Title Page

Prediction of Transporter-Mediated Rosuvastatin Hepatic Uptake Clearance and Drug Interaction in Humans Using Proteomics-Informed REF Approach

Vineet Kumar, Mengyue Yin, Kazuya Ishida, Laurent Salphati, Cornelis E. C. A. Hop, Christopher Rowbottom, Guangqing Xiao, Yurong Lai, Anita Mathias, Xiaoyan Chu, W. Griffith Humphreys, Mingxiang Liao, Zsuzsanna Nerada, Nóra Szilvásy, Scott Heyward and Jashvant D. Unadkat

Department of Pharmaceutics, University of Washington, Seattle, Washington (VK, MY, KI & and JDU)

& Present address: Gilead Sciences, Inc., Foster City, California

Drug Metabolism and Pharmacokinetics, Genentech, Inc., South San Francisco, California (LS and CECAH)

DMPK, Biogen Idec, Cambridge, Massachusetts (CR and GX #)

# Present address: Sunovion Pharmaceuticals, Inc., Marlborough, Massachusetts

Clinical Pharmacology (AM), and Drug Metabolism (YL), Gilead Sciences, Inc., Foster City, California

Pharmacokinetics, Pharmacodynamics and Drug Metabolism, Merck & Co. Inc., Kenilworth, New Jersey (XC)
Bristol-Myers Squibb Company, Princeton, New Jersey (WGH* )

* Present address: Aranmore Pharma Consultant, Trenton, New Jersey

Takeda Pharmaceuticals International Co., Cambridge, Massachusetts (ML$ )

$ Present address: Clovis Oncology, San Francisco, California

SOLVO Biotechnology, Gyár utca 2, Budaörs, 2040, Hungary (ZN, NS)

BioIVT, Baltimore, Maryland (SH)
Running Title Page

Running Title: IVIVE of rosuvastatin hepatic clearance in human

Corresponding author: *Department of Pharmaceutics, University of Washington, Seattle, P.O. Box 357610, WA 98195, USA. Phone: +1-206-685-2869. Fax: +1-206-543-3204.

Email: jash@uw.edu

Number of text pages: 42 (including tables, figure legends, and references):

Number of tables: 1

Number of figures: 5

Number of references: 34

Number of words in the Abstract: 250

Number of words in the Introduction: 666

Number of words in the Discussion: 1853

ABBREVIATIONS: BCA, Bicinchoninic acid assay; CLb, Biliary clearance; ER, Endoplasmic reticulum; DTT, Dithiothreitol; HBSS, Hank's balanced salt solution; IVIVE, In vitro to in vivo extrapolation; IVCIVH, In vitro cells to in vivo hepatocytes extrapolation; IAA, Iodoacetamide; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LT, Liver tissue; PH, Plated hepatocytes; PMA, Plasma membrane abundance; SCHH, Sandwich-cultured human hepatocytes; SDS, Sodium dodecyl sulfate; SIL, Stable isotope labeled; SH, Suspended hepatocytes; Sulfo-NHS-SS-Biotin, Sulfosuccinimidyl-2-(biotinamido) ethyl-1, 3-
dithiopropionate; DDI, drug-drug interaction; HSA, human serum albumin; BSA, bovine serum albumin.
Abstract (250/250)

Suspended (SH), plated (PH) or sandwich-cultured human hepatocytes (SCHH) are routinely used for *in vitro* to *in vivo* extrapolation (IVIVE) of transporter-mediated hepatic clearance (CL) of drugs. However, these hepatocyte models have been reported to underpredict transporter-mediated *in vivo* hepatic uptake CL ($CL_{\text{uptake, in vivo}}$) of some drugs. Therefore, we determined if transporter-expressing cells (TEC) can accurately predict the $CL_{\text{uptake, in vivo}}$ of drugs. To do so, we determined the uptake CL ($CL_{\text{int,uptake,cells}}$) of rosuvastatin (RSV) by TEC (OATPs/NTCP) and then scaled it to that *in vivo* by REF (the ratio of transporter abundance in human livers and TEC) determined by LC-MS/MS-based quantitative proteomics. Both the TEC and hepatocyte models did not meet our pre-defined success criteria of predicting within 2-fold the RSV $CL_{\text{uptake, in vivo}}$ value obtained from our PET imaging. However, the TEC performed better than the hepatocyte models. Interestingly, using REF, TEC successfully predicted RSV $CL_{\text{int,uptake,hep}}$ obtained by the hepatocyte models, suggesting that the underprediction of RSV $CL_{\text{uptake, in vivo}}$ by TEC and hepatocytes is due to an endogenous factor(s) not present in these *in vitro* models.

Therefore, we determined if inclusion of plasma (or albumin) in TEC uptake studies, improved IVIVE of RSV $CL_{\text{uptake, in vivo}}$. It did, but our predictions still fell shy of our pre-defined 2-fold lower boundary. Thus, additional studies are needed to improve transporter-mediated IVIVE of hepatic uptake CL of drugs. However, using REF and TEC, we successfully predicted the magnitude of PET-imaged inhibition of RSV $CL_{\text{uptake, in vivo}}$ by cyclosporine A.
Significance Statement

We showed that the in vivo transporter-mediated uptake CL of rosuvastatin, determined by PET imaging, cannot be accurately predicted from in vitro studies in transporter-expressing cells (scaled using REF) or human hepatocytes (scaled based on mg of protein per g of liver). This conclusion held irrespective of whether albumin or plasma was included in the in vitro studies. Thus, additional studies are needed to improve IVIVE of transporter-mediated drug CL.
Introduction

In drug discovery, predicting human pharmacokinetics (PK) of a drug is critical in the selection of new molecular entities with desired PK properties. With increased understanding of the absorption, distribution, metabolism, and excretion (ADME) processes, transporters are well recognized for their significant role in the ADME of drugs and their metabolites. For example, transporters play an important role in the hepatobiliary clearance (CL) and therefore drug-drug interaction (DDI) and hepatic concentrations of many drugs, including drug used for hypercholesterolemia, diabetes and HCV infection (Endres et al., 2006; Giacomini et al., 2010; Kock and Brouwer, 2012; Shebley et al., 2017).

While in vitro and in silico methods (e.g. physiologically based pharmacokinetic (PBPK) models) have been developed to successfully predict hepatic metabolic CL of drugs (Sager et al., 2015), such methods have had limited success in predicting transporter-mediated CL and DDI. Currently, hepatocyte models, such as suspended (SH) or plated (PH) hepatocytes are used for in vitro to in vivo extrapolation (IVIVE) of transporter-mediated hepatic CL of drugs. However, these hepatocyte models are widely reported to underpredict the in vivo hepatic CL of some drugs (Abe et al., 2008; Li et al., 2010; Jones et al., 2012; Zou et al., 2013). In addition, they are costly and show considerable inter-lot variability in transporter activity (Vildhede et al., 2014; Izumi et al., 2017). To overcome these shortcomings, we have hypothesized that the in vivo transporter-mediated CL of drugs can be predicted by first measuring the transporter-mediated CL of the drug in transporter-expressing cells (TEC). Then, this CL can be scaled by relative expression factor (REF), that is the relative abundance of the transporter in cells vs. human tissue as determined by LC-MS/MS-based quantitative targeted proteomics. As a proof-of-concept, we have shown that this TEC/REF approach can successfully predict the renal secretory CL of
metformin in humans (Kumar et al., 2018). Moreover, we have recently shown that this proteomics-informed REF approach can successfully predict the hepatic uptake CL of rosuvastatin (RSV) and the magnitude of its inhibition by rifampin in the rat (Ishida et al., 2018a and 2018b).

Here we report a study to determine if TEC/REF approach can successfully predict the in vivo hepatic uptake CL of RSV in humans that we have determined using PET imaging (Billington et al., 2019). As before, we set our success criteria as being able to predict the hepatic uptake CL of RSV within 2-fold of the observed value (Kumar et al., 2017; Ishida et al., 2018a). Since human hepatocytes are widely used to predict the hepatic uptake CL of drugs, we compared the predicted hepatic uptake CL of RSV using the TEC/REF approach with that based on traditional hepatocyte models, namely suspended (SH), plated (PH) or sandwich-cultured human hepatocytes (SCHH) (scaled using mg of protein per g of liver) (Fig. 1). We also determined if RSV uptake CL in SH, PH or SCHH can be predicted from transporter-expressing cells using the REF approach (i.e.). The results of this aim would be informative in case neither approach (i.e. TEC nor human hepatocytes) successfully predicted the in vivo RSV hepatic uptake CL. The REF approach has traditionally been based on the total transporter abundance in both the transporter-expressing cells and in human tissue. However, only transporters present in the plasma membrane actively transport drugs. Therefore, we determined if the use of plasma membrane abundance (PMA) vs. total abundance of transporters improves the predictions of the REF approach. Recent studies have shown that plasma or albumin can increase the intrinsic hepatic uptake CL of drugs mediated by OATPs (Kim et al., 2019; Bowman et al., 2020). Therefore, we also determined if the inclusion of plasma or albumin in our in vitro uptake studies using TEC improves the predictions of the TEC/REF approach. Last, but not least, we
determined if the magnitude of inhibition of the RSV hepatic uptake CL by cyclosporine A (CsA), observed in our PET imaging study, can be predicted by using the TEC/REF approach.
Materials and Methods

Chemicals and Reagents

Synthetic signature peptides for OATP1B1, OATP2B1, OATP1B3, and NTCP were obtained from New England Peptides (Boston, MA). The corresponding stable isotope labeled (SIL) peptides for the above transporters, dithiothreitol (DTT), iodoacetamide (IAA), mass spectrometry grade trypsin, William's E Medium (no glutamine), cryopreserved hepatocyte recovery medium, total protein quantification bicinchoninic acid assay (BCA) kit, Hank's balanced salt solution with calcium and magnesium (HBSS), HBSS without calcium and magnesium, human serum albumin (HSA), bovine serum albumin (BSA) and Pierce cell surface protein isolation kit were obtained from Thermo Scientific (Rockford, IL). Na\(^{+}\)-free HBSS was prepared by replacing NaCl and NaHCO\(_3\) with choline chloride and potassium bicarbonate respectively from HBSS constituents. Pierce cell surface protein isolation kit contains sulfo-NHS-SS-biotin, quenching solution (100 mM glycine), lysis buffer, neutravidin agarose gel, wash buffer, column accessory pack, DTT, phosphate buffer and Tris buffer. HPLC-grade acetonitrile and sodium dodecyl sulfate (SDS) were purchased from Fischer Scientific (Fair Lawn, NJ). Cyclosporine A and formic acid was purchased from Sigma-Aldrich (St. Louis, MO). All reagents were analytical grade. 24-well collagen-coated plates and Matrigel® were purchased from Corning (Kennebunk, ME). Human hepatocyte thaw medium, INVITROGRO CP medium, INVITROGRO HI medium, and TORPEDO antibiotic mix were obtained from BioIVT (Westbury, NY).

Radiolabeled [\(^3\)H]RSV (> 98% purity) and unlabeled RSV were purchased from American Radiolabeled Chemicals (Saint Louis, MO) and Toronto Research Chemicals (North York, ON, Canada) respectively. Human plasma was purchased from Bloodworks NW (Seattle, WA).
Procurement of Human Hepatocytes and Transporter-Expressing Cells

Human liver frozen tissues and cryopreserved human hepatocytes, which were obtained from the same donor, were obtained from BioIVT (Westbury, NY). The demographics of the human liver donors (n = 4, ADR, FEA, JEL, and YTW) were as described before (Kumar et al., 2019). OATP1B1-expressing CHO cells were generously provided by Dr. Bruno Stieger, University Hospital Zurich, Switzerland. OAPT1B1-expressing HEK293, OATP1B3-expressing HEK293, OATP2B1-expressing MDCKII, and NTCP-expressing HEK293 cells were generously provided by SOLVO Biotechnology, Hungary.

RSV Uptake Study in OATP1B1/2B1/1B3 or NTCP Transporter-Expressing Cells

[3H]RSV transport studies were conducted in OATP1B1-expressing CHO cells, OATP2B1-expressing MDCKII cells, OATP1B1-expressing HEK293 cells, OATP1B3-expressing HEK293 cells and NTCP-expressing HEK293 cells. Transporter-expressing cells were grown in 24-well poly-D-lysine-coated plates, at a density of 0.5 million cells per well with 1 ml of DMEM (CHO cells: low glucose DMEM, HEK293 cells and MDCKII cells: high glucose DMEM) medium for 24 hr. After 24 hr, cells were washed twice with 1 ml/well DPBS buffer. Then, they were incubated at 37 °C with Ca2+ and Mg2+- containing HBSS buffer (hereafter referred as HBSS buffer) containing 30 nM [3H]RSV and 70 nM unlabeled RSV with or without 200 µM bromsulphthalein (BSP) (OATP cells) or with HBSS or Na+-free HBSS buffer (NTCP cells) for 5-60 seconds (within linear range) (Bi et al., 2013). After incubation, the [3H]RSV solution was removed, and the cells were washed by ice-cold HBSS buffer three times (1ml each). The uptake CL was calculated by the uptake at 5 sec when the uptake was proportional to time. [3H]RSV uptake at 5 and 60 sec (within linear phase) in the presence of 200 µM BSP and in the absence of sodium was used to estimate [3H]RSV passive diffusion clearance. Then, the cells were lysed at
37 °C for 2 hours with 1 ml 2% SDS. Forty microliters of this lysate were used for total protein determination using the BCA Protein Assay Kit, and 700 µl was used to quantify total radioactivity by Tri-Carb Liquid Scintillation Counters (PerkinElmer, Waltham, MA). To determine the inhibitory effect of CsA, the [³H]RSV uptake study in the presence of 0.3µM CsA were conducted in OATP1B1-, OATP2B1-, OATP1B3- and NTCP-expressing cells as described above except that the cells were preincubated with 0.3µM CsA for 45 minutes before conducting the uptake study. This is analogous to our in vivo PET study where we measured the hepatic uptake CL of RSV after ~45 min of CsA infusion (Billington et al., 2019).

**RSV uptake by TEC in the presence and absence of albumin or human plasma**: 100% human plasma (HP) or 5% human serum albumin (HSA) was used for these studies and the unbound RSV concentration was maintained at ~1 µM in the presence/absence of proteins. Hence, due to the large cost of RSV radioactivity, RSV uptake in these studies was quantified using LC-MS/MS. First, the OATP1B1-, OATP1B3- and NTCP-expressing cells were pre-incubated with HBSS buffer or 100% HP or 5% HSA for 10 minutes. Then, the uptake of RSV by TEC was conducted in the presence of HBSS buffer or 100% HP or 5% HSA as described above. The cell pellets were suspended in 500 µL of acetonitrile:water (80:20) containing 20nM cerivastatin as an internal standard to precipitate the protein. After centrifuge at 18,000g for 20 minutes, 100 µL supernatant was transferred into 96-well microplate for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Extra wells in every plate were added into 1mL 2%SDS to lyse the cells for protein quantification. The protein content in the lysate was determined using BCA Protein Assay Kit as described above.

**LC-MS/MS Analysis**
The supernatant was assayed by AB Sciex Triple Quad 6500 tandem mass spectrometer with coupled with Waters Acquity UPLC system (Waters, Hertfordshire, UK). The injection volume was 10 μL. The LC separations were performed using an Acuity UPLC BEH C18 column (1.7μm, 2.1mm × 50mm) with a corresponding C18 VanGuard Precolumn (1.7μm, 2.1mm × 5mm) (Waters Technologies, Milford, MA, USA). The LC mobile phases were: (A) 0.1% formic acid in water; (B) 0.1% formic acid in acetonitrile. The solvent gradient profile (min/%B) was 0/[10%]- 0.20[10%]-1.50[95%]-2.20[95%]-2.30[10%]-3.0[10%]. The samples were analyzed in the positive ionization mode. The transitions (Q1 >Q3) for RSV and cerivastatin (internal standard) were 482.3 > 258.2 and 460.3 > 356.3, respectively. The lower limit of RSV quantification was 0.20nM. Declustering potential was 80V and 100V for RSV and cerivastatin, respectively. Collision energy was 30V and 40V for RSV and cerivastatin, respectively.

**RSV Unbound Fraction in 100% Human Plasma or 5% Human Serum Albumin**

Five hundred μL of the RSV solution used in the above uptake experiment was used to determine the unbound fraction of RSV using the Centrifree® Ultrafiltration Device (EMD Millipore Corporation, Billerica, MA). The HBSS solution and the plasma/albumin solution were used for non-specific binding and protein-binding, respectively. The unbound fraction of RSV in 100% HP or 5% HSA was calculated by determining the amount of RSV in the samples before filtration and in the filtrate and was corrected for non-specific binding of RSV.


*SH:* Cryopreserved human hepatocytes were thawed and processed as described before (Kumar et al., 2019). Briefly, the hepatocytes were centrifuged at 1000 g for 5 mins at 4 °C to remove cryopreserved hepatocyte recovery medium. Then, the hepatocytes were reconstituted in Ca²⁺-
containing or Na\textsuperscript{+}-free HBSS medium and the medium containing 0.35×10\textsuperscript{6} viable hepatocytes (50 µl) were transferred to a 1.5 ml centrifuge tube and placed in a 37 °C water bath. The transport study was started by the addition of 50 µl of Ca\textsuperscript{2+}-containing or Na\textsuperscript{+}-free HBSS medium containing 60 nM \textsuperscript{3}H RSV and 140 nM RSV with or without 400 µM BSP to achieve a final concentration of 30 nM \textsuperscript{3}H RSV, 70 nM RSV and 200 µM BSP. At 2 and 10 min (within linear phase), the transport was terminated, as follows, using the oil-spin method. First, 150 µl of ice-cold Na\textsuperscript{+}-free HBSS was added to the hepatocyte incubation. Then, 200 µl of the medium containing the hepatocytes were transferred to the top of the oil layer (containing 200 µl of 3% cesium chloride with 200 µl of the mixture of silicone oil: mineral oil (5:1)) and centrifuged for 15 sec at 12000 g. The supernatant was immediately aspirated and the tube containing the pellet was cut, and the pellet was transferred and dissolved in 1 ml of 2% SDS. The total radioactivity in SH lysate (700 µl) was measured using a liquid scintillation counter (PerkinElmer, Waltham, MA). In addition, 40 µL of SH lysate was used to measure total protein using the BCA Protein Assay Kit as described above.

\textit{PH:} Cryopreserved human hepatocytes were thawed and plated as described before (Kumar et al., 2019). About 0.35×10\textsuperscript{6} hepatocytes were plated per well in a 24-well collagen-coated plate for 5 hours with 0.5 ml/well TORPEDO containing INVITROGRO CP medium (1:9 (v/v)). \textsuperscript{3}H RSV uptake (at 2 and 10 min; within linear phase) by the plated human hepatocytes incubated with Ca\textsuperscript{2+}-containing or Na\textsuperscript{+}-free HBSS medium containing 30 nM labeled \textsuperscript{3}H RSV and 70 nM unlabeled RSV was measured, as described above, in the presence or absence of Na\textsuperscript{+} or 200 µM BSP. Immediately afterwards, 1 ml of 2% SDS was added to each well to lyse the cells (uptake phase) and processed as described above.
SCHH: Cryopreserved human hepatocytes were thawed and sandwich-cultured as described before (Kumar et al., 2019). SCHH protocol was adopted from Liu et al., and Pfeifer et al., (Liu et al., 1999; Pfeifer et al., 2013). Briefly, on day 4 after sandwich-culturing the SCHH (0.35×10^6 hepatocytes/well) were washed twice with 1 ml of Ca^{2+}-containing HBSS buffer and then pre-incubated for 10 minutes at 37 °C with 500 µl of Ca^{2+}-containing HBSS. Then, the SCHH were incubated at 37 °C with 500 µl of 30 nM [^3H]RSV and 70 nM unlabeled RSV for 0.5, 2, and 5 minutes (linear phase) in Ca^{2+}-containing HBSS with or without 200 µM BSP (to assess OATP-mediated uptake) or Na^+ (to assess NTCP-mediated uptake). Uptake was terminated by washing the SCHH with ice-cold Ca^{2+}- and Mg^{2+}- containing HBSS. Immediately afterwards, 1 ml of 2% SDS was added to each well to lyse the cells (uptake phase) and processed as described above.

**Quantification of Total or Plasma Membrane Transporter Abundance in Transporter-Expressing Cell Lines, Human Liver Tissues, Suspended, Plated, and Sandwich-Cultured Human Hepatocytes**

Data on total or PMA of OATP1B1/2B1/1B3 or NTCP transporter abundance in the transporter-expressing cells, human liver tissues (n = 39) and hepatocyte homogenates were obtained from our previous publications (Wang et al., 2016; Kumar et al., 2017; Kumar et al., 2019).

**Data and Statistical Analyses**

**Determination of Transporter-Mediated and Passive Diffusion CL_{int} of RSV**

OATP1B1/2B1/1B3 or NTCP Transporter-Expressing Cells: The OATP or NTCP transporter-mediated intrinsic uptake CL (CL_{int,uptake,cells,1B1}, CL_{int,uptake,cells,1B3}, CL_{int,uptake,cells,2B1}, CL_{int,uptake,cells,NTCP}, hereafter referred to as CL_{int,uptake,cells}) of [^3H]RSV was determined first by taking the difference in [^3H]RSV uptake into the cells in the presence and absence of BSP or
Na⁺, respectively. Then, the OATP- or NTCP-mediated uptake CL\text{int} of [\textsuperscript{3}H]RSV into these cells was calculated as the ratio of the rate of transporter-mediated uptake of [\textsuperscript{3}H]RSV and [\textsuperscript{3}H]RSV concentration in the medium (30 nM). The passive diffusion CL\text{int} (CL\text{int,passive,cells}) of [\textsuperscript{3}H]RSV into the cells was calculated as above except that the rate of uptake of [\textsuperscript{3}H]RSV uptake into the cells in the presence of 200 µM BSP or in the absence of sodium was used. The CL\text{int,passive,cells} used was the average of passive diffusion CL obtained in CHO, MDCKII, and HEK293 cells.

**SH, PH and SCHH:** The total transporter-mediated CL\text{int} of [\textsuperscript{3}H]RSV in hepatocyte models (CL\text{int,uptake,hep}) was calculated in the presence of 200 µM BSP (an OATP and NTCP inhibitor) as above. Of this, the OATP-mediated CL\text{int} of [\textsuperscript{3}H]RSV was calculated as above, but the passive diffusion CL\text{int} (CL\text{int,passive,hep}) was determined by using only the data on uptake of [\textsuperscript{3}H]RSV in Na⁺-free HBSS buffer and in the presence of BSP. NTCP-mediated CL\text{int} of [\textsuperscript{3}H]RSV was calculated as above but by using only the data on uptake of [\textsuperscript{3}H]RSV in Ca\textsuperscript{2+}-containing and Na⁺-free HBSS buffer.

\[
CL_{\text{int,uptake,hep}} = CL_{\text{int,OATP,hep}} + CL_{\text{int,NTCP,hep}} + CL_{\text{int,passive,hep}} \quad \text{Eq. 1}
\]

Where, \(CL_{\text{int,OATP,hep}}, CL_{\text{int,NTCP,hep}}, \) and \(CL_{\text{int,passive,hep}}\) are OATP-mediated, NTCP-mediated and passive diffusion CL\text{int} of RSV in the hepatocyte models respectively.

**IVIVE of Intrinsic Hepatic Uptake CL of RSV from Transporter-Expressing Cells (CL\text{int,in vivo,pred,cells}), in the Presence or Absence of Plasma Proteins, Using REF**

The \(CL_{\text{int,in vivo,pred,cells}}\) from the transporter-expressing cells was calculated as follows as the sum of the contribution of uptake CL of [\textsuperscript{3}H]RSV by each transporter (using plasma membrane abundance, Table 1) and by passive diffusion:
\[ CL_{\text{int,in vivo,pred,cells}} = CL_{\text{int,in vivo,pred,1B1}} + CL_{\text{int,in vivo,pred,1B3}} + CL_{\text{int,in vivo,pred,2B1}} + \\
CL_{\text{int,in vivo,pred,NTCP}} + CL_{\text{int,in vivo,pred,passive}} \quad \text{Eq. 2} \]

Where \( CL_{\text{int,in vivo,pred,passive}} \) was scaled as in equation 4.

The contribution of each transporter in prediction of RSV \( CL_{\text{int,in vivo,pred,cells}} \) was calculated using equation 3 as described below for OATP1B1 transporter.

\[ CL_{\text{int,in vivo,pred,1B1}} = CL_{\text{int,uptake,cells,1B1}} \times [\text{OATP1B1}]_{\text{avg}} \quad \text{Eq. 3} \]

Where \( [\text{OATP1B1}]_{\text{avg}} \) represents average total OATP1B1 transporter abundance in human liver samples (n=39). Since, the cell-surface biotinylation methodology to estimate PMA of transporters cannot be used with intact tissue (Kumar et al., 2019), all the transporters quantified in liver tissue were assumed to be present in the plasma membrane. The RSV uptake experiments in the presence of HP or HSA were conducted only with OATP1B1, OATP1B3 and NTCP-expressing cells since these three transporters account for more than 90% active uptake of RSV. Of note, since our data showed that RSV OATP2B1 and passive diffusion CL in TEC in absence of proteins accounted for only 7.6% and 0.6% of total RSV uptake, the sensitivity of our instrument was insufficient to estimate these CL in the presence of proteins due to the extensive protein-binding of RSV. Therefore, we assumed OATP2B1-mediated and passive diffusion CL were identical with or without plasma proteins.

**IVIVE of Intrinsic Hepatic Uptake CL (CL\text{int,in vivo,pred,hep}) of RSV from Hepatocyte Models:**

\( CL_{\text{int,uptake,hep}} \) of RSV obtained from hepatocyte models was scaled to human liver as follows:

\[ CL_{\text{int,in vivo,pred,hep}} = CL_{\text{int,uptake,hep}} \times \frac{\text{CL}_{\text{int,uptake,hep}} (\mu l/min/mg \text{ protein}) \times \text{liver weight (g)} \times \text{Total protein per unit liver weight}}{\text{Eq. 4}} \]
Where, $CL_{\text{int, in vivo, pred, hep}}$ is the predicted in vivo intrinsic hepatic uptake CL from data in each hepatocyte model. Liver weight is 1500 g and the total protein per unit liver weight is 88 mg/g of liver (Karlgren et al., 2012).

**Prediction of In vivo Total Hepatic Uptake CL ($CL_{\text{in vivo, pred}}$) of RSV based on Transporter-Expressing Cells ($CL_{\text{in vivo, pred, cells}}$) and Hepatocyte Models ($CL_{\text{in vivo, pred, hep}}$):**

RSV in vivo hepatic uptake CL was computed from its hepatic intrinsic uptake CL based on TEC/REF or hepatocytes as follows:

$$CL_{\text{in vivo, pred}} = \frac{Q_{\text{hep}} \times f_{u,b} \times CL_{\text{int,in vivo,pred,cells/hep}}}{Q_{\text{hep}} + f_{u,b} \times CL_{\text{int,in vivo,pred,cells/hep}}} \quad \text{Eq. 5}$$

Where ($Q_{\text{hep}}$) is the liver blood flow and $f_{u,b}$ is the RSV fraction unbound in blood (0.1) estimated using equation 6.

$$f_{u,b} = \frac{f_{u,p}}{B:P} \times (1 - H) \quad \text{Eq. 6}$$

$f_{u,p}$ is the RSV fraction unbound in plasma (0.12) (Martin et al., 2003), and $B:P$ is the RSV blood to plasma ratio (0.69 (Martin et al., 2003)) and H is the hematocrit value (0.4).

**Prediction of $CL_{\text{int, uptake, hep}}$ of $[^3H]RSV$ in Hepatocyte Models from TEC (IVCIVH):**

The $CL_{\text{int, uptake, hep}}$ of $[^3H]RSV$ in each human hepatocyte model was predicted from $CL_{\text{int, uptake, cells}}$ as follow:

$$CL_{\text{int, hep, predicted}} = CL_{\text{int, uptake, cells,OATP1B1}} \times [OATP1B1]_{hep} + CL_{\text{int, uptake, cells,OATP1B3}} \times [OATP1B3]_{hep} + CL_{\text{int, uptake, cells,OATP2B1}} \times [OATP2B1]_{hep} + CL_{\text{int, uptake, cells,NTCP}} \times [NTCP]_{hep} + CL_{\text{int, passive, cells}} \quad \text{Eq. 7}$$
Where, $[\text{Transporter}]_{\text{hep}}$ represents total or PMA of the transporter (picomole/mg protein) in the hepatocyte model (Kumar et al., 2019).

**IVIVE of inhibition of RSV $CL_{\text{in vivo,uptake}}$ by CsA based on TEC/REF:**

$$C_{\text{L, int, in vivo, pred, transporter(i)}}^{CsA(+)} = C_{\text{L, int, uptake, cells, transporter(i)}}^{CsA(+)} \times [\text{Transporter(i)}]_{\text{avg}} \quad \text{Eq. 8}$$

$$C_{\text{L, int, in vivo, pred, cells}}^{CsA(+)} = \sum C_{\text{L, int, in vivo, pred, transporter(i)}}^{CsA(+)} + C_{\text{L, int, in vivo, pred, passive}}^{CsA(+)} \quad \text{Eq. 9}$$

The predicted % inhibitory effect of 0.3μM CsA on total RSV hepatic uptake CL was calculated as follows:

$$\text{Total %Inhibitory Effect} = \frac{Q_{\text{hep}} \times f_{u,b} \times C_{\text{L, int}}^{CsA(+)}}{Q_{\text{hep}} + f_{u,b} \times C_{\text{L, int}}^{CsA(+)}} \frac{C_{\text{L, int}}^{CsA(-)}}{Q_{\text{hep}} + f_{u,b} \times C_{\text{L, int}}^{CsA(-)}} \quad \text{Eq. 10}$$

**Statistical analysis:**

Wilcoxon matched-pair signed rank statistical test (using Prism 7, version 7.03) was used to compare the observed $CL_{\text{int,uptake,total}}$, $CL_{\text{int,OATP,hep}}$, $CL_{\text{int,NTCP,hep}}$, and $CL_{\text{int,passive,hep}}$ of $[^3H]$RSV across the various hepatocyte models (Fig. 2). For analyses of the rest of the data shown in Figs. 2, 3 and S1, the Tukey's multiple comparison test was used.
Results

OATP1B1, OATP1B3, OATP2B1 and NTCP-Mediated RSV $CL_{int,uptake,cells}$ Based on Their Total or PMA in Transporter-Expressing Cells

When expressed with respect to mg of cellular protein, except for OATP2B1, the transporter-mediated [3H]RSV $CL_{int,uptake,cells}$ was similar across all the transporters (Table 1). However, when [3H]RSV $CL_{int,uptake,cells}$ was expressed per pmol of total or PMA of each transporter, the picture changed. In this case, the NTCP-mediated [3H]RSV was ~2-fold greater than by OATPs irrespective of whether $CL_{int,uptake,cells}$ was obtained using total or the PMA of the transporter (Table 1). Except for OATP2B1, the majority (>60%) of each transporter were found to be expressed in the PM. The estimated $CL_{int,passive,cells}$ of [3H]RSV in CHO, MDCKII and HEK293 cells was similar across the cells (mean: 0.19±0.04 µl/min/mg cellular protein, range: 0.15 to 0.24 µl/min/mg cellular protein) and statistically not different from the corresponding passive diffusion CL in hepatocyte models ($CL_{int,passive,hep}$) (SH, PH and SCHH: 0.58±0.24, 0.30±0.20 and 0.67±0.75 µl/min/mg protein, respectively) (Fig. S1).

A Comparison of [3H]RSV $CL_{int,uptake,hep}$ in SH, PH and SCHH

The total uptake of [3H]RSV in all lots of hepatocytes was about the same except for JEL where it was consistently higher than the other three lots due to greater OATP-mediated uptake (Fig. 2). Overall, $CL_{int,uptake,hep}$, $CL_{int,OATP,hep}$, $CL_{int,NTCP,hep}$, and $CL_{int,passive,hep}$ of RSV were not significantly different between the hepatocyte models (Fig. 2D). In addition, the average contribution of OATPs was higher than NTCP (56% vs. 40%) to total [3H]RSV $CL_{int,uptake,hep}$ (Fig. 2D). Of note, the average $CL_{int,passive,hep}$ of [3H]RSV in SH, PH and SCHH was only 5.6±3.2%, 2.2±1.0% and 7.4±8.0% of the total [3H]RSV $CL_{int,uptake,hep}$ respectively (Fig. 2A-D).
Prediction of RSV $CL_{uptake, \text{in } vivo}$ from Transporter-Expressing Cells ($CL_{\text{in } vivo,pred,cells}$) and Hepatocyte Models ($CL_{\text{in } vivo,pred,hep}$) (IVIVE)

$CL_{uptake, \text{in } vivo}$ predicted using REF and transporter-expressing cells (OATP1B1/1B3/2B1, NTCP and passive diffusion) fell outside our pre-defined acceptance criteria of being within 2-fold of the observed hepatic uptake blood $CL$ ($CL_{uptake, \text{in } vivo}$). The predicted RSV hepatic $CL_{\text{in } vivo,pred,cells}$ was 25.1% (303.2±100.6 ml/min) of the mean observed hepatic $CL_{uptake, \text{in } vivo}$ (mean 1205.6 ml/min, 95% CI 802-1609 ml/min) (Billington et al., 2019). Of the predicted total RSV $CL_{uptake, \text{in } vivo}$ by transporter-expressing cells, ~99% was via transporters and ~1% via passive diffusion (Fig. 3). Of the total predicted RSV $CL_{\text{int,in } vivo,pred,cells}$, the contribution of OATP1B1, OATP1B3, OATP2B1, NTCP and passive diffusion clearance was 37.9, 15.5, 7.6, 38.3 and 0.6% respectively (Fig. 3). The $CL_{\text{int,in } vivo,pred,hep}$ based on SH, PH, and SCHH was about 16% (138.9±53.8 ml/min), 21% (175.2±71.2 ml/min), and 14% (121.5±31.3 ml/min) of the observed value respectively (Fig. 3). The $CL_{\text{int,in } vivo,pred,cells}$ of RSV based on transporter-expressing cells was 3934.2±1634.2 ml/min (OATP1B1: 1489.8±726.8 ml/min, OATP1B3: 610.8±306.6 ml/min, OATP2B1: 300.9±112.9 ml/min, and NTCP: 1507.6±667.5 ml/min, passive diffusion: 25.1 ml/min). The average $CL_{\text{int,in } vivo,pred,hep}$ of RSV based on SH, PH and SCHH (1551±672.6, 2022.6±965.6 and 1328.7±376.2 ml/min respectively) was ~50-70% lower than that based on transporter-expressing cells.

In the presence of 100% HP, the unbound RSV $CL_{\text{int,uptake,cells}}$ into OATP1B1, OATP1B3 and NTCP-expressing cells increased by 1.77±0.19, 1.67±0.06 and 1.49±0.20 fold, respectively. In the presence of 5% HSA, unbound RSV $CL_{\text{int,uptake,cells}}$ into OATP1B1, OATP1B3 and NTCP-expressing cells increased by 2.07±0.75, 1.47±0.26, 1.60±0.05 fold, respectively. Of note, the non-specific binding of RSV to the apparatus (measured in HBSS) was 0.08±0.06. After
correcting for this, the unbound fraction of RSV in 100% HP and 5% HSA was 0.15±0.01 and 0.13±0.01, respectively.

**Prediction of RSV $CL_{int,uptake,hep}$ from Transporter-Expressing Cells (IVCIVH)**

The total transporter-mediated $CL_{int,uptake,hep}$ in hepatocyte models predicted from cell lines (i.e. the sum of $CL_{int}$ predicted by the individual transporter-expressing cells, OATP1B1/1B3/2B1 and NTCP as well as passive diffusion CL) was not significantly different from the observed values, irrespective of whether the predictions were based on the total or PMA of the transporters in the cells (Fig. 4). The average contribution of OATP1B1, OATP1B3, OATP2B1, NTCP and passive diffusion to the [$^3$H]RSV uptake in hepatocyte models was about 42±8, 9±1, 9±1, 33±0.3, and 7±7% respectively. The [$^3$H]RSV $CL_{int,passive,hep}$ and $CL_{int,passive,cells}$ were not significantly different (Fig. S1).

**IVIVE of the magnitude of inhibition of RSV Hepatic Uptake CL by 0.3µM CsA based on Transporter-Expressing Cells**

The %inhibition of [$^3$H]RSV uptake into OATP1B1, OATP1B3, OATP2B1 and NTCP cells by CsA (0.3µM) was 56.5%±6.1%, 47.5%±12.5%, 56.6%±32.0% and 21.9%±6.7% respectively. This translated to CsA (0.3µM) inhibiting the total $CL_{uptake, in vivo}$ of RSV by 36.0%.

In our PET imaging study, the in vivo % inhibitory effect of 3µM CsA ($f_{u,b}$=0.1) on [$^{11}$C]RSV hepatic uptake in 3 subjects was 40.3%±6.8%, with the 95% confidence interval 23.4~57.2% (Billington et al., 2019).

**Transporter Abundance-Activity Correlation in Human Hepatocyte Models**

The observed total $CL_{int,uptake,hep}$ of [$^3$H]RSV in the human hepatocyte models was well correlated with total transporter abundance of OATP1B1 ($R^2 = 0.80$; Fig 5A) but not with total abundance
of OATP1B3, OAPT2B1, or NTCP (Fig. 5B-D). This conclusion did not change if the PMA of the transporters was used as the independent variable (data not shown).
Discussion

We designed our study to incorporate several unique features, hitherto not considered when conducting IVIVE of transporter-based CL. First, we did not assume, as others have (Li et al., 2010; Kim et al., 2019), that *in vivo* CL of OATP substrates, like RSV, is rate-determined by its uptake into the liver. This assumption can only be made if the sinusoidal efflux CL of the drug is much less than its metabolic plus biliary CL (Patilea-Vrana and Unadkat, 2018). Our PET imaging data have confirmed that this assumption is not correct for RSV (Billington et al., 2019) and, as discussed below, will result in biased evaluation of the CL\textsubscript{in vivo,pred}. Here, we correctly compared the *in vivo* RSV hepatic uptake CL determined by PET imaging with that predicted based on TEC/REF and hepatocytes without any assumptions of the rate-determining step. Second, we studied RSV uptake in multiple hepatocyte models (using cells isolated from the same donor) in which we previously quantified the total and PMA abundance of OATPs and NTCP (Kumar et al., 2018). Hence, we were able to test which hepatocyte model (SH, PH or SCHH) better predict *in vivo* hepatic uptake CL of RSV and compared these predictions with that obtained from transporter-expressing cells. Third, quantifying RSV uptake CL into cells and hepatocytes allowed us to determine if there was an *in vitro* to *in vitro* (IVCIVH) agreement in the predicted transporter-based hepatic uptake CL of RSV. This comparison is important (as described below) as both *in vitro* models under-estimated the *in vivo* hepatic uptake CL of RSV, albeit to a different extent. Fourth, as there is limited data on IVIVE of transporter-mediated DDI, we determined whether the magnitude of *in vivo* inhibition of hepatic uptake CL of RSV by CsA observed in our PET imaging study (Billington et al., 2019) could be predicted from *in vitro* uptake studies in TEC.
The predicted RSV $CL_{in\,vivo,\,pred,\,cells}$ by transporter-expressing cells was 303.2 ml/min when plasma/albumin was not included in the uptake studies, a value much less than (~25%) of the observed value (1205.6 ml/min; calculated from Billington et al., 2019). Thus, IVIVE of RSV $CL_{in\,vivo,\,pred,\,cells}$ based on TEC/REF does not meet our pre-defined success criteria of being within 2-fold of the observed value, a criterion widely accepted for wide therapeutic index drugs. The goal of IVIVE of CL is not to accurately predict CL of a drug, but to provide an estimate of the eventual CL of the drug likely to be expected in a Phase 1 trial of the drug. If the % PMA of transporters in liver tissue is similar to that in the hepatocytes (and not 100% as assumed), our predicted $CL_{in\,vivo,\,pred,\,cells}$ will not change substantially (though it will be slightly lower) because the major contributors to the uptake of RSV (OATP1B1 and NTCP) were predominately present in the plasma membrane of the hepatocytes and the transporter-expressing cells.

Since human hepatocytes are routinely used to predict transporter-mediated CL in vivo, we next examined whether the various human hepatocytes models (SH, PH, or SCHH) could better predict RSV $CL_{uptake,\,in\,vivo}$ than the TEC/REF approach. They did not; all the models considerably underpredicted (by about 5 to 10-fold) RSV $CL_{uptake,\,in\,vivo}$, among which the PH showed the best performance while the SCHH showed the worst. This finding is consistent with previous literature reports that hepatocytes underpredict (sometimes drastically) transporter-mediated hepatic CL of drugs (Jones et al., 2012). Based on the above discrepancy (~52%), we asked if the RSV $CL_{int,\,uptake,\,cells}$ by transporter-expressing cells could predict $CL_{int,\,uptake,\,hep}$ by hepatocyte models using the proteomics-informed approach (IVCIVH). Indeed, it could, irrespective of whether the total or PMA of the transporters was used as REF (Fig. 4). In addition, the RSV $CL_{int,\,passive,\,cells}$ predicted by the TEC, was not significantly different from that observed in hepatocytes ($CL_{int,\,passive,\,hep}$) (Fig. S1). This result immediately poses the question:
why was the RSV $CL_{\text{in vivo, pred, cells}}$ significantly greater than that predicted from the hepatocytes ($CL_{\text{in vivo, pred, hep}}$) (Fig. 3)? This was because we used PMA-based REF to predict $CL_{\text{in vivo, pred, cells}}$, whereas $CL_{\text{in vivo, pred, hep}}$ did not take into consideration PMA of transporters. Instead, it used the total protein in human liver as a scaling factor and assumed 100% PMA of the transporters in the hepatocyte models even though it was only 60-80% (OATPs and NTCP) (Kumar et al., 2019). Indeed, when a hybrid (REF and total protein) approach was used to scale $CL_{\text{int, hep, predicted}}$ (Table 1, Fig. 4), the $CL_{\text{in vivo, pred, hep}}$ was not significantly different from $CL_{\text{in vivo, pred, cells}}$ (data not shown).

The above IVCIVH comparison was informative to address the question as to why both approaches (TEC/REF and hepatocytes) under-estimated RSV $CL_{\text{uptake, in vivo}}$ the hepatocytes more so than the cells. A possible explanation is that the in vitro models are not adequately capturing the in vivo transporter (OATP/NTCP)-mediated or the passive hepatic uptake clearance of RSV or both. This could be due to differences in the in vivo RSV affinity ($K_m$) or $k_{\text{cat}}$ (catalytic turnover) of the drug by the transporters is different from that in vitro.

Mechanistically, this could be due to a difference in post-translational modifications of the transporter protein(s) between in vitro and in vivo resulting in either a changed $k_{\text{cat}}$, or the affinity ($K_m$) for the drug. In vitro to in vivo differences in $k_{\text{cat}}$ or $K_m$ could also be caused by endogenous factors present in in vivo but absent in in vitro models such as plasma proteins (Bowman et al., 2019). However, when we adjusted the transporter-mediated $CL_{\text{int, uptake, cells}}$ of RSV for the plasma or HSA-mediated increase in RSV $CL_{\text{int, uptake, cells}}$, the predicted hepatic $CL_{\text{int, in vivo, pred, cells}}$ improved but it still fell short of our success criteria (Fig 2B). Thus, other explanations need to be invoked to explain the IVIVE discrepancy in prediction of RSV $CL_{\text{int, uptake, cells}}$. One possibility is that our PET imaging study over-estimated the true $CL_{\text{uptake, in vivo}}$ as PET imaging does not allow us to distinguish between RSV present in the hepatocytes vs. that in the
canaliculi. Interestingly, the above explanations for under-estimation of \( \text{in vivo} \) RSV hepatic uptake CL by \( \text{in vitro} \) systems must be species-dependent as we have previously shown that TEC/REF approach successfully and accurately predicted the \( \text{in vivo} \) transporter-mediated RSV hepatic uptake CL in rats obtained by PET imaging (Ishida et al., 2018a).

To illustrate why comparing the predicted RSV \( CL_{\text{in vivo, pred, cells}} \) in TEC to the observed \( \text{in vivo} \) systemic RSV blood CL is erroneous, let us consider the following. Our predicted (480.1±128.6 ml/min with HP; 520.3±133.0 ml/min with HSA) value is well within the 2-fold of the observed \( \text{in vivo} \) systemic RSV blood CL (850.5 ml/min; Martin et al., 2003). This is because when hepatic uptake CL is not the rate-determining step, the \( \text{in vivo} \) systemic CL of a drug will always be less than the hepatic uptake CL of the drug. Hence comparing the hepatic uptake CL, predicted based on TEC or hepatocyte studies, even though an underestimation of the true hepatic uptake CL, may fortuitously fall within the 2-fold boundary of the observed \( \text{in vivo} \) systemic CL of the drug (Fig. S2). Thus, previous reports of success with such comparisons should be viewed with great caution (Kim et al., 2019).

Although NTCP-mediated RSV \( CL_{\text{int, uptake, cells, NTCP}} \) was about twice that by OATP1B1 (Table 1), due to the greater hepatic/hepatocyte abundance of OATP1B1, the latter was the dominant contributor (33 - 48%) to the total RSV \( CL_{\text{int, uptake, hep}} \). The contribution of OATP1B1 (33 - 48%) and NTCP (33%) to RSV \( CL_{\text{int, uptake, hep}} \) was higher than that of OATP2B1 (8 - 10%) or OATP1B3 (8 - 9%). Thus, it is not surprising that OATP1B1 total abundance (or PMA) showed good correlation \( (R^2 \geq 0.80) \) with total RSV \( CL_{\text{int, uptake, hep}} \) into SH, PH and SCHH (Fig. 5). The poorer correlation of total RSV \( CL_{\text{int, uptake, hep}} \) with NTCP abundance is surprising and needs further exploration.
REF based on PMA of a transporter is expected to better predict transporter-mediated clearance than REF based on total cellular transporter abundance. This was not the case here because the major contributors to RSV $CL_{\text{int,uptake,hep}}$, OATP1B1 and NTCP, were found to be predominately in the plasma membrane of cells and hepatocytes (Kumar et al., 2018). Nevertheless, as shown previously for OCT2 (Kumar et al., 2018), it is important that PMA of transporters along with the in vivo transport mechanism be considered in IVIVE using TEC/REF approach.

One goal of IVIVE of hepatobiliary CL of drugs is to predict the hepatic concentrations of the drug when the site of toxicity or efficacy is within the liver. Obviously, predicting the in vivo hepatic CL of a drug is insufficient to predict the hepatic concentration of a drug. This is because all in vivo hepatobiliary clearances (sinusoidal uptake CL ($CL_{\text{s,uptake}}$), metabolic CL ($CL_{\text{metabolism}}$), biliary efflux CL ($CL_{\text{b,efflux}}$), sinusoidal efflux CL($CL_{\text{s,efflux}}$)) need to be estimated or predicted in vitro. While the SCHH have been suggested as a tool to estimate these clearances, as previously shown by us (Kumar et al., 2020) and the data presented here on the shortfall of the SCHH model, alternative methods are needed to estimate all these hepatobiliary clearances. One alternative method is to use proteomics-based REF for IVIVE of $CL_{\text{s,efflux}}$ and $CL_{\text{b,efflux}}$.

$CL_{\text{metabolism}}$ can readily be obtained by using human liver microsomes/cytosol or S9 fraction. As to whether this proteomics-informed REF approach can successfully predict these clearances and tissue concentration of drugs in humans is yet to be determined. However, initial success in IVIVE of hepatic $^{11}$C-RSV concentrations in our rat study demonstrates the potential of this approach (Ishida et al., 2018a).

In our PET imaging study, we found that 0.3µM CsA reduced RSV hepatic $CL_{\text{uptake, in vivo}}$ in 3 of the 4 subjects (Billington et al., 2019). In those where it did, the in vivo %inhibition by CsA was 40.3%±6.8%. Our TEC successfully predicted the %inhibition (36.0%) of $CL_{\text{uptake, in vivo}}$ because
it fell within the 95% confidence interval (23.4%~57.2%) of the % inhibition observed in vivo. This observation adds to the growing evidence that in vivo transporter-mediated DDI can be predicted from in vitro studies (Ishida et al., 2018b).

In summary, we compared the ability of two in vitro systems, TEC and human hepatocyte models, to predict in vivo RSV hepatic CL\textsubscript{uptake, in vivo} in humans. We show that the TEC/REF approach resulted in a better prediction of the RSV hepatic CL\textsubscript{uptake, in vivo} compared with the hepatocyte models. However, both in vitro models failed to accurately predict (within 2-fold) the observed RSV CL\textsubscript{uptake, in vivo}. The reason for this in vitro to in vivo discrepancy remains unclear and needs further investigation. Clearly, inclusion of albumin or plasma in the in vitro uptake studies did not bridge this discrepancy. Interestingly, this result is in contrast to our previous success using TEC/REF to predict the in vivo hepatic CL of RSV in rats (Ishida et al., 2018a) and renal secretory CL of metformin in humans (Kumar et al., 2018). Collectively, our data suggest that the TEC/REF approach appears to be superior to hepatocytes in IVIVE of transporter-mediated hepatic CL of drugs. In addition, as we have previously shown (Ishida et al., 2018a), the REF approach can successfully predict the magnitude of in vivo DDI.
Acknowledgments

The authors thank Tot Bui Nguyen (University of Washington) for her support in human hepatocytes, transporter-expressing cell line culture and LC-MS/MS proteomics. The authors also thank Dr. Bhagwat Prasad (University of Washington) for his support in LC-MS/MS proteomics and Dr. Bruno Stieger (University of Zurich) for providing OATP1B1-expressing CHO cells.
Authorship Contributions

Participated in research design: Kumar, Yin, Ishida, Salphati, Hop, Rowbottom, Xiao, Lai, Mathias, Chu, Humphreys, Liao, Heyward and Unadkat.

Conducted experiments: Kumar, Yin

Contributed new reagents or analytic tools: Nerada, Szilvásy, and Heyward.

Performed data analysis: Kumar, Yin, Ishida and Unadkat.

Wrote or contributed to the writing of the manuscript: Kumar, Yin, Ishida, Salphati, Hop, Rowbottom, Xiao, Lai, Mathias, Chu, Humphreys, Liao, Nerada, Szilvásy, Heyward and Unadkat.
References


Footnotes

VK and MY were supported in part by the Simcyp Grant & Partnership Scheme and University of Washington Research Affiliate Program on Transporters (UWRAPT) funded by Genentech, Biogen, Gilead, Merck, Bristol-Myers Squibb, Pfizer and Takeda.
Figure Legends

Figure 1. Schematic of our approach for IVIVE of RSV Hepatic Uptake CL using the Proteomics-Informed REF Approach or the Traditional Physiological Scaling Approach

TEC – transporter-expressing cells, HSA – human serum albumin, HP – human plasma, REF-relative expression factor, WSHM – well-stirred hepatic model, HPPGL – total hepatic protein per gram of liver, IVICIVH – extrapolation of in vitro CL_{int,uptake,cells} to CL_{int,uptake.hep}

Figure 2. [^{3}\text{H}]RSV CL_{int,uptake.hep} in SH, PH and SCHH. [^{3}\text{H}]RSV CL_{int,uptake.hep} was determined in 4 lots of SH (A), PH (B) or SCHH (C). The average contribution to [^{3}\text{H}]RSV CL_{int,uptake.hep} followed the order OATPs> NTCP > passive diffusion (D). [^{3}\text{H}]RSV CL_{int,uptake.hep}, CL_{int,OATP,hep}, CL_{int,NTCP,hep}, and CL_{int,passive,hep} were not significantly different between the hepatocyte models (Wilcoxon matched-pair signed rank test). The average data from panel A, B and C are shown in panel D and they are mean±SD of 4 lots of hepatocytes, each conducted in triplicate.

Figure 3. IVIVE of RSV hepatic CL_{uptake, in vivo} based on transporter-expressing cells or the hepatocyte models, SH, PH or SCHH in HBSS buffer (A) or adjusted for the protein-mediated uptake of RSV in transporter-expressing cells (B). The transporter-expressing cells predicted the hepatic CL_{uptake, in vivo} within 3-fold of the observed value (1205.6 ml/min). In contrast, the hepatocyte models underpredicted RSV CL_{uptake, in vivo} by 5- to 10-fold. OATPs were the major contributors to the total RSV CL_{int,in vivo,pred,cells} and CL_{int,in vivo,pred,hep} followed by NTCP with smallest contributor being passive diffusion CL. The solid and dashed lines show the 95% CI of the observed hepatic CL_{uptake, in vivo} and 2-fold lower limit of the mean observed hepatic
$CL_{\text{uptake, in vivo}}$ (603 ml/min). RSV $CL_{\text{int,in vivo,pred,cells}}$ was significantly higher (Tukey’s multiple comparison test) than $CL_{\text{int,in vivo,pred,hep}}$ obtained from any of the hepatocyte model. Data shown are mean±SD of 4 lots of hepatocytes, each conducted in triplicate. (A). When the transporter-mediated $CL_{\text{int,uptake,cells}}$ was adjusted for the increase in the RSV uptake in the presence of 100% HP or 5%HSA, the transporter-expressing cells better predicted hepatic $CL_{\text{uptake, in vivo}}$ (480 ml/min with 100% HP; 520 ml/min in 5%HSA) but still fell outside of 2-fold of the observed value (B).

**Figure 4. IVCIVH of [${}^3$H]RSV $CL_{\text{int,uptake,hep}}$.** The predicted [${}^3$H]RSV $CL_{\text{int,uptake,hep}}$ from transporter-expressing cells was not significantly different from the observed [${}^3$H]RSV $CL_{\text{int,uptake,hep}}$, irrespective of whether the predictions were made based on the total or PMA of the uptake transporters. NS: Not significantly different based on Wilcoxon matched-pair signed rank statistical test, data shown are mean ±SD of 4 lots of hepatocytes, each conducted in triplicate.

**Figure 5. Correlation of [${}^3$H]RSV $CL_{\text{int,uptake,hep}}$ and total transporter protein abundance in hepatocyte models.** The [${}^3$H]RSV $CL_{\text{int,uptake,hep}}$ was highly correlated with the total abundance of OATP1B1. However, the correlation with OATP1B3, OATP2B1 and NTCP transporter abundance was poor. Similar results were obtained if the x-axis was PMA of the respective transporter. Data shown are mean of triplicates in 4 lots of hepatocytes models.
### Tables

**Table 1.** OATP1B1/2B1/1B3- and NTCP-mediated $CL_{int,uptake,cells}$ of $[^3]H$RSV based on their total or PMA in the transporter-expressing cells

<table>
<thead>
<tr>
<th>Transporter (cells)</th>
<th>Transporter-mediated $CL_{int,uptake,cells}$ of RSV (µL/min/mg cellular protein)*</th>
<th>Total transporter abundance (pmol/mg cellular protein) in cells#</th>
<th>Transporter-mediated RSV $CL_{int,uptake,cells}$ (µL/min/pmol total transporter protein)</th>
<th>PMA of transporter (% total)$^a$</th>
<th>Transporter-mediated RSV $CL_{int,uptake,cells}$ (µL/min/pmol PM transporter protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1 (CHO cells)</td>
<td>40.1</td>
<td>7.53</td>
<td>5.32</td>
<td>79.7±4.7$^a$</td>
<td>6.68</td>
</tr>
<tr>
<td>OATP1B3 (HEK293 cells)</td>
<td>28.6</td>
<td>6.23</td>
<td>4.60</td>
<td>63.2±1.6$^a$</td>
<td>7.27</td>
</tr>
<tr>
<td>OATP2B1 (MDCKII cells)</td>
<td>1.2</td>
<td>0.95</td>
<td>1.22</td>
<td>37.1±15.7$^a$</td>
<td>3.29</td>
</tr>
<tr>
<td>NTCP (HEK293 cells)</td>
<td>34.8</td>
<td>3.29</td>
<td>10.58</td>
<td>77.4</td>
<td>13.65</td>
</tr>
</tbody>
</table>

$^a$from Kumar et al., (Kumar et al., 2017).  
$^b$ Data shown are mean of triplicates or mean±SD of three independent experiments (except NTCP, which is mean of 2 independent determinations). 
$^c$ Data are mean of duplicate determinations from a single experiment.  
* Data from a single experiment mean of triplicate determinations (except NTCP, average of duplicate determinations). The $CL_{int,uptake,OATP1B1}$ of RSV obtained in OATP1B1-expressing CHO cells was confirmed with $CL_{int,uptake,OATP1B1}$ of RSV in OATP1B1-expressing HEK293 cells.
Uptake Assay
Transporter-Expressing Cells (TEC)

CL_{int,uptake,cells} +5\% HSA/100\% HP

Transporter Quantification
LC-MS/MS Proteomics

IVIVE using REF & the WSHM

Predicted
CL_{int,uptake,in vivo}

Liver Weight
HPPGL

IVIVE using physiological scaling & the WSHM

Predicted
CL_{int,uptake,in vivo}

Compare

PET Imaging Study
in vivo CL_{int,uptake}
Figure 2.
In-vivo observed hepatic uptake CL (mean, 95% CI): (1205.6, 801.8 – 1609.4 ml/min) Billington et al., 2019
**B**

In-vivo observed hepatic uptake CL (mean, 95% CI): (1205.6, 801.8 – 1609.4 ml/min) Billington et al., 2019

![Graph showing RSV scaled hepatic CL uptake, in vivo](image)
Figure 4.

<table>
<thead>
<tr>
<th>[3H]RSV CL&lt;sub&gt;in&lt;/sub&gt;,uptake,hep (μl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
</tr>
<tr>
<td>Predicted – based on total abundance</td>
</tr>
<tr>
<td>Predicted – based on PM abundance</td>
</tr>
</tbody>
</table>

SH | PH | SCHH |
---|----|------|
10 | 15 | 16   |
20 | 25 | NS   |
30 | NS | NS   |

Figure 4.
Figure 5.

A

\[ y = 5.4416x + 3.7281 \]
\[ R^2 = 0.8082 \]

OATP1B1 total abundance in SH, PH and SCHH
(pmol/mg protein)

B

\[ y = 29.202x + 2.7679 \]
\[ R^2 = 0.1906 \]

OATP1B3 total abundance in SH, PH and SCHH
(pmol/mg protein)

C

\[ y = -6.5729x + 17.671 \]
\[ R^2 = 0.0682 \]

OATP2B1 total abundance in SH, PH and SCHH
(pmol/mg protein)

D

\[ y = 7.0278x + 8.1519 \]
\[ R^2 = 0.0518 \]

NTCP total abundance in SH, PH and SCHH
(pmol/mg protein)