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

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

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Current address (H.C.): Analytical Sciences, GlaxoSmithKline, King of Prussia, PA

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Corresponding author: Yurong Lai

Route 206 & Provinceline Rd

Princeton, NJ 08540

ph: (609) 252-6365

fax: (609) 252-7354

yurong.lai@bms.com

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Abbreviations: AUC, area under the concentration-time curve; Cl_{up}, unbound active uptake clearance; fu, fraction unbound; fu_{cell}, intracellular unbound fraction; Kp, total tissue partition coefficient; $Kp_{u,u}$, unbound tissue partition coefficient; LC-MS/MS, liquid chromotography-mass spectrometry; OATP, organic anion transporting polypeptide; PS_{diff}, unbound passive diffusion clearance

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 unbound liver partitioning ($Kp_{u,u}$) of rosuvastatin, a substrate of hepatic organic anion transporting peptides (OATPs), in cynomolgus monkeys and compare to 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, intracellular unbound fraction and $Kp_{u,u}$. In vitro $Kp_{u,u}$ under-predicted 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, 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 under-predicted using hepatocytes in vitro. Modeling approaches that allow integrating corrections from passive diffusion or protein binding on active uptake could improve estimation of in vivo intracellular partitioning of this OATP substrate. 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. While 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 reliably be 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 using rat or human hepatocytes for uptake of substrates for the hepatic organic anion transporting polypeptides (OATPs) (Paine et al., 2008, Yabe et al., 2011, Nordell et al., 2013, Pfeifer et al., 2013, Shitara et al., 2013, Keemink et al., 2015). Approaches

which have compared in vitro partitioning in rat hepatocytes to that in the rat liver in situ or in vivo conclude generally good prediction for select compounds (Paine et al., 2008, Pfeifer et al., 2013). However, as mentioned, rat orthologs of the OATP family differ considerably in amino acid sequence and tissue distribution compared with the human isoforms (Hagenbuch and Meier, 2004) and uptake into human hepatocytes has been demonstrated considerably lower than that in rat for a number of OATP substrates (Poirier et al., 2009, Menochet et al., 2012b). Additionally, in vitro-to-in vivo extrapolation in rats is generally assessed using fresh hepatocytes, for which human materials are at best impractical in drug discovery and development. These discrepancies beg the question of the suitability of methods validated for prediction of partitioning in rat for prediction of liver partitioning using human cryopreserved hepatocytes. Recently, Shen et al. cloned and characterized OATP1B1, 1B3, and 2B1 in cynomolgus monkeys, demonstrating these isoforms share >90% amino acid identity with the human OATPs along with similar 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 similar in vivo disposition and OATP-mediated interactions for rosuvastatin in humans were quantitatively reproduced using monkeys (Shen et al., 2013, Shen et al., 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 vitro-to-in vivo extrapolation of rosuvastatin unbound liver partitioning.

Materials and Methods

Chemicals. Rosuvastatin was purchased from Toronto Research Chemicals (North York, ON, Canada). Carbamazepine and rifamycin 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. Monkeys (n=3, 6-8 kg) were fasted overnight starting at 3pm, then the following morning 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/timepoint) 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 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 injected SQ at the biopsy site. With the monkey in a dorsal recumbent position, hair was clipped from the right cranial abdomen and the abdomen sterile prepped. 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 visualized at the appropriate biopsy site then the spring-loaded biopsy apparatus was discharged to obtain a core of liver tissue (~4-8 mg). At completion of the procedure, ultrasound

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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 timepoint (n=1/timepoint). Three weeks later the procedure was repeated in the same animals. Blood samples were again collected 1, 6, and 24 hours post-dose in all animals and one liver sample was again collected from one animal at 1, 6, and 24 hours (n=1/timepoint). The liver sampling was staggered so that the same timepoint was not collected from the same animal on the two study days. On each study day the animals were fed at approximately 8 hours post-dose. Liver samples were snap-frozen on dry ice and all samples were stored at -80°C until rosuvastatin concentrations were determined by liquid chromotography-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 # 012-1407) were purchased from In Vitro ADMET Laboratories, LLC. (Columbia, MD). Hepatocytes were thawed at 37°C then placed in InVitroGRO HT medium. Cells were spun down and reconstituted in KHB to a density of $2x10^6$ viable cells/mL. Cell viability was >90% as determined by trypan blue staining. Rosuvastatin uptake was evaluated from 0.25-15 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 rifamycin SV from 0.25-1.5 minutes. All time points were evaluated in triplicate. For evaluation at 37°C, cells were pre-warmed 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 2x the rosuvastatin concentration was added. Aliquots were taken at specified timepoints and added to centrifuge tubes containing 100 µL 5:1 silicone:mineral oil (density=1.015) and 50 µL 2M ammonium acetate then spun on 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. 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 homogenate. Cryopreserved hepatocytes were thawed at 37°C in InVitroGRO HT and reconstituted to 15 x 10^6 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 x 10^6 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 cut-off 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, 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 matrix sample and 50 µL of buffer were taken; matrix sample was added to 50 µL blank buffer and buffer added to 20 µL blank matrix to maintain similar composition between buffer and samples for 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, dilution

factor was determined by using a value of 1 mg protein/10^6 cells which was compared to 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 (dH_2O). 100 µL homogenized sample was mixed with 200 µL acetonitrile containing internal standard (100 nM rosuvastatin-d6). Samples were then vortexed and centrifuged for 10 min at 3500 rpm. The supernatant was then dried under nitrogen gas and reconstituted in 50 µL mobile phase A. Plasma and liver standard curves were prepared by adding 10 µL of 10x standard into 90 µL blank plasma or liver homogenate, then precipitated, dried, and reconstituted similarly to samples. In vivo samples were run on an HPLC system consisting of a Shimadzu LC-10AD VP pump (Shimadzu Corp., 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). Five uL of sample was injected onto an Atlantis dC18 column (5 um, 2.1 x 10 mm)(Waters Corp., Millford, 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 3-3.5 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 4-4.1 minutes, 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 positive mode. The calibration curves for plasma and liver ranged from 2-1000 nM (r^2=0.999 and r^2=0.997 for plasma and liver, respectively).

In vitro samples

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Hepatocyte pellets were cut from microcentrifuge tubes and sonicated following addition of 100 µL dH_2O for 15 minutes. 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, then treated similarly to hepatocyte samples. A 225 µL aliquot of standard and samples were filtered through a 0.45 µm hydrophilic low-binding PTFE filter plate, then the filtrates dried under nitrogen gas and reconstituted in 150 µL 4:1 mobile phase A:B. Plasma, liver, and hepatocyte lysate samples from in vitro binding experiments were quantitated with a standard curve made in similar matrix (5:2, blank buffer:matrix). To the 70 µL of each sample or standard, 200 µL of acetonitrile containing internal standard (100 nM carbamazepine) was added and samples vortexed. A 200 µL aliquot of each was then filtered through a 0.45 µm hydrophilic low-binding PTFE filter plate, then the filtrates dried under nitrogen gas and reconstituted in 150 µL 4:1 mobile phase A:B. In vitro samples were run on a Shimadzu UPLC system (Shimadzu Corp., Japan) connected to an AB Sciex 6500 Qtrap mass spectrometer (MDS Sciex, Concord, ON). Five uL of sample was injected onto an Atlantis dC18 column (5um, 2.1 x 10 mm). Mobile phases were 0.1% formic acid in water (A) and acetonitrile (B). Elution of rosuvastatin and internal standard was achieved with the following gradient: 15% B to 60% B over 2 minutes, 60% B to 95% B from 2-2.5 minutes, 95% B maintained from 2.5-3 minutes, return to 15% B from 3-3.1 minutes, 15% B maintained from 3.1-5 minutes. Multiple reaction monitoring mass transitions were 482.2→258.2 and 237.1→194.0 for rosuvastatin and carbamazepine, respectively, in positive mode. The calibration curves for all matrices ranged from 1-1000 nM (r^2=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 equations (1) and (2), respectively:

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$$Kp = \frac{C_{liver}}{C_{plasma}} \quad (1)$$

$$Kp = \frac{AUC_{liver}}{AUC_{plasma}} \quad (2)$$

where C_{liver} and C_{plasma} represent the total rosuvastatin concentration in the liver and plasma. The area under the concentration-time curve (AUC) values of rosuvastatin were calculated from 0-24 hrs using the Bailer method in Phoenix WinNonLin 6.3 (Certara L.P., Princeton, NJ). Unbound liver partitioning ($Kp_{u,u}$) was determined with equation (3):

$$Kp_{u,u} = Kp \cdot \frac{fu_{liver}}{fu_{plasma}} \quad (3)$$

Where fu_{liver} and fu_{plasma} represent the unbound fraction in monkey liver and plasma, respectively, as determined by equilibrium dialysis.

Mathematical modeling of in vitro rosuvastatin uptake was performed using a 2-compartment model, similar to those previously described (Poirier et al., 2008, Menochet et al., 2012a). 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/10^6 cells (Reinoso et al., 2001). As changes in rosuvastatin medium concentrations over time were not expected in these experiments, 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 Figure 1. Model equations are as below for uptake at 37°C:

$$dC_{medium}/dt = \frac{-C_{medium} \cdot fu_{medium} \cdot (Cl_{up} + PS_{diff}) + C_{cell} \cdot fu_{cell} \cdot PS_{diff}}{V_{medium}} \quad (4)$$

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$$dC_{cell}/_{dt} = \frac{C_{medium} \cdot fu_{medium} \cdot (Cl_{up} + PS_{diff}) - C_{cell} \cdot fu_{cell} \cdot PS_{diff}}{V_{cell}} \quad (5)$$

and at 4°C and at 37°C with rifamycin SV:

$$dC_{medium}/_{dt} = \frac{-C_{medium} \cdot fu_{medium} \cdot PS_{diff} + C_{cell} \cdot fu_{cell} \cdot PS_{diff}}{V_{medium}} \quad (6)$$

$$dC_{cell}/_{dt} = \frac{C_{medium} \cdot fu_{medium} \cdot PS_{diff} - C_{cell} \cdot fu_{cell} \cdot PS_{diff}}{V_{cell}} \quad (7)$$

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, fu_{medium} and fu_{cell} represent the unbound fraction in the medium and intracellular compartments, respectively, CL_{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 1,000 µL/10^6 cells, respectively. Rosuvastatin was presumed to be completely unbound in the medium and fu_{medium} was fixed to 1. Fitted parameters included PS_{diff}, Cl_{up}, and fu_{cell}. We further evaluated the effect of varying fu_{cell} values on the fitted clearance parameters by fixing fu_{cell} from 0.05-1 and fitting CL_{up} and PS_{diff}, which could be estimated using only data at 37°C when fu_{cell} was fixed. Initial conditions for the intracellular compartment were fixed at the intercept at time=0 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 $Kp_{u,u}$, which were determined as below, using C_{cell} and C_{medium} at 15 minutes, after steady-state was obtained:

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$$Kp = \frac{C_{cell}}{C_{medium}} \quad (8)$$

$$Kp_{u,u} = Kp \cdot fu_{cell} \quad (9)$$

Following the estimation of in vitro parameters, simulations using the 2-compartment model were performed to test various hypotheses to explain observed in vitro-to-in vivo discrepancy in $Kp_{u,u}$. 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 2-compartment model were scaled up to that of whole liver similarly using physiologic values for a 5 kg cynomolgus monkey and published scaling factor for hepatocellularity (Bischoff et al., 1971, Houston, 1994). Fraction unbound in extracellular and intracellular compartments was fixed to those currently measured in plasma and liver. The initial concentration in the extracellular compartment was arbitrarily fixed at 1 µM and that of the intracellular compartment 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 was used to scale Cl_{up} 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 1B3 and 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 negligible in monkeys compared to OATP1B1 and 1B3. Equation 10 was used to calculate liver active uptake clearance:

$$Clup_{liver} = Clup_{hepatocyte(in\ vivo)} \frac{OATP1B1/1B3\ expression\ in\ liver}{OATP1B1/1B3\ expression\ in\ hepatocytes} \quad (10)$$

where $Clup_{hepatocyte(in\ vivo)}$ represents in vivo unbound Cl_{up} scaled directly from in vitro hepatocyte uptake and $Clup_{liver}$ represents the scaled in vivo unbound Cl_{up} after accounting for hepatocyte-

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to-liver differences in transporter expression. Simulations were then carried out by replacing the value for Clup with Clliver in the model. A scaling factor for PS_{diff} was also considered. Using rat hepatocytes, PS_{diff} for 7 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 PS_{diff}. 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 Cl_{up} in model equations.

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Results

In vivo rosuvastatin liver Kp, binding and $Kp_{u,u}$ in monkeys.

Individual plasma and liver rosuvastatin concentrations following oral administration in monkeys are displayed in Figure 2. Liver concentrations greatly exceeded plasma concentrations with individual Kp values for each set of liver and corresponding plasma sample ranging from 69-145. Partitioning was similar at each timepoint, indicating hepatic transport had reached equilibrium at the timepoints assessed. Using overall AUC values, the value for total Kp was 118. Calculated rosuvastatin fraction unbound in plasma and liver were both 0.12, resulting in an in vivo $Kp_{u,u}$ value of 118. Calculated fraction unbound in hepatocytes at 15 x 10^6 cells/mL was similar to that in liver (0.13). Undiluted fraction unbound values at lower hepatocytes concentrations were considered invalid, as undiluted fraction unbound becomes imprecise when measured fraction unbound approaches 80% (Pfeifer et al., 2013) and these values were 77% and 86% at 1 and 5 x 10^6 cells/mL, respectively.

Prediction of in vivo liver partitioning from in vitro hepatocyte uptake.

As shown in Figure 3A, uptake at 4°C and that with rifamycin were overlapping, indicating passive diffusion of rosuvastatin is similar at 4°C and 37°C. In vitro model fitting is shown in Figure 3B, indicating good fit of data at 37°C and 4°C by the 2-compartment model. Simulated medium concentrations are shown in Figure 3C, verifying little change over time as presumed, assuming mass-balance is maintained. The fitted in vitro values for fu_{cell}, Cl_{up}, and PS_{diff} were 0.50 (7%), 54.5 (8%) µL/10^6 cells/min, and 4.48 (13%) µL/10^6 cells/min, respectively. Coefficient of variation (CV%) for each parameter in parentheses indicate the model was able to estimate Cl_{up}, PS_{diff} and fu_{cell} simultaneously with good precision. Using this fitted fu_{cell} value, the calculated value for in vitro $Kp_{u,u}$ was 12, underestimating in vivo Kp_{uu} by ~10-fold.

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The effect of varying fu_{cell} values on fitted clearance parameters is displayed in Figure 4. When fu_{cell} was fixed, CL_{up} and PS_{diff} could be reliably estimated using only the 37°C data (CV<20% for both parameters). As shown, imprecision of fu_{cell} when the value is high (≥0.5) has little effect on rosuvastatin Cl_{up} or PS_{diff}; however, imprecision in this parameter at low values appears to greatly affect both clearance parameter estimations. Fixing the rosuvastatin fu_{cell} to a lower value than the true value in the in vitro system would underestimate Cl_{up}, overestimate PS_{diff}, and therefore underestimate $Kp_{u,u}$. Fixing fu_{cell} to the highest value of 1 resulted in a $Kp_{u,u}$ of 25, still underpredicting in vivo $Kp_{u,u}$ by ~5-fold.

Model simulations to hypotheses underlying the underprediction of in vivo Kpu,u

As shown in Table 1, when 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 to 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 $Kp_{u,u}$ was still similarly underestimated by ~10-fold. Correcting CL_{up} for reported hepatocyte-to-liver OATP expression made no significant improvement on this under-prediction. Corrections on PS_{diff} and assuming active transport is not limited by protein binding improved $Kp_{u,u}$ estimation to similar 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 which affected $Kp_{u,u}$ significantly affected the extracellular (plasma) steady-state concentrations with little effect on those in the intracellular (liver) compartment.

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. While 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 plasma concentrations, whether by metabolism or transport, underestimation by in vitro methods is a well-known issue in drug development (Galetin, 2014). Specifically, using physiologically-based pharmacokinetic modeling, prediction of in vivo plasma uptake clearance for OATP substrates in humans from in vitro hepatocyte uptake requires empirical scaling factors to account for under-prediction in vitro (Poirier et al., 2009, Jones et al., 2012). Previous estimates evaluate prediction of human plasma data only, however, and conclusions about in vitro-to-in vivo prediction of hepatic accumulation cannot be made, while 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 collection of plasma and liver over time in monkeys, and report an under-prediction of unbound steady-state hepatic partitioning for rosuvastatin using cryopreserved hepatocytes, in a species with similar OATP homology and activity as that in humans (Shen et al., 2013).

While 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 $Kp_{u,u}$ from Kp. 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 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 fu_{liver} is supported by

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the similar fraction unbound determined in hepatocyte lysate (at density: 15 x 10^6 cells/mL). Multiple approaches have been described for determination of in vitro fu_{cell} using hepatocytes (Yabe et al., 2011, Menochet et al., 2012a, Shitara et al., 2013). The in vitro value estimated currently with the 2-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 fu_{cell} estimate in this work is higher than the measured fu_{liver}. Due to saturation of nonspecific binding sites in vitro, the in vitro fu_{cell} may overestimate the true value in tissue (Zamek-Gliszczynski et al., 2013). The importance of understanding the in vitro fu_{cell} parameter is emphasized in Figure 4, in which applying too low a value, such as that directly measured in tissue, could greatly affect both active and passive values, and further underestimate the $Kp_{u,u}$. In the current analysis, even assuming the largest in vitro fu_{cell} value of 1 for rosuvastatin, the in vitro data still under-predict the in vivo $Kp_{u,u}$ in vivo by 5-fold, indicating that even in the uncertainty of in vitro fu_{cell} there still exists an in vitro-to-in vivo discrepancy in prediction of unbound hepatocyte partitioning.

The utility of modeling of 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. While our data cannot rule out the involvement of other tissues, we can confirm with the current data that hepatic uptake is

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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 integrating 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 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 PS_{diff} 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 PS_{diff} using a previously reported difference for 7 drugs in suspension vs. short-term plated hepatocytes. With the application of this scaling factor, the in vitro-to-in vivo disconnect on $Kp_{u,u}$ was almost eliminated. Another assumption in all clearance prediction is that only 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; e.g. for most statins hepatic extraction is ≥70% while 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 protein binding effects was completely able to reconcile the under-prediction of in vivo $Kp_{u,u}$ 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 $Kp_{u,u}$ could hypothetically be reconciled through scaling of any of multiple parameters, it must be noted that the observed underprediction for rosuvastatin may not quantitatively be 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

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different scalars or scaling of different parameters may be needed for substrates of other hepatic transporters, such as OCT1, 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 underestimation of unbound partitioning in vitro, including evaluation of a wider range of transporter substrates.

In summary, we present novel methodology for 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. While in vitro hepatocyte uptake predicted the presence of active uptake and a $Kp_{u,u}$ greater than unity, current in vitro methods were not able to quantitatively predict unbound partitioning in vivo, with a 10-fold under-prediction. Multiple explanations for this in vitro under-prediction 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 $Kp_{u,u}$ is similar in monkeys for substrates of other hepatic transporters, or OATP substrates undergoing multiple processes including enzyme metabolism.

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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, Humpheys, Cai

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Footnotes

a) This research was supported by Bristol-Myers Squibb, Co.

b) This research was previously presented as an abstract: Prediction of In Vivo Rosuvastatin Liver Partitioning in Cynomolgus Monkeys using In Vitro Hepatocyte Uptake, American Association of Pharmaceutical Scientists/International Transporter Consortium Joint Workshop on Transporters, Baltimore, MD April 2015.

c) Reprint requests should be sent to:

Bridget L. Morse

Route 206 & Provinceline Rd

Princeton, NJ 08540

Bridget.Morse@bms.com

Figure Legends

Figure 1. In vitro model structure.

Model parameters (Cl_{up}, PS_{diff}, and fu_{cell}) were fit to measured intracellular rosuvastatin concentrations using hepatocyte uptake data at 37°C and 4°C, simultaneously. *Cl_{up} included only for data at 37°C, as indicated in model equations under Materials and Methods. C_{medium}, fu_{medium}, amd V_{medium} represent the rosuvastatin concentration, fraction unbound and volume in the medium compartment, respectively; C_{cell}, fu_{cell}, and V_{cell} represent the rosuvastatin concentration, fraction unbound and volume in the intracellular compartment, respectively. Cl_{up} and PS_{diff} represent unbound active and unbound diffusion clearance, respectively.

Figure 2. Rosuvastatin liver and plasma concentrations following oral administration to cynomolgus monkeys.

Monkeys (n=3) were administered rosuvastatin 3 mg/kg by oral gavage on two study days. On each study day, plasma was collected at 1, 6, and 24 hours from all monkeys and one liver sample from one monkey was taken per study day. Data presented are corresponding plasma and liver samples taken over two study days.

Figure 3. Rosuvastatin uptake in monkey hepatocytes.

A) Comparison of rosuvastatin uptake at 37°C and 4°C with that at 37°C with and without 100 uM rifamycin SV (mean ±SD). Closed and open symbols represent two different experiments. B) Observed (symbols, mean) and fitted (lines) rosuvastatin uptake at 37°C and 4°C. All data were fit simultaneously. C) Simulated medium concentrations from resulting fitted model parameters.

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Figure 4. Effect of fixed fu_{cell} values on fitted clearance parameters.

The parameter fu_{cell} was fixed to values ranging from 0.05-1 and at each fixed fu_{cell} value, Cl_{up} and PS_{diff} parameters were estimated using the 2-compartment model described in Figure 1, using uptake at 37°C only.

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Tables

Table 1. Values used for model simulations and resulting predicted steady-state rosuvastatin concentrations and partitioning.

	PS_{diff} (mL/min)	Cl_{up} (mL/min)	fu_{EC}	fu_{IC}	C_{EC} (μM)	C_{IC} (μM)	Kp	$Kp_{u,u}$
Scaled in vitro model parameters	82	1010	0.12	0.12	0.11	1.4	12	12
Corrected for transporter expression	82	1147	0.12	0.12	0.10	1.5	15	15
Corrected for cell surface area	14	1010	0.12	0.12	0.011	1.6	74	74
Active uptake not limited by binding	82	1010	0.12*	0.12	0.015	1.6	106	106

Clearance parameters were scaled from those determined in the in vitro model to represent those in the liver of a 5 kg monkey. Volumes for the extracellular and intracellular compartments were fixed to those of plasma and liver, respectively, of a 5 kg monkey. Extracellular and intracellular fraction unbound (fu) were fixed to those measured in monkey plasma and liver, respectively. The initial condition for C_{EC} was arbitrarily fixed to 1 μM and to 0 for C_{IC}.

* removed from Cl_{up}

PS_{diff}=unbound passive diffusion clearance

Cl_{up}=unbound active uptake clearance

fu=fraction unbound

C=concentration

EC=extracellular

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IC=intracellular

Kp=total liver partition coefficient

$Kp_{u,u}$ =unbound liver partition coefficient

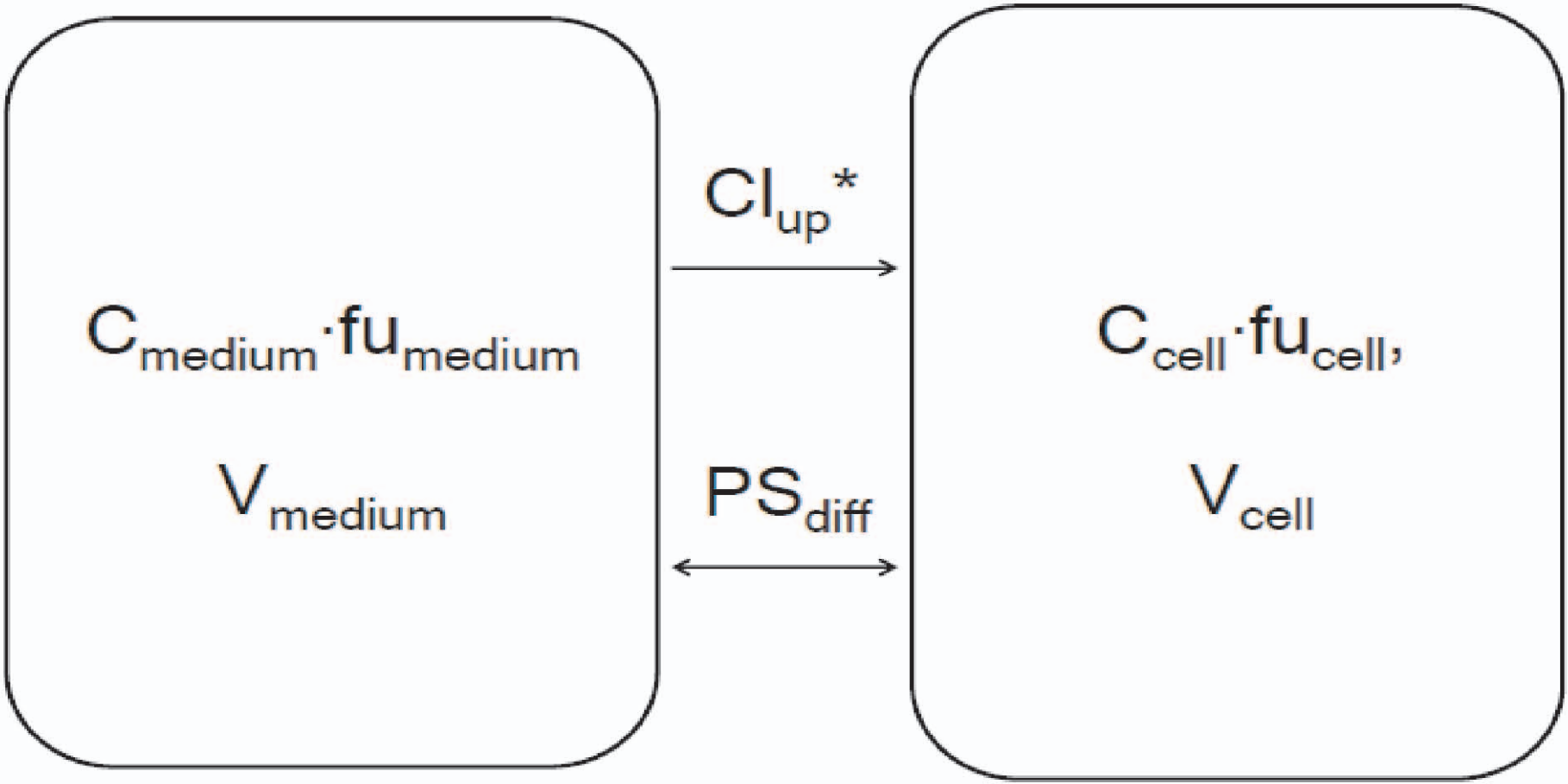
$C_{medium} \cdot fu_{medium}$
V_{medium}
Cl_{up}*
PS_{diff}
$C_{cell} \cdot fu_{cell}$,
V_{cell}

Fig 1

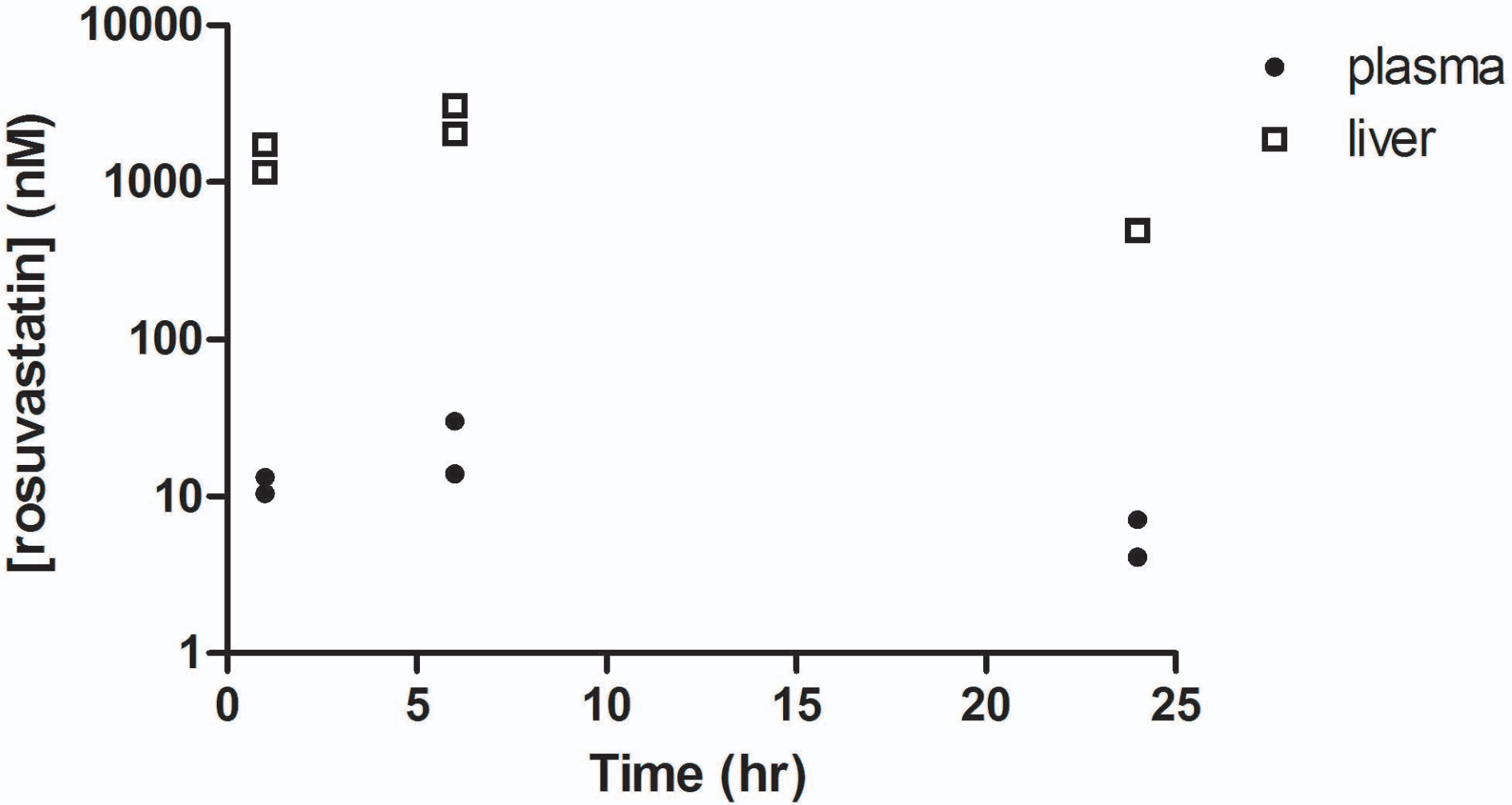
10000
1000
100
10
1
[rosuvastatin] (nM)
0
5
10
15
20
25
Time (hr)
plasma
liver

Fig 2

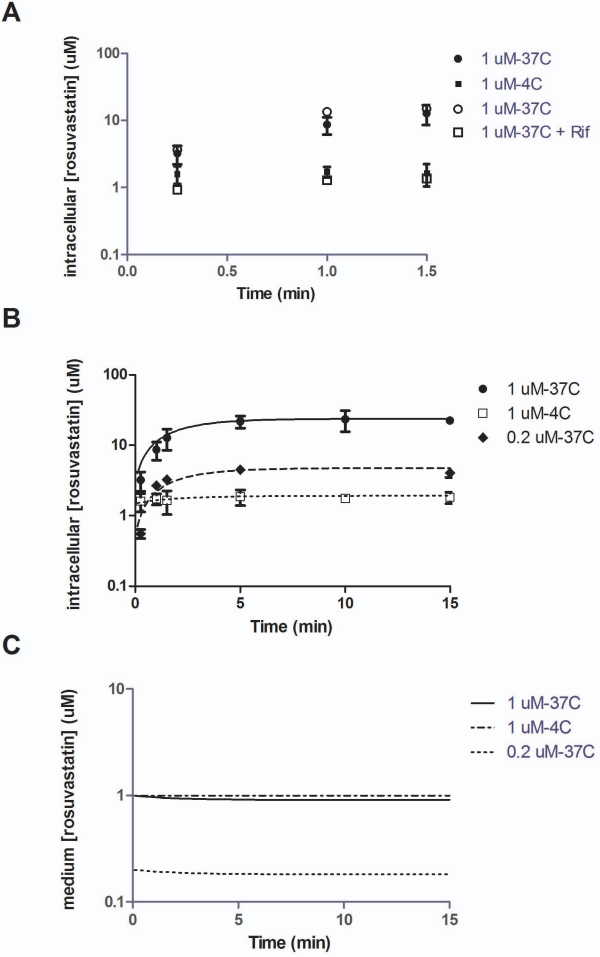
A
intracellular [rosuvastatin] (uM)
100
10
1
0.1
0.0
0.5
1.0
1.5
Time (min)
1 uM-37C
1 uM-4C
1 uM-37C
1 uM-37C + Rif
B
intracellular [rosuvastatin] (uM)
100
10
1
0.1
0
5
10
15
Time (min)
1 uM-37C
1 uM-4C
0.2 uM-37C
C
medium [rosuvastatin] (uM)
10
1
0.1
0
5
10
15
Time (min)
1 uM-37C
1 uM-4C
0.2 uM-37C

Fig 3

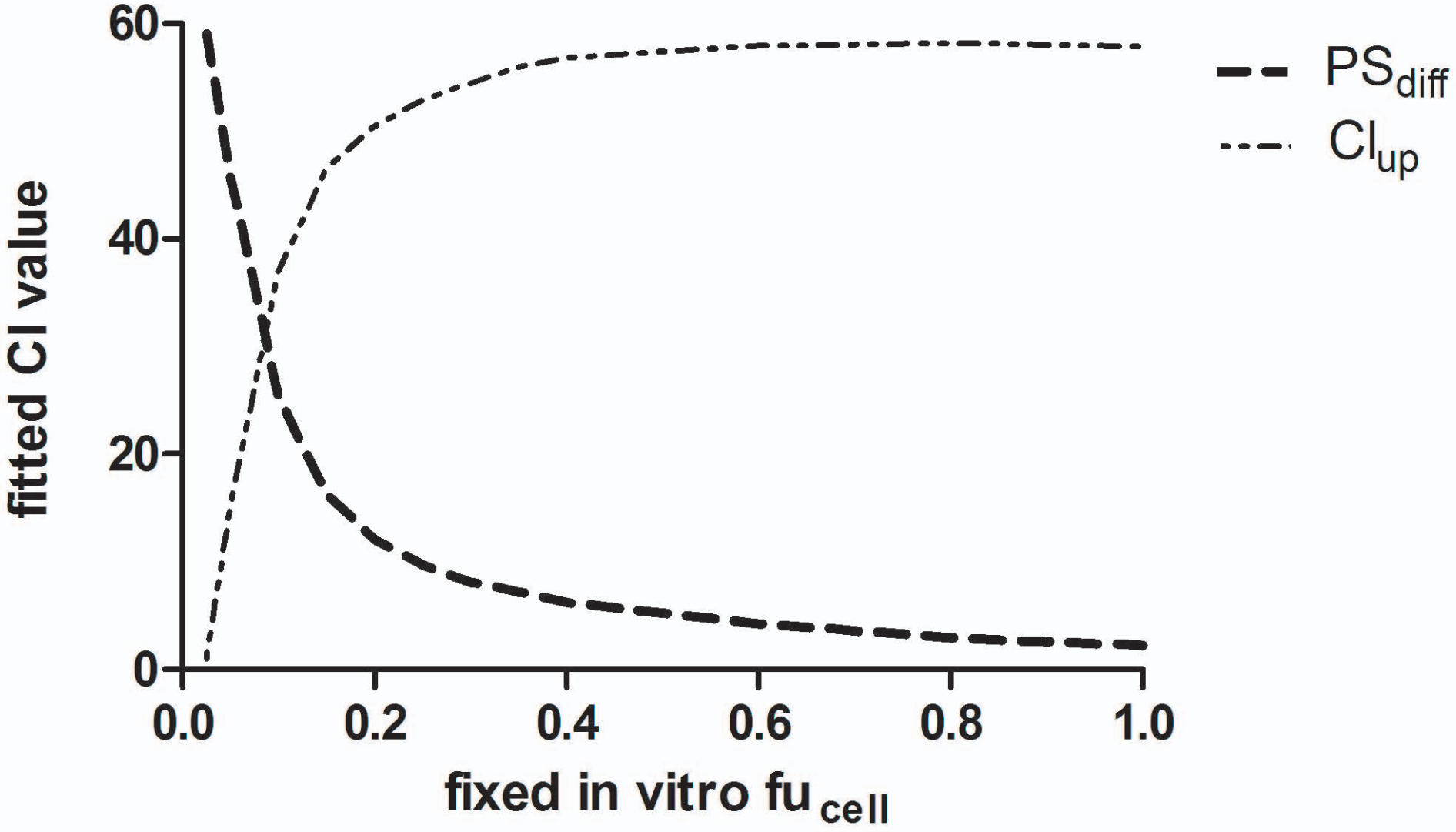
60
40
20
0
fitted Cl value
0.0
0.2
0.4
0.6
0.8
1.0
fixed in vitro fu cell
PS diff
Cl up

Fig 4