Successful prediction of in vivo hepatobiliary clearances and hepatic concentrations of rosvastatin using sandwich-cultured rat hepatocytes, transporter-expressing cell lines, and quantitative proteomics

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d) Nonstandard Abbreviations: AUC, area under the concentration-time profile; BEI, biliary excretion index; CHO cells, Chinese hamster ovary cells; $CL_{bile}$, biliary clearance; $CL_{de-lac}$, de-lactonization clearance; $CL_{lac}$, lactonization clearance; $CL_{met}$, remainder metabolic
clearance; $CL_{passive}$, passive diffusion uptake clearance; $CL_{s,efflux}$, sinusoidal efflux clearance; $CL_{s,uptake}$, sinusoidal uptake clearance; $CL_{s,uptake}^{Oatp+CHO}$, total $CL_{s,uptake}$ estimated from Oatp-expressing cells (active) and CHO-mock cells (passive); $CL_{s,uptake}^{Oatp+HEK}$, total $CL_{s,uptake}$ estimated from Oatp-expressing cells (active) and HEK293-mock cells (passive); $CL_{s,uptake}^{Oatp+SCRH}$, total $CL_{s,uptake}$ estimated from Oatp-expressing cells (active) and SCRH (passive); $CL_{s,uptake}^{SCRH}$, total $CL_{s,uptake}$ estimated from SCRH not corrected for Oatp protein expression; $CL_{s,uptake}^{PET}$, total $CL_{s,uptake}$ in our PET imaging study; CI, confidence interval; CV, coefficient of variation; DDIs, drug-drug interactions; HBSS buffer, Hank’s balanced salt solution buffer; HEK293 cells, human embryo kidney 293 cells; IVIVE, in vitro-to-in vivo extrapolation; KH buffer, Krebs-Henseleit buffer; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Ntcp, sodium taurocholate co-transporting polypeptide; Mrp, multidrug resistance-associated protein; Oatp, organic anion transporting polypeptide; PET, positron emission tomography; RAF, relative activity factor; RSV, rosuvastatin; SCH, sandwich-cultured hepatocytes; SCRH, sandwich-cultured rat hepatocytes; SD rat, Sprague-Dawley rat; SDS, sodium dodecyl sulfate; TCA, taurocholic acid
**Abstract**

We determined whether *in vivo* transporter-mediated hepatobiliary clearance and hepatic concentrations of rosuvastatin (RSV) in the rat could be predicted by transport activity in sandwich-cultured rat hepatocytes (SCRH) and/or transporter-expressing cell lines scaled by differences in transporter protein expression between SCRH, cell lines, and rat liver. The predicted hepatobiliary clearances and hepatic concentrations of RSV were compared to our previously published PET imaging data. Sinusoidal uptake clearance (*CL*$_{s,uptake}$) and efflux (canalicular and sinusoidal) clearances of [³H]-RSV in SCRH were evaluated in the presence and absence of Ca²⁺ and in the absence and presence of 1 mM unlabeled RSV (to estimate passive diffusion clearance). *CL*$_{uptake}$ of RSV into cells expressing Organic anion transporting polypeptide (Oatp) 1a1, 1a4, and 1b2 was also determined. Protein expression of Oatps in SCRH and Oatp-expressing cells was quantified by LC-MS/MS. SCRH well predicted the *in vivo* RSV sinusoidal and canalicular efflux clearances, but significantly underestimated *in vivo* *CL*$_{s,uptake}$. Oatp expression in SCRH was significantly lower than that in the rat liver. *CL*$_{s,uptake}$, based on RSV *CL*$_{uptake}$ into Oatp-expressing cells (active transport) plus passive diffusion clearance in SCRH, scaled by the difference in protein expression in Oatp cells vs. SCRH vs. rat liver, was within the 2-fold of that observed in SCRH or *in vivo*. *In vivo* hepatic RSV concentrations were well predicted by Oatp-expressing cells after correcting *CL*$_{s,uptake}$ for Oatp protein expression. This is the first demonstration of successful prediction of *in vivo* hepatobiliary clearance and hepatic concentrations of RSV using transporter-expressing cells and SCRH.
Keywords: hepatobiliary clearance; sandwich-cultured hepatocytes; in vitro-in vivo extrapolation; rosvastatin; quantitative proteomics; organic anion transporting polypeptides; Oatp1a1, Oatp1a4, Oatp1b2
Introduction

Hepatobiliary clearance of drugs is determined by transporter-mediated uptake and efflux, metabolism, or a combination of all three. Predicting in vivo hepatobiliary clearance of drugs from in vitro data (in vitro-in vivo extrapolation, IVIVE) is important for drug development including determining first in human dose and anticipating drug-drug interactions (DDIs). While IVIVE of metabolic clearance of drugs has been successful (Ke et al., 2014; Rostami-Hodjegan and Tucker, 2007; Rowland et al., 2011; Soars et al., 2007), prediction of transporter-mediated clearance remains a challenge (Abe et al., 2008; Zou et al., 2013).

Sandwich-cultured hepatocytes (SCH) are considered the gold standard for estimating in vivo transporter-mediated hepatobiliary clearances of drugs (Pfeifer et al., 2014; Swift et al., 2010). However, such predictions typically underestimate the drug’s in vivo hepatic clearance (Jones et al., 2012; Watanabe et al., 2009; Zou et al., 2013), requiring empirical scaling factors (Jones et al., 2012; Kimoto et al., 2017; Ménochet et al., 2012; Zou et al., 2013). This empirical scaling factor is drug-dependent and therefore cannot be generalized. Others have proposed the relative activity factor (RAF) approach when using human hepatocytes to predict transporter-mediated in vivo clearance of drugs (Chapy et al., 2015). However, to date, the RAF approach has been used primarily to determine the fraction of drug transported by hepatocytes (Hirano et al., 2004; 2006; Kitamura et al., 2008; Kunze et al., 2014; Nakakariya et al., 2008a; Nakakariya et al., 2008b; Yamashiro et al., 2006). This is because the RAF can only be used when transporter-specific substrates are available for use both in vitro and in vivo. This requirement is a significant limitation for predicting organic anion transporting polypeptide (OATP)-dependent drug clearance as most OATP substrates (e.g. rosuvastatin) are transported by multiple OATPs. Therefore, alternative approaches are required to predict transporter-mediated clearance of drugs.
For this reason, we and others have proposed that transporter-mediated hepatobiliary clearance of drugs may be possible to estimate directly from transporter-expressing cell lines (Bosgra et al., 2014; Li et al., 2010; Prasad and Unadkat, 2015; Vildhede et al., 2016). The disadvantage of this approach is that the absolute abundance of the transporter protein in both the cell lines and liver tissue must be quantified. With the development of targeted quantitative proteomics, this disadvantage has been overcome (Bosgra et al., 2014; Ohtsuki et al., 2011; Prasad and Unadkat, 2014; Wang et al., 2015). Indeed, Bosgra et al. showed that the systemic clearance of rosvastatin (RSV) in humans was well predicted using suspended primary human hepatocytes, OATP-expressing cell lines, and quantitative proteomics (Bosgra et al., 2014).

Besides systemic clearance, it is also important to predict drug concentrations in the target tissue (in the case of RSV, hepatic concentration) to predict drug efficacy/toxicity and the prediction of DDIs. As stated above, our ultimate goal is to predict transporter-mediated hepatobiliary clearance of drugs and hepatic concentrations directly from transporter-expressing cell lines. Therefore, as a first step towards achieving this goal, using RSV as our model drug, we determined whether Oatp transporter-expressing cell lines, quantitative proteomics combined with sandwich-cultured rat hepatocytes (SCRH) can predict the in vivo transporter-mediated hepatobiliary clearance and hepatic concentrations of RSV observed in our PET imaging study (He et al., 2014).
Materials and Methods

Materials. RSV (acid form, hereafter referred to as RSV) was purchased from Cayman Chemicals (Ann Arbor, MI). [³H]-RSV sodium salt (acid form, 20 mCi/mmol, radiochemical purity 99%) was purchased from American Radiolabeled Chemicals (St. Louis, MO). RSV lactone was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). All other chemicals were of reagent or analytical grade and purchased from other commercial suppliers.

SCRH. Freshly isolated SCRH (adult male SD rats) plated into 24-well plate were purchased from Triangle Research Labs (TRL), LLC (Durham, NC) or Qualyst Transporter Solutions, LLC (Durham, NC). According to vendors’ instruction, after receiving SCRH, the shipping medium was changed to maintenance medium (purchased from the vendors). Then, the maintenance medium (purchased from the vendors) was changed every 24 hr. The uptake and efflux experiments were conducted at about 96 hr after plating.

Uptake and Efflux of RSV in SCRH. The uptake and efflux of [³H]-RSV was evaluated as previously described (B-CLEAR® technology, Qualyst Transporter Solutions) (Pfeifer et al., 2013b) with minor modifications. Briefly, after washing SCRH (0.35-0.4 × 10⁶ cells/well) twice with pre-warmed 500 µL Ca²⁺/Mg²⁺-containing Hank’s balanced salt solution (HBSS, hereafter referred to as Ca²⁺-containing HBSS) or 500 µL Ca²⁺/Mg²⁺-free HBSS with 1 mM EDTA (hereafter referred to as Ca²⁺-free HBSS), the SCRH were first pre-incubated with 500 µL of Ca²⁺-containing HBSS or Ca²⁺-free HBSS (to disrupt the canalicular tight junctions) for 10 min at 37 °C. The SCRH were then incubated with 500 µL of 0.5 µM RSV containing 0.08-0.2 µCi/well [³H]-RSV in Ca²⁺-containing HBSS at 37 °C. Then, at 2-, 5-, 10-, and 20-min incubation with 0.5 µM RSV, the Ca²⁺-containing HBSS was collected, and SCRH were washed twice with ice-cold Ca²⁺-containing or Ca²⁺-free HBSS. Then, 1 mL of 2% sodium dodecyl
sulfate (SDS) was added to each well to lyse the cells (uptake phase). To evaluate the efflux of RSV from the SCRH, after 10-min pre-incubation with Ca$^{2+}$-containing or Ca$^{2+}$-free HBSS, the SCRH were incubated with 500 µL of 0.5 µM RSV containing 0.08-0.2 µCi/well [$^3$H]-RSV in Ca$^{2+}$-containing HBSS at 37 °C for 20 min. Then, Ca$^{2+}$-containing HBSS was aspirated, and SCRH were washed twice (500 µL) with ice-cold Ca$^{2+}$-containing or Ca$^{2+}$-free HBSS. Then, SCRH were incubated with 500 µL of RSV-free Ca$^{2+}$-containing or Ca$^{2+}$-free HBSS for 2, 5, 10, and 15 min at 37 °C. An aliquot of the Ca$^{2+}$-containing and Ca$^{2+}$-free HBSS was collected, and the SCRH were washed twice (500 µL) with ice-cold Ca$^{2+}$-containing or Ca$^{2+}$-free HBSS. Then, 1 mL of 2% SDS was added to each well to lyse the SCRH (efflux phase). The total radioactivity in the samples was measured using liquid scintillation counter (Perkin Elmer, Waltham, MA). To inhibit all uptake transporters and to determine passive diffusion uptake clearance of the RSV into the hepatocytes, SCRH were incubated with and without 1 mM of unlabeled RSV throughout the above time periods. The total protein concentration of cell lysate was measured with Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL), according to the manufacture’s instruction. Uptake and efflux experiments in the absence and presence of 1 mM unlabeled RSV were performed in 6 and 5 lots of SCRH respectively.

**Determination of RSV and RSV Lactone in SCRH Samples by HPLC Fractionation.** Fifty to hundred µL of SCRH samples (both cell lysates and buffer) were mixed with the same volume of acetonitrile (containing 20 µM unlabeled RSV and 20 µM of RSV lactone). After centrifuging the samples at 16,000 × g and 4 °C for 5 min, the supernatant (50-100 µL) was analyzed by HPLC (Alliance 2695, Waters Corporation, Milford, MA). RSV and RSV lactone were separated on an Agilent ZORBAX SB-C8 column (3.5 µm, 4.6 × 100 mm) using a mobile phase (1 mL/min) consisting of 20 mM KH$_2$PO$_4$ (pH 2.5) (A) and acetonitrile (B). The gradient
was: 40% B (0-6 min); 40-55% B (6-10 min); 55% B (10-20 min); 55-80% (20-22 min); 80% B (22-27 min); 80-40% B (27-29 min); and 40% B (29-30 min). UV absorbance of the analytes was monitored at 243 nm, and fractions were collected every minute for liquid scintillation counting. RSV and its lactone eluted at ~12 and ~17 min, respectively. To determine the percent of RSV and RSV lactone in the samples, the radioactivity associated with the unlabeled RSV and RSV lactone was expressed as a percent of total radioactivity collected in the fractions.

**Estimation of the Hepatobiliary Clearances of RSV in SCRH.** To estimate the hepatobiliary clearances of [\(^3\)H]-RSV in SCRH in both the uptake and efflux phase, the following model (Eq. 1-6) was fitted to the data using the nonlinear regression package, Phoenix® (Certara, Princeton, NJ). The Poisson error model was used as the residual error model.

\[
\frac{dX_{buffer}}{dt} = CL_{s,flux} \times \frac{X_{cell}}{V_{cell}} - CL_{s,uptake} \times \frac{X_{buffer}}{V_{buffer}} + K_{flux} \times X_{bile} 
\]

(1)

\[
\frac{dX_{buffer}}{dt} = CL_{s,flux} \times \frac{X_{cell}}{V_{cell}} + CL_{bile} \times \frac{X_{cell}}{V_{cell}} - CL_{s,uptake} \times \frac{X_{buffer}}{V_{buffer}} 
\]

(2)

\[
\frac{dX_{cell}}{dt} = CL_{s,uptake} \times \frac{X_{buffer}}{V_{buffer}} - CL_{s,flux} \times \frac{X_{cell}}{V_{cell}} - CL_{bile} \times \frac{X_{cell}}{V_{cell}} - CL_{lac} \times X_{cell} + CL_{de-lac} \times \frac{X_{fac.cell}}{V_{cell}} - CL_{met} \times \frac{X_{cell}}{V_{cell}} 
\]

(3)

\[
\frac{dX_{cell}}{dt} = CL_{s,uptake} \times \frac{X_{buffer}}{V_{buffer}} - CL_{s,flux} \times \frac{X_{cell}}{V_{cell}} - CL_{bile} \times \frac{X_{cell}}{V_{cell}} - CL_{lac} \times X_{cell} + CL_{de-lac} \times \frac{X_{fac.cell}}{V_{cell}} - CL_{met} \times \frac{X_{cell}}{V_{cell}} 
\]

(4)

\[
\frac{dX_{bile}}{dt} = CL_{bile} \times \frac{X_{cell}}{V_{cell}} - K_{flux} \times X_{bile} 
\]

(5)

\[
\frac{dX_{bile+cell}}{dt} = \frac{dX_{bile}}{dt} + \frac{dX_{cell}}{dt} 
\]

(6)

where \(X_{buffer}^+\) and \(X_{buffer}^-\) indicate the amount of [\(^3\)H]-RSV in the Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free HBSS, respectively. \(X_{cell}^+\) and \(X_{cell}^-\) indicate the amount of [\(^3\)H]-RSV in the cells incubated with Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free HBSS, respectively. \(X_{bile}\) is the amount of [\(^3\)H]-
RSV in the bile pockets of SCRH incubated with Ca\(^{2+}\)-containing HBSS. \(X_{\text{lac,cell}}^+\) and \(X_{\text{lac,cell}}^-\) indicate the amount of [\(^3\)H]-RSV lactone in the cells incubated with Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free HBSS, respectively. \(CL_{\text{s,uptake}}\), \(CL_{\text{s,efflux}}\), and \(CL_{\text{bile}}\) are the sinusoidal uptake, the sinusoidal efflux, and the biliary clearance of [\(^3\)H]-RSV respectively. \(CL_{\text{lac}}\), \(CL_{\text{de-lac}}\), and \(CL_{\text{met}}\) are the metabolic clearance of [\(^3\)H]-RSV to the lactone, the metabolic clearance of [\(^3\)H]-RSV lactone to [\(^3\)H]-RSV, and the remainder metabolic clearance of [\(^3\)H]-RSV, respectively.

RSV is converted to the lactone form (RSV lactone), and RSV lactone is back-converted to the acid form (Nezasa et al., 2002). In addition, RSV is metabolized to the pentenoic acid derivative (minor metabolite; remainder metabolic clearance) in the rat liver (Nezasa et al., 2002; Pfeifer et al., 2013a). \(V_{\text{cell}}\) and \(V_{\text{buffer}}\) represent the volume of SCRH (7.4 µL/mg protein) (Pfeifer et al., 2013b) and buffer (500 µL), respectively. \(K_{\text{flux}}\) represents the rate constant of efflux of bile from bile pocket due to “pulsing” of the bile canaliculi (Pfeifer et al., 2013b). If the 95% confidence interval (CI) of a parameter encompassed zero, that parameter was fixed to zero. In addition, \(CL_{\text{lac}}\), \(CL_{\text{de-lac}}\), \(CL_{\text{met}}\), and \(K_{\text{flux}}\) were assumed to be invariant for the two concentration of unlabeled RSV (0.5 µM and 1 mM).

**RSV Uptake into CHO-Oatp1a1, HEK293-Oatp1a4, and HEK293-Oatp1b2 Expressing Cells.** CHO-Oatp1a1, HEK293-Oatp1a4, HEK293-Oatp1b2, and their corresponding mock-transfected cells were a gift from SOLVO Biotechnology (Budaörs, Hungary). CHO-Oatp1a1 and CHO-mock cells, grown in 75 cm\(^2\) flasks, were harvested using trypsin, and were plated at the density of 0.25 × 10\(^6\) cells/cm\(^2\) in 24-well plates. These cells were incubated with 5 mM of sodium butyrate about 24 hr before the transport experiments. HEK293-Oatp1a4, HEK293-Oatp1b2, and HEK293-mock cells, grown in 75 cm\(^2\) flask with 3 µg/mL puromycin, were harvested using trypsin, and plated at the density of 0.25 × 10\(^6\) cells/cm\(^2\)
in 24-well poly-D-lysine coated plates. These cells were also incubated with 3 µg/mL puromycin 24 hr before the transport experiments. The cells were washed three times with pre-warmed Krebs-Henseleit (KH) buffer, then pre-incubated with the KH buffer at 37 °C for 10 min. After pre-incubation, the cells were incubated with 500 µL of 0.07 µM RSV containing 0.2 µCi/well [³H]-RSV at 37 °C for 5 sec (Oatp1a1) or 15 sec (Oatp1a4 and Oatp1b2), a time period over which the uptake of [³H]-RSV was assumed to be linear (Oatp1a1) or found to linear (Oatp1a4 and Oatp1b2; data not shown). The KH buffer containing RSV was aspirated, and the cells were washed three times with ice-cold KH buffer, and then 1 mL of 2% SDS was added to lyse the cells. The total radioactivity and total protein concentration in the samples were measured as described above. The transporter-mediated uptake was calculated by subtracting the uptake of the drug in the mock cells from that in the transfected cells. The Oatp-mediated uptake clearance of RSV into each Oatp cell line was calculated as the ratio of the rate of transporter-mediated uptake (unlabeled plus labeled RSV; in pmol/min/mg total protein) and the total concentration of RSV in the medium (0.07 µM). The passive diffusion-mediated uptake clearance of RSV was calculated as above but using the uptake clearance in mock-transfected cell lines. We also evaluated RSV uptake into CHO-Ntcp cells. However, RSV was not transported by Ntcp (data not shown).

**Quantification of Transporters in SCRH and Transporter-expressing Cell Lines using Surrogate Peptides and Quantitative Proteomics.** The total cell membranes of SCRH (only sufficient cells were available for three out of 6 lots) and Oatp-expressing cell lines were isolated using Calbiochem® ProteoExtract® Native Membrane Protein Extraction Kit (EMD Millipore Corporation, Billerica, MA) according to the manufacture’s instruction. Cell membranes were then reduced, denatured, alkylated, and digested as previously described with minor
modifications (Wang et al., 2015). That is, 80 µL of total membranes (0.3-0.6 mg/mL) were incubated with 10 µL of 250 mM dithiothreitol, 20 µL of 10% sodium deoxycholate, 10 µL of 10 mg/mL human serum albumin, and 40 µL of 100 mM ammonium bicarbonate (pH 7.8) at 95 °C for 10 min. Then, the mixture was incubated with 20 µL of 500 mM iodoaceticamide for 30 min at room temperature in the dark. To this mixture were added, 0.5 mL of methanol, 0.1 mL of chloroform, and 0.4 mL of Milli-Q water. After centrifuging the samples at 16,000 × g and 4 °C for 5 min, the pellet was washed once with 1.0 mL of methanol, and re-suspended in 60 µL of 50 mM ammonium bicarbonate. The extracted proteins were digested with 20 µL of 0.16 µg/µL trypsin at 37 °C for 18 hr. The digestion was stopped by adding 20 µL of chilled labeled peptide internal standard cocktail (in 80% acetonitrile solution with 0.2% formic acid, Supplemental Table 2), and the samples were centrifuged at 5,000 × g and 4 °C for 5 min. Finally, 5 µL of the supernatant were injected onto a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system to quantify the expression of the Oatp transporters in total cell membranes (expressed as fmol/µg membrane protein).

Scaling CL\text{uptake} from In Vitro to In Vivo. The intrinsic uptake clearance CL\text{uptake} of RSV in the transporter-expressing cell lines were scaled to that in SCRH (in vitro-to-in vitro extrapolation, Eq. 7) or in vivo (IVIVE, i.e., in liver tissue, Eq. 8) as follows:

\[ CL_{\text{scaled_{in vitro-to-in vitro}}} = \sum \left( CL_{\text{uptake,Oatp,i}} \times \frac{[\text{Transporter_{SCRH}}]}{[\text{Transporter_{cells}}]} \right) + CL_{\text{passive}} \quad (7) \]

\[ CL_{\text{scaled_{in vitro-to-in vivo}}} = \sum \left( CL_{\text{uptake,Oatp,i}} \times \frac{[\text{Transporter_{tissue}}]}{[\text{Transporter_{cells}}]} \right) + CL_{\text{passive}} \quad (8) \]

where \( CL_{\text{uptake,Oatp,i}} \) indicates \( CL_{\text{uptake}} \) into the Oatp transporter-expressing cell line (i.e., Oatp 1a1, 1a4, or 1b2). \( CL_{\text{passive}} \) indicates the passive diffusion uptake clearance estimated from mock-transfected cell lines or SCRH (in the presence of 1 mM unlabeled RSV). As to
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which $CL_{\text{passive}}$ provides the best estimate of the observed in vitro (SCRH) or in vivo $CL_{\text{s.uptake}}$ was also examined (see Results). $[\text{Transporter}_{\text{tissue}}]$, $[\text{Transporter}_{\text{SCRH}}]$, and $[\text{Transporter}_{\text{cell}}]$ indicate the expression of each Oatp in SD rat liver, SCRH, and transporter-expressing cells, respectively. Scaling Oatp $CL_{\text{uptake}}$ in cell lines to SCRH and then to in vivo using transporter expression data is equivalent to directly scaling cell line $CL_{\text{uptake}}$ to that in vivo because the expression in SCRH cancels out as follows:

$$CL_{\text{uptake}}(\text{scaled in vitro to in vivo}) = \sum (CL_{\text{uptake, Oatp.j}} \times \frac{\text{Transporter expression, tissue}}{\text{Transporter expression, cell}} \times \frac{\text{Transporter expression, tissue}}{\text{Transporter expression, scan}}) + CL_{\text{passive}}$$

(9)

Then, all passive and active clearances (in mL/min/mg protein) estimated from SCRH or mock-transfected cell lines (CHO and HEK293 cells), were expressed as mL/min/kg body weight, assuming 200 mg of total protein of SCRH or mock-transfected cells/g liver and 40 g liver/kg body weight (Abe et al., 2008; Wolf et al., 2010). In addition, to compare the predicted and in vivo observed $CL_{\text{s.uptake}}$, the predicted $CL_{\text{s.uptake}}$ (in mL/min/kg body weight) was scaled to that in vivo using fraction unbound in plasma (0.039) and blood:plasma ratio (0.67) of RSV in the rat (Fukuda et al., 2008). The total $CL_{\text{s.uptake}}$ of RSV, calculated as the Oatp-mediated $CL_{\text{uptake}}$ ($CL_{\text{Oatp}}^{\text{Oatp}}, \text{summation of } CL_{\text{uptake}}$ in each Oatp-expressing cells), plus $CL_{\text{passive}}$ (estimated from SCRH in the presence of 1 mM unlabeled RSV, CHO-mock, or HEK293-mock cells) are indicated as $CL_{\text{s.uptake}}^{\text{Oatp+SCRH}}, CL_{\text{s.uptake}}^{\text{Oatp+CHO}}, \text{and } CL_{\text{s.uptake}}^{\text{Oatp+HEK}}$, respectively. Statistically significant differences between the various groups were determined by the Welch’s t-test (corrected for multiple comparisons).

**Simulation of Hepatic Concentrations of RSV Using HepatobiliaryClearances of RSV Estimated from Oatp-expressing Cells and SCRH.** The hepatic concentrations of RSV were simulated using mean $[^{3}\text{H}]$-RSV $CL_{\text{efflux}}$ and $CL_{\text{bile}}$ estimated from SCRH and $CL_{\text{s.uptake}}^{\text{Oatp+SCRH}}$,
and compared to our previous *in vivo* PET imaging data (He et al., 2014). The hepatic concentrations of RSV were simulated using the following equation and SAAMII (The Epsilon Group, Charlottesville, VA):

\[
\frac{dX_{\text{liver}}}{dt} = CL_{\text{s,uptake}}^{\text{Oatp+SCRH}} \cdot \frac{X_{\text{blood}}}{V_{\text{blood}}} - CL_{\text{s,efflux}} \cdot \frac{X_{\text{liver}}}{V_{\text{liver}}} - CL_{\text{bile}} \cdot \frac{X_{\text{liver}}}{V_{\text{liver}}}
\]  

(10)

where \(X_{\text{liver}}\), \(X_{\text{blood}}\), \(V_{\text{liver}}\), and \(V_{\text{blood}}\) indicate the amount of RSV in the liver, the amount of RSV in blood, the volume of the liver, and the volume of blood, respectively. The lag time from blood to liver and liver to bile as well as \(V_{\text{liver}}\) and \(V_{\text{blood}}\) were fixed as reported previously (He et al., 2014).
Results

Cellular Accumulation and Efflux of $[^3\text{H}]-\text{RSV}$ and Estimation of Hepatobiliary Clearance of RSV in SCRH. The morphology of SCRH was good (Supplemental Figure 1), and the biliary excretion index (BEI) values of taurocholic acid (TCA) were reasonable or lower than expected (Supplemental Table 3) (Lee et al., 2010; Li et al., 2010). The mean percent of $[^3\text{H}]-\text{RSV}$ and $[^3\text{H}]-\text{RSV}$ lactone (in the absence of 1 mM unlabeled RSV) was 67-78% and 13-21% in cell lysate samples (Supplemental Table 4). The percent of RSV in SCRH was similar to that reported previously (Nezasa et al., 2002). In contrast, all the radioactivity in the buffer samples in the absence of 1 mM unlabeled RSV was in the acid form (Supplemental Table 4). However, due to much lower total cellular radioactivity in the presence of 1 mM unlabeled RSV, we could not quantify the percentage of $[^3\text{H}]-\text{RSV}$ and $[^3\text{H}]-\text{RSV}$ lactone in the cell lysates or the buffer samples (data not shown). Mean cellular content of $[^3\text{H}]-\text{RSV}$ and $[^3\text{H}]-\text{RSV}$ lactone in the SCRH were used to estimate the hepatobiliary clearances of RSV described below.

Figure 1 and Supplemental Figure 2 show the cellular accumulation of $[^3\text{H}]-\text{RSV}$ and $[^3\text{H}]-\text{RSV}$ lactone and efflux of $[^3\text{H}]-\text{RSV}$ into the buffer. In three lots of SCRH (RSD279, RSD288, and Rs14Dec15T), the cellular accumulation of $[^3\text{H}]-\text{RSV}$ in the absence of 1 mM unlabeled RSV with Ca$^{2+}$-containing HBSS was modestly higher than that with Ca$^{2+}$-free HBSS (Figure 1 and Supplemental Figure 2) while in the remaining lots there was no difference. Therefore, $CL_{\text{bile}}$ could be estimated in only these three lots of SCRH (Table 1 and Supplemental Table 3). The cellular accumulation of $[^3\text{H}]-\text{RSV}$ and $[^3\text{H}]-\text{RSV}$ lactone and $[^3\text{H}]-\text{RSV}$ efflux into the buffer in the presence of 1 mM unlabeled RSV were much lower than that in the absence of 1 mM unlabeled RSV (Figure 1 and Supplemental Figure 2; note the difference in scale of the Y-axis). $CL_{\text{s,uptake}}$ of $[^3\text{H}]-\text{RSV}$ was significantly decreased by 1 mM unlabeled RSV, but
surprisingly $CL_{\text{s,efflux}}$ of $[^3\text{H}]}$-RSV was significantly increased by 1 mM unlabeled RSV (Table 1 and Supplemental Table 3). In addition, because the cellular accumulation of $[^3\text{H}]}$-RSV in the presence of 1 mM unlabeled RSV with $\text{Ca}^{2+}$-containing HBSS was the same as that with $\text{Ca}^{2+}$-free HBSS, $CL_{\text{bile}}$ in the presence of 1 mM unlabeled RSV could not be estimated and was fixed to 0.

**Comparison of Hepatobiliary Clearance of RSV and Oatp Expression in SCRH with That Observed In Vivo.** Consistent with previous findings (Jones et al., 2012; Watanabe et al., 2009), the in vivo $CL_{\text{s,uptake}}$ of RSV was under-predicted by SCRH (5.50-fold) (Figure 2). In contrast, in vivo $CL_{\text{s,efflux}}$ and $CL_{\text{bile}}$ were exceedingly well predicted by SCRH ($p>0.05$; Figure 2). Since only the in vivo $CL_{\text{s,uptake}}$ was underestimated by SCRH, we determined if this was due to difference in expression of Oatps in our SCRH vs. SD liver tissue. Indeed, the expression of all the Oatps was significantly lower in SCRH than that in SD liver tissue (Figure 3).

**Scaling RSV $CL_{\text{uptake}}$ in Oatp-expressing Cells to That in SCRH (in vitro-to-in vitro) or That In Vivo Based on Oatp Transporter Expression.** After correcting for transporter protein expression, the transporter-mediated intrinsic RSV $CL_{\text{uptake}}$ by Oatp1a1 was higher than that by Oatp1a4 or Oatp1b2 (Table 2). These individual $CL_{\text{uptake}}$ values were scaled to SCRH using their respective protein expression in SCRH, and then these scaled-up $CL_{\text{uptake}}$ and $CL_{\text{passive}}$ estimated from SCRH and mock cells (Table 3) were summed to recreate in silico the intrinsic $CL_{\text{s,uptake}}$ of RSV in SCRH (in vitro-to-in vitro extrapolation, Figure 4A). The in silico $CL^{\text{Oatp+HEK}}_{\text{s,uptake}}$ and $CL^{\text{Oatp+SCRH}}_{\text{s,uptake}}$ were not significantly different from the observed $CL^{\text{SCRH}}_{\text{s,uptake}}$ (not corrected for Oatp protein expression) (Figure 4A). In contrast, the in silico $CL^{\text{Oatp+CHO}}_{\text{s,uptake}}$ was
greater than $CL_{s,uptake}^{SCRH}$ (Figure 4A).

The prediction of total (active plus passive) in vivo $CL_{s,uptake}$ estimated from Oap-expressing cell lines after correcting for Oatp protein expression was significantly improved compared with $CL_{s,uptake}$ estimated from SCRH data (without correcting for transporter protein expression; Figure 2 and 4B). When compared with $CL_{s,uptake}^{Oatp+CHO}$, $CL_{s,uptake}^{Oatp+SCRH}$ or $CL_{s,uptake}^{Oatp+HEK}$, better approximated our PET imaging data ($CL_{s,uptake}^{PET}$) (Figure 4B). However, these three clearances were within 2-fold of the observed value in our PET imaging study ($CL_{s,uptake}^{PET}$), irrespective of whether $CL_{passive}$ was estimated from SCRH, CHO-mock cells or HEK293-mock cells (Figure 4B).

Simulation of Hepatic RSV Concentrations Predicted by Transporter-based Scaling of RSV Hepatobiliary Clearance. The mean hepatic concentrations of RSV predicted based on $CL_{s,efflux}$ and $CL_{bile}$ from SCRH and $CL_{s,uptake}^{Oatp+SCRH}$ fell within the 95% CI of the concentrations in our previous PET imaging study, suggesting no significant difference between the observed and predicted hepatic concentrations (Figure 5).
Discussion

As we have described before, the hepatic clearance of a drug when transporters are involved is determined by the extended clearance model (Patilea-Vrana and Unadkat, 2016; Sirianni and Pang, 1997):

$$CL_H = \frac{Q_H f_{up}CL_{s,uptake}(CL_{met} + CL_{bile})}{Q_H (CL_{s,efflux} + CL_{met} + CL_{bile}) + f_{up}CL_{s,uptake}(CL_{met} + CL_{bile})}$$ (11)

where $Q_H$, $f_{up}$, and $CL_{met}$ are unbound fraction in plasma and metabolic clearance, respectively. Only when $CL_{s,efflux} \ll CL_{met} + CL_{bile}$, $CL_H = Q_H \cdot f_{up} \cdot CL_{s,uptake}$, i.e. $CL_{s,uptake}$ is the rate-determining step (Patilea-Vrana and Unadkat, 2016). When $CL_{s,efflux}$ is NOT $\ll CL_{met} + CL_{bile}$, $CL_H$ will be determined by all clearance processes, that is $CL_{s,uptake}$, $CL_{s,efflux}$, and $CL_{bile}$. Therefore, if $CL_{s,efflux}$ is assumed to be $\ll CL_{met} + CL_{bile}$, when it is not (as in the case of RSV, Table 1), such an assumption could lead to poor prediction of $CL_H$ of the drug. For this reason, to correctly predict the in vivo hepatic clearance of RSV from SCRH, one must estimate all the relevant clearances (i.e. $CL_{s,uptake}$, $CL_{s,efflux}$, and $CL_{bile}$). Therefore, we estimated all the clearances involved in hepatobiliary clearance of RSV in SCRH. Since we had estimates of all these values from our PET imaging study, we were able to compare the values obtained from SCRH with those observed in vivo. Our goal was to predict the hepatobiliary clearances of RSV within 2-3-fold of the observed value, since such range of prediction is widely accepted as adequate for IVIVE (Rostami-Hodjegan and Tucker, 2007; Rowland et al., 2011).

$CL_{s,uptake}$ and $CL_{s,efflux}$ are each a summation of both passive diffusion and/or transporter-mediated clearances, and quantifying the contribution of both pathways is important for IVIVE. In the present study, to estimate passive $CL_{s,uptake}$ clearance of RSV in SCRH, unlabeled 1 mM RSV was included in the SCRH studies to inhibit all uptake transporters.
Although there are no data on the Km values of RSV uptake for the rat hepatic transporters, these values for human transporters are less than 70 µM (Bosgra et al., 2014; Ho et al., 2006; Kitamura et al., 2008; Kumar et al., 2015). Therefore, we assumed that 1 mM of unlabeled RSV completely inhibited all the rat hepatic transporters. Indeed, this 1 mM unlabeled RSV drastically reduced $CL_{s,uptake}$ of $[^3H]$-RSV in SCRH. However, surprisingly, $CL_{s,efflux}$ of $[^3H]$-RSV was significantly increased by 1 mM unlabeled RSV (Table 1 and Supplemental Table 3). We do not have a good explanation for this increase. We speculate that 1) the higher concentration of the drug in the SCRH may have saturated intracellular binding of the drug and therefore $CL_{s,efflux}$ (based on total hepatocyte drug concentration) was increased; 2) we assumed that the metabolism of the drug was not saturated even in the presence of 1 mM unlabeled RSV. If the RSV metabolite(s) was not a substrate of transporters, inhibition of RSV metabolism might result in an apparent increase in $CL_{s,efflux}$ of $[^3H]$-RSV; 3) metabolite(s) of RSV in SCRH may have stimulated sinusoidal efflux transport (if any) of RSV. RSV is reported to be a substrate of human multidrug resistance-associated protein (MRP) 4, but there are no reports whether rat sinusoidal efflux transporters, such as Mrp3 and Mrp4, can transport RSV. In addition, we have previously reported that Mrp4 in the rat liver tissue and cryopreserved hepatocytes was not detectable by LC-MS/MS (Wang et al., 2015). Further studies are needed to characterize the sinusoidal efflux of RSV and its metabolite(s) in SCRH.

$CL_{s,efflux}$ and $CL_{bile}$ of RSV determined in SCRH were not significantly different from those observed in vivo (Figure 2). $CL_{bile}$ could be determined in only three lots of SCRH (Figure 2) as the remaining lots showed poor biliary clearance (Figure 1, Supplemental Figure 2, Table 1, and Supplemental Table 3). Thus, we used data from the lots where $CL_{bile}$ was observed based on the assumption that formation of canaliculic junctions may not have been
adequate in the lots that showed on RSV $CL_{bile}$. Although these $CL_{bile}$ values were smaller than those reported by Pfeifer et al. (Pfeifer et al., 2013b), interestingly they are closer to those observed in vivo. In contrast, $CL_{s,uptake}$ of 0.5 µM RSV estimated from SCRH was significantly lower than that observed in vivo (Figure 2). Others have also reported that sandwich-cultured human hepatocytes under-predict the observed hepatic clearance of drugs (Jones et al., 2012; Kotani et al., 2011). We speculated that this might be due to a decrease in expression of transporters when the hepatocytes are cultured. Indeed, others have reported that the protein expression of sinusoidal uptake transporters is lower than that in the liver tissue (Badee et al., 2015; Bi et al., 2013). The protein expression of Oatp1a1, Oatp1a4, and Oatp1b2 in SCRH was significantly lower than that in the SD rat liver tissue (Figure 3). This lower expression completely explained the under-prediction of the in vivo $CL_{s,uptake}$ by SCRH. Of note, the $CL_{s,uptake}$ were similar and not significantly different between the SCRH lots where we had measurable RSV $CL_{bile}$ (RSD279, RSD288, and Rs14Dec15T) and where it was not measurable (RSD270, RSD294, and Rs18Jan16T).

Next, we estimated uptake $CL_{passive}$ using SCRH, mock-transfected CHO or HEK293 cells. $CL_{passive}$ estimated from these cell lines was higher than sinusoidal $CL_{passive}$ from SCRH (Table 3). We do not know the reason(s) for this over-estimation, but $CL_{passive}$ in the corresponding mock cells was not dependent on the concentration of the unlabeled RSV used and was not affected by the presence of 100 µM rifamycin SV (data not shown). In fact, the CHO cells drastically overestimated $CL_{passive}$ uptake in SCRH. Again, we do not know the reason(s) for this difference, but it might be due to differences between cells in surface area, membrane composition, and binding of RSV either to the cell surface or to intracellular proteins. Nevertheless, because the Oatp-mediated transport of RSV was the dominant contributor to the
sinusoidal uptake of RSV, the \( \text{in silico } CL_{\text{S,uptake}} \) (\( \text{scaled}_{\text{in vitro} \text{ to } \text{in vitro}} \)), was similar irrespective of whether \( CL_{\text{passive}} \) was estimated from SCRH or HEK293-mock cells and not significantly different from the observed \( CL_{\text{S,uptake}}^{\text{SCRH}} \) (Figure 4A). In contrast, \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{CHO}} \) over-predicted the observed \( CL_{\text{S,uptake}}^{\text{SCRH}} \).

The \( \text{in vivo } CL_{\text{S,uptake}} \) predicted using Oatp-expressing cells and \( CL_{\text{passive}} \) from cell lines or SCRH was remarkably consistent with that observed \( \text{in vivo} \) (within 2-fold) irrespective of whether \( CL_{\text{passive}} \) was estimated from SCRH or CHO-mock cells or HEK293-mock cells (Figure 4B). As for \( \text{in vitro} \text{-to-} \text{in vitro} \) extrapolation discussed above, prediction of \( \text{in vivo} \) \( CL_{\text{S,uptake}} \) based on \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{SCRH}} \) or \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{HEK}} \) better predicted \( CL_{\text{S,uptake}}^{\text{PET}} \). Prediction based on \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{CHO}} \) were within 2-fold because Oatp transport of RSV was the dominant process with passive diffusion playing a minor role. However, for another drug, this might not be the case. Thus, we propose that until additional data are obtained to the contrary, for drugs where the \( CL_{\text{passive}} \) is significant, that this value be estimated from SCH (using selective inhibitors of the uptake transporter) or HEK293-mock cells.

Besides, \( CL_{\text{S,uptake}}^{\text{PET}} \), the observed hepatic concentrations of RSV were well predicted by \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{SCRH}} \). The predicted values fell within 95% CI of the observed values, indicating no significant difference between these values (Figure 5). Although the predicted highest hepatic concentration of RSV (\( C_{\text{max}} \)) was greater than the observed value, this discrepancy was expected as the mean value of the \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{SCRH}} \) was greater than that observed \( \text{in vivo} \) (\( CL_{\text{S,uptake}}^{\text{PET}} \)). In addition, the half-life of the drug estimated from SCRH was shorter than that in our previous \( \text{in vivo} \) PET imaging study, because the mean value of \( CL_{\text{S,efflux}} \) and \( CL_{\text{bile}} \) in SCRH were greater than those observed \( \text{in vivo} \) (Figure 2). Since the values of \( CL_{\text{S,uptake}}^{\text{Oatp}+\text{SCRH}} \) and
are almost identical, the latter would also equally well predict the observed hepatic concentrations of RSV in our PET imaging study.

Our proposed approach has some limitations. First, it requires that the quantitative targeted proteomics method be available. Second, it assumes that the transporter abundance measured reflects functional transporters present on the cell surface. The latter is partially overcome by quantifying cell surface expression of the transporter using a refined biotinylation method that we have recently published (Kumar et al., 2017).

In conclusion, this is the first report of successful prediction of *in vivo* hepatic concentrations as well as hepatobiliary clearance of a drug from studies in transporter-expressing cell lines (scaled by Oatp protein abundance) and SCRH. Our data clearly demonstrate that the under-prediction of $CL_{s,uptake}$ of RSV was due to the lower expression of Oatps in SCRH vs. liver tissue. We speculate that such lower expression of transporters may also explain the under prediction of hepatic clearance of other drugs based on sandwich-cultured hepatocyte studies. Thus, we recommend that transporter expression is measured in *in vitro* systems used to predict *in vivo* hepatobiliary clearance of drugs. In addition, our studies demonstrate the promise of utilizing transporter-expressing cells to directly predict $CL_{s,uptake}$ of drugs. As noted earlier, scaling Oatp $CL_{uptake}$ in cell lines to SCRH and then to *in vivo* using transporter expression data is equivalent to directly scaling $CL_{uptake}$ from cell lines to *in vivo* (Eq. 9, see Method).

There is no reason why this approach could not be used to also predict sinusoidal and canalicular efflux clearance of drugs thus avoiding the use of SCH and the disadvantage of the RAF approach inherent to OATP substrates. Therefore, we propose that studies be conducted to determine if our approach can be generalized to successfully predict hepatobiliary clearance and hepatic concentrations of drugs other than RSV, especially in humans. This, of course, will
require that data from imaging studies in humans is available. Fortunately, such data are increasingly becoming available (Shimizu et al., 2012; Takashima et al., 2012).
Acknowledgement

We would like to acknowledge Dr. Bhagwat Prasad and Dr. Sarah Billington for their technical help in LC-MS/MS protein quantification.
Conflict of Interest

The authors have no conflict of interest.
DMD #76539

Authorship Contributions

Participated in research design: Ishida, Ullah, Unadkat

Conducted experiments: Ishida

Performed data analysis: Ishida

Contributed new reagents: Tóth, Juhasz

Contributed to the writing of the manuscript: Ishida, Ullah, Tóth, Juhasz, Unadkat
References


Hirano M, Maeda K, Shitara Y and Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *J Pharmacol Exp Ther* **311**:139-146.


Footnotes

a) This work is supported by a Postdoctoral Fellowship to KI from F. Hoffmann-La Roche Ltd.
DMD #76539

Legends to Figures

Figure 1. Typical Observed and Predicted Cellular Accumulation and Efflux Profiles of [3H]-RSV in Two Lots of SCRH, Each Showing Modest (RSD288) or No Biliary Efflux of RSV (RSD294).

Circles/solid line and triangles/dashed line indicate the observed/predicted [3H]-RSV and [3H]-RSV lactone cellular accumulation (A, B, E, F) or efflux of [3H]-RSV into the buffer (C, D, G, H) with Ca2+-containing and Ca2+-free HBSS, respectively. The small (RSD288) or no difference (RSD294) in cellular accumulation of [3H]-RSV between Ca2+-containing and Ca2+-free HBSS indicate modest or no biliary efflux of [3H]-RSV. Panels B, D and F, H show the [3H]-RSV profiles in the presence of unlabeled 1 mM RSV.

Figure 2. Comparison of In Vivo Hepatobiliary Clearance of RSV Predicted from SCRH with Our Previous PET Imaging Study

RSV $CL_{s,uptake}$ in SCRH was significantly lower than that observed in our PET imaging study (He et al., 2014). In contrast, the in vivo RSV $CL_{s,efflux}$ and $CL_{bile}$ were well predicted by SCRH. The inset shows RSV $CL_{s,efflux}$ and $CL_{bile}$ estimated using SCRH.

Data shown are mean ± standard deviation from 3 rats (from our previous PET imaging study), 6 lots of SCRH for $CL_{s,uptake}$ and $CL_{s,efflux}$, or 3 lots of SCRH for $CL_{bile}$.

*p<0.05 - significantly different from our previous PET imaging study.

Figure 3. Oatp Protein Expression in the SD Rat Liver and SCRH

Expression of Oatp transporter proteins in SCRH was significantly lower than that in the rat liver (Data are from Wang et al., 2015).
Data shown are mean ± standard deviation of 6 SD rat livers and 3 lots of SCRH.

*p<0.05 - significantly different from SD rat livers.

**Figure 4. Comparison of Recreated In Silico CL_{s,uptake} of RSV with CL_{s,uptake} in SCRH or Our Previous PET Imaging Study**

The recreated in silico RSV CL^{Oatp+SCRH} or CL^{Oatp+HEK} better estimated the observed CL_{s,uptake} in SCRH (panel A) or in vivo (panel B). In addition, the recreated in silico CL^{Oatp+SCRH}, CL^{Oatp+CHO}, CL^{Oatp+HEK} of RSV were within the 2-fold of CL_{s,uptake} estimated from SCRH (panel A) or in vivo CL_{s,uptake} (panel B).

CL^{SCRH}_{s,uptake}, CL^{Oatp+SCRH}_{s,uptake}, CL^{Oatp+CHO}_{s,uptake}, CL^{Oatp+HEK}_{s,uptake}, and CL^{PET}_{s,uptake} indicate total CL_{s,uptake} estimated from SCRH not corrected for the Oatp protein expression, the recreated in silico CL_{s,uptake} from Oatp-expressing cells (active) and SCRH (passive), the recreated in silico CL_{s,uptake} from Oatp-expressing cells (active) and CHO-mock cells (passive), the recreated in silico CL_{s,uptake} from Oatp-expressing cells (active) and HEK293-mock cells (passive), and total CL_{s,uptake} in our previous in vivo PET imaging study, respectively.

Data shown are the mean ± standard deviation of 3 lots of SCRH, 3 rats (from our previous PET imaging study, He et al., 2014), and 6 rat livers (Wang et al., 2015).

†p<0.05 - significantly different from CL^{PET}_{s,uptake}.

**Figure 5. Simulated RSV Hepatic Concentrations based on Hepatobiliary Clearances of the Drug Estimated from Oatp-expressing Cells and SCRH.**

The mean predicted hepatic concentration of RSV (orange solid line), generated from the
estimated $CL_{Oatp+SCRH}^{uptake}$, $CL_{efflux}$ and $CL_{bite}$ from SCRH (see Methods), fell within the 95% confidence interval (CI) of the observed concentrations in our previous in vivo PET imaging study (blue circles and gray dashed lines, (He et al., 2014)), suggesting an excellent agreement between the observed and predicted hepatic RSV concentrations.
### Table 1

Estimates of [$^3$H]-RSV Hepatobiliary Clearances in SCRH in the presence of unlabeled RSV (0.5 µM or 1 mM)

<table>
<thead>
<tr>
<th></th>
<th>Clearance (µL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 µM</td>
</tr>
<tr>
<td>$CL_{s,uptake}$</td>
<td>27.7 ± 7.18</td>
</tr>
<tr>
<td>$CL_{s,efflux}$</td>
<td>0.56 ± 0.40</td>
</tr>
<tr>
<td>$CL_{bile}$</td>
<td>0.32 ± 0.24$^a$</td>
</tr>
<tr>
<td>$CL_{iac}$</td>
<td>32.1 ± 14.2</td>
</tr>
<tr>
<td>$CL_{de-lac}$</td>
<td>142 ± 70</td>
</tr>
<tr>
<td>$CL_{met}$</td>
<td>0.097 ± 0.062$^c$</td>
</tr>
<tr>
<td>$K_{flux}$ (min⁻¹)</td>
<td>0.108$^d$</td>
</tr>
</tbody>
</table>

$^a$ $CL_{bile}$, in the presence of 0.5 µM unlabeled RSV was detected in only 3 lots of SCRH.

$^b$ $CL_{bile}$ in the presence of 1 mM unlabeled RSV was virtually zero and therefore could not be estimated. Consequently, this value was fixed as 0.

$^c$ $CL_{met}$ could be estimated in only 5 lots of SCRH.

$^d$ $K_{flux}$ could be estimated in only 2 lots of SCRH; therefore, only mean value of $K_{flux}$ are shown.

Data are mean ± standard deviation of 6 (in the presence of 0.5 µM unlabeled RSV) or 5 (in the presence of 1 mM unlabeled RSV) lots of SCRH.

*p<0.05 - significantly different from the clearance in the presence of 0.5 µM.
Table 2 Protein Expression of Oatps and Transport-mediated Intrinsic $CL_{uptake}$ of RSV in CHO-Oatp1a1, HEK293-Oatp1a4, and HEK293-Oatp1b2 Cells

<table>
<thead>
<tr>
<th>Oatp Isoform</th>
<th>Transporter Expression (fmol/µg membrane protein)</th>
<th>Intrinsic $CL_{uptake}$</th>
<th>Intrinsic $CL_{uptake}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µL/min/mg total protein)</td>
<td>(µL/min/fmol transporter)</td>
</tr>
<tr>
<td>1a1</td>
<td>6.72 ± 0.63</td>
<td>459</td>
<td>0.113</td>
</tr>
<tr>
<td>1a4</td>
<td>2.61 ± 0.25</td>
<td>54.0</td>
<td>0.043</td>
</tr>
<tr>
<td>1b2</td>
<td>18.1 ± 1.79</td>
<td>143</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation of triplicate determination.

Transporter-mediated uptake clearance of RSV was calculated as the ratio of RSV transporter-mediated uptake (uptake in transfected cells minus that in mock cells) over 5 sec (Oatp1a1) or 15 sec (Oatp1a4 and Oatp1b2) and the total buffer RSV concentration (0.07 µM).
Table 3  $CL_{\text{passive}}$ estimated from SCRH, CHO-mock Cells, and HEK293-mock Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>$CL_{\text{passive}}$ (µL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCRH</td>
<td>2.14 ± 0.49</td>
</tr>
<tr>
<td>CHO-mock</td>
<td>47.6 ± 10.1</td>
</tr>
<tr>
<td>HEK293-mock</td>
<td>5.68 ± 0.21</td>
</tr>
</tbody>
</table>

Data shown are the mean ± standard deviation of 5 lots (SCRH), 3 independent experiments (CHO-mock), or 6 independent experiments (HEK293-mock cells).
Figure 2

- Previous PET imaging study
- SCR H not corrected for Oatp expression
Figure 3

The figure shows the expression levels of Oatp1a1, Oatp1a4, and Oatp1b2 in SD rat liver and SCRH samples. The expression levels are measured in fmol/µg membrane protein. The bars indicate the mean expression levels with standard deviations. Asterisks denote statistically significant differences compared to the control group.
Figure 4
Figure 5
Successful prediction of *in vivo* hepatobiliary clearance and hepatic concentrations of rosvastatin using sandwich-cultured rat hepatocytes, transporter-expressing cell lines, and quantitative proteomics

Kazuya Ishida, Mohammed Ullah, Beáta Tóth, Viktoria Juhasz, and Jashvant D. Unadkat

Supplemental Materials
Legends to Supplemental Figures

Supplemental Figure 1. Microscopic Images of SCRH.

A: RSD288, B: Rs14Dec15T, C: Rs18Jan16T. These images were provided by TRL (A) and Qualyst (B and C) respectively. The images were taken at 96 hr after plating. Images for RSD270, RSD279, and RSD294 were not available. The magnification was 20× (A) and 40× (B and C) respectively.

Supplemental Figure 2. Observed and Predicted Cellular Accumulation and Efflux Profiles of [3H]-RSV in the Remaining Four Lots of SCRH.

Circles/solid line and triangles/dashed line indicate the observed/predicted [3H]-RSV and [3H]-RSV lactone cellular accumulation (A, B, E, F, I, K, L) or efflux of [3H]-RSV into the buffer (C, D, G, H, J, M, N) with Ca²⁺-containing and Ca²⁺-free HBSS, respectively. The small (RSD270 and Rs18Jan16T) or no difference (RSD279 and Rs14Dec15T) in cellular accumulation of [3H]-RSV between Ca²⁺-containing and Ca²⁺-free HBSS indicate modest or no biliary efflux of [3H]-RSV. Panels B, D, F, H, L, and N show the [3H]-RSV profiles in the presence of unlabeled 1 mM RSV.
### Supplemental Table 1. LC Condition for Surrogate Peptide Quantification

<table>
<thead>
<tr>
<th>Column</th>
<th>Acquity UPLC® HSS T3, 1.8 µm, 2.1 × 100 mm, Waters Corporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guard column</td>
<td>Security Guard column (C18, 4 × 20 mm, Phenomenex, Torrance, CA)</td>
</tr>
<tr>
<td>Runtime</td>
<td>27 min</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: water with 0.1% formic acid</td>
</tr>
<tr>
<td></td>
<td>B: acetonitrile with 0.1% formic acid</td>
</tr>
<tr>
<td>Mobile phase gradient</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Flow rate (mL/min)</td>
</tr>
<tr>
<td>0-4</td>
<td>0.3</td>
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<tr>
<td>4-8</td>
<td>0.3</td>
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<tr>
<td>8-18</td>
<td>0.3</td>
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<tr>
<td>18-22.9</td>
<td>0.3</td>
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<td>22.9-23.0</td>
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</tr>
<tr>
<td>23.0-23.1</td>
<td>0.3</td>
</tr>
<tr>
<td>23-27</td>
<td>0.3</td>
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## Supplemental Table 2. Surrogate Peptides of Rat Hepatic Oatps and Their MS/MS Parameters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Surrogate peptide sequence</th>
<th>Peptide type</th>
<th>Parent ion</th>
<th>Fragment ion</th>
<th>Cone voltage</th>
<th>Collision energy</th>
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<tbody>
<tr>
<td>Oatp1a1</td>
<td>EENLGITK</td>
<td>Light</td>
<td>452.2</td>
<td>418.1</td>
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<tr>
<td>Oatp1a1</td>
<td>EENLGITK</td>
<td>Heavy</td>
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<td>50</td>
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<tr>
<td>Oatp1a4</td>
<td>TFQFPGDIESSK</td>
<td>Light</td>
<td>678.55</td>
<td>832.35</td>
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<tr>
<td>Oatp1a4</td>
<td>TFQFPGDIESSK</td>
<td>Heavy</td>
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<tr>
<td>Oatp1b2</td>
<td>SVQPELK</td>
<td>Light</td>
<td>400.73</td>
<td>486.29</td>
<td>614.35</td>
<td>60</td>
</tr>
<tr>
<td>Oatp1b2</td>
<td>SVQPELK</td>
<td>Heavy</td>
<td>404.74</td>
<td>494.3</td>
<td>622.36</td>
<td>60</td>
</tr>
</tbody>
</table>
### Supplemental Table 3. Estimates of [\(^3\)H]-RSV Hepatobiliary Clearances in SCRH in the presence of unlabeled RSV (0.5 µM or 1 mM) and the corresponding Biliary Excretion Index (BEI) of RSV and Taurocholic acid (TCA)

<table>
<thead>
<tr>
<th></th>
<th>Clearance (µL/min/mg protein)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSD270</td>
<td>RSD279</td>
<td>RSD288</td>
<td>RSD294</td>
<td>Rs14Dec15T</td>
<td>Rs18Jan16T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 µM</td>
<td>1 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CL(_{s,uptake})</strong></td>
<td>23.4</td>
<td>2.38</td>
<td>19.4</td>
<td>2.83</td>
<td>28.8</td>
<td>1.75</td>
<td>36.7</td>
</tr>
<tr>
<td><strong>CL(_{s,efflux})</strong></td>
<td>1.30</td>
<td>2.11</td>
<td>0.709</td>
<td>3.42</td>
<td>0.242</td>
<td>1.18</td>
<td>0.375</td>
</tr>
<tr>
<td><strong>CL(_{bile})</strong></td>
<td>0(^a)</td>
<td>0(^a)</td>
<td>0.067</td>
<td>0(^a)</td>
<td>0.336</td>
<td>0(^a)</td>
<td>0(^a)</td>
</tr>
<tr>
<td><strong>CL(_{lac})</strong></td>
<td>24.5</td>
<td>13.7</td>
<td>49.8</td>
<td>33.1</td>
<td>33.1</td>
<td>24.1</td>
<td>47.4</td>
</tr>
<tr>
<td><strong>CL(_{de-lac})</strong></td>
<td>112</td>
<td>63.1</td>
<td>242</td>
<td>138</td>
<td>138</td>
<td>86.7</td>
<td>208</td>
</tr>
<tr>
<td><strong>CL(_{met})</strong></td>
<td>0.045</td>
<td>0(^a)</td>
<td>0.174</td>
<td>0.028</td>
<td>0.139</td>
<td>0.098</td>
<td></td>
</tr>
<tr>
<td><strong>K(_{flux}) (min(^{-1}))</strong></td>
<td>0(^a)</td>
<td>0(^a)</td>
<td>0.091</td>
<td>0(^a)</td>
<td>0.125</td>
<td>0(^a)</td>
<td></td>
</tr>
<tr>
<td>BEI of RSV (%)(^b)</td>
<td>9.97(^c)</td>
<td>10.5(^c)</td>
<td>24.1(^c)</td>
<td>12.6(^c)</td>
<td>36.4(^c)</td>
<td>0.03(^c)</td>
<td></td>
</tr>
<tr>
<td>BEI of TCA (%)(^b)</td>
<td>15.9(^c)</td>
<td>17.2(^c)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>51.8(^d)</td>
<td>37.9(^d)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Since the 95% CI of the parameter encompassed zero, this parameter was fixed to zero.

\(^b\)BEI (%) = \(100 \times \left(\text{drug accumulation}_{\text{Ca}^{2+}-\text{containing HBSS}} - \text{drug accumulation}_{\text{Ca}^{2+}-\text{free HBSS}}\right)/\text{drug accumulation}_{\text{Ca}^{2+}-\text{containing HBSS}}\)

\(^c\)Estimated from the 10-min uptake of RSV (0.5 µM) or TCA (0.5 µM) into SCRH evaluated with Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free HBSS.

\(^d\)These data were provided by Qualyst. Estimated from the 10-min uptake of TCA (1 µM) into SCRH evaluated with Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free HBSS.

N.D.: not determined.
Supplemental Table 4. The Percentage of Total Radioactivity Associated with RSV and RSV Lactone in SCRH Incubated with 0.5 µM RSV (data are mean ± SD and range of 3 lots of SCRH)

<table>
<thead>
<tr>
<th>Time</th>
<th>Phase</th>
<th>% RSV</th>
<th>% RSV lactone</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;-containing</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;-free</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;-containing</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;-free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-containing</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-free</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-containing</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-free</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-containing</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-free</td>
</tr>
<tr>
<td>Cell lysate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>Uptake</td>
<td>77.7 %&lt;sup&gt;a&lt;/sup&gt; (73.1-82.2%)</td>
<td>68.0%&lt;sup&gt;a&lt;/sup&gt; (64.1-71.8%)</td>
<td>18.0%&lt;sup&gt;a&lt;/sup&gt; (17.8-18.2%)</td>
<td>24.0%&lt;sup&gt;a&lt;/sup&gt; (10.9-37.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>Uptake</td>
<td>71.6 ± 3.90% (67.2-74.5%)</td>
<td>74.6 ± 4.22% (69.8-77.4%)</td>
<td>15.3 ± 8.13% (8.05-24.1%)</td>
<td>15.9 ± 3.75% (11.6-18.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>Uptake</td>
<td>71.3 ± 5.75% (67.0-77.8%)</td>
<td>71.3 ± 10.4% (59.8-79.9%)</td>
<td>15.1 ± 2.38% (12.3-16.7%)</td>
<td>15.6 ± 5.45% (10.9-21.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>Uptake</td>
<td>70.7 ± 1.41% (69.6-72.3%)</td>
<td>68.6 ± 3.80% (64.2-71.0%)</td>
<td>17.3 ± 2.24% (15.6-19.8%)</td>
<td>14.2 ± 1.36% (13.3-15.7%)</td>
<td></td>
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</tr>
<tr>
<td>2 min</td>
<td>Efflux</td>
<td>70.2 ± 4.01% (67.8-74.8%)</td>
<td>74.9 ± 4.36% (71.4-79.8%)</td>
<td>15.9 ± 5.69% (11.6-22.4%)</td>
<td>12.8 ± 2.89% (9.72-15.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>Efflux</td>
<td>67.2 ± 6.23% (62.0-74.1%)</td>
<td>71.6 ± 4.82% (66.6-76.2%)</td>
<td>13.3 ± 4.70% (7.92-16.3%)</td>
<td>13.3 ± 1.90% (11.9-15.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>Efflux</td>
<td>62.6 ± 13.6% (47.1-72.2%)</td>
<td>71.9 ± 6.97% (66.3-79.7%)</td>
<td>20.7 ± 11.7% (12.3-34.1%)</td>
<td>16.6 ± 3.73% (13.8-20.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>Efflux</td>
<td>66.6%&lt;sup&gt;a&lt;/sup&gt; (64.2-69.0%)</td>
<td>69.1%&lt;sup&gt;a&lt;/sup&gt; (61.7-76.6%)</td>
<td>17.0%&lt;sup&gt;a&lt;/sup&gt; (16.4-17.6%)</td>
<td>16.3%&lt;sup&gt;a&lt;/sup&gt; (13.3-19.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>Efflux</td>
<td>103 ± 2.16% (101-105%)</td>
<td>109 ± 9.38% (97.9-115%)</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>Efflux</td>
<td>102 ± 4.45% (96.2-105%)</td>
<td>95.9 ± 2.55% (92.9-97.4%)</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>Efflux</td>
<td>97.3 ± 6.66% (90.1-103%)</td>
<td>103 ± 2.80% (100-106%)</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>Efflux</td>
<td>95.8%&lt;sup&gt;a&lt;/sup&gt; (93.0-98.6%)</td>
<td>99.2%&lt;sup&gt;a&lt;/sup&gt; (96.0-102%)</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D.: not detected; a – SD values are not provided because n = 2.
Supplemental Figure 1
Supplemental Figure 2

A

- RSV, 0.5 µM, Ca (+)
- RSV, 0.5 µM, Ca (-)
- RSV lactone, 0.5 µM, Ca (+)
- RSV lactone, 0.5 µM, Ca (-)

B

- RSV, 1 mM, Ca (+)
- RSV, 1 mM, Ca (-)
- RSV lactone, 1 mM, Ca (+)
- RSV lactone, 1 mM, Ca (-)

C

- RSV, 0.5 µM, Ca (+)
- RSV, 0.5 µM, Ca (-)

D

- RSV, 1 mM, Ca (+)
- RSV, 1 mM, Ca (-)

E

- RSV, 0.5 µM, Ca (+)
- RSV, 0.5 µM, Ca (-)
- RSV lactone, 0.5 µM, Ca (+)
- RSV lactone, 0.5 µM, Ca (-)

F

- RSV, 1 mM, Ca (+)
- RSV, 1 mM, Ca (-)

G

- RSV, 0.5 µM, Ca (+)
- RSV, 0.5 µM, Ca (-)

H

- RSV, 1 mM, Ca (+)
- RSV, 1 mM, Ca (-)