data analysis:** Morse, Cai, L. Zhang, Y. Zhang, Shen, Shu, Lai.

**Wrote or contributed to the writing of the manuscript:** Morse, Lai, MacGuire, Marathe, Humphes, Cai.

### References


### TABLE 1

Values used for model simulations and resulting predicted steady-state rosuvastatin concentrations and partitioning

<table>
<thead>
<tr>
<th>Parameter</th>
<th>m/min</th>
<th>m/min</th>
<th>μM</th>
<th>μM</th>
<th>Kp</th>
<th>Kp,u</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSdiff</td>
<td>82</td>
<td>1010</td>
<td>0.12</td>
<td>0.12</td>
<td>0.11</td>
<td>1.4</td>
</tr>
<tr>
<td>Corrected for transporter expression</td>
<td>82</td>
<td>1147</td>
<td>0.12</td>
<td>0.12</td>
<td>0.10</td>
<td>1.5</td>
</tr>
<tr>
<td>Corrected for cell surface area</td>
<td>14</td>
<td>1010</td>
<td>0.12</td>
<td>0.12</td>
<td>0.011</td>
<td>1.6</td>
</tr>
<tr>
<td>Active uptake not limited by binding</td>
<td>82</td>
<td>1010</td>
<td>0.12</td>
<td>0.12</td>
<td>0.015</td>
<td>1.6</td>
</tr>
</tbody>
</table>

IC = extracellular; IC = intracellular.

*$^*$Removed from CIC,q.


Address correspondence to: Yurong Lai, Route 206 and Provinceline Rd, Princeton, NJ 08540. E-mail: yurong.lai@bms.com.