**In vitro** evaluation of hepatic transporter-mediated clinical drug-drug interactions: hepatocyte model optimization and retrospective investigation

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Running title page

Running title: In vitro evaluation of OATP-mediated DDI

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Abbreviation: SCHH: Sandwich cultured human hepatocyte; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; NTCP, sodium taurocholate cotransporting polypeptide. DDI, drug-drug interaction
Abstract:

To assess the feasibility of using sandwich cultured human hepatocytes (SCHH) as a model for characterizing transport kinetics for in vivo pharmacokinetic prediction, the expression of OATP proteins in SCHH, along with biliary efflux transporters (Li et al., 2009), were quantitatively confirmed by LC-MS/MS. Rifamycin SV (Rif SV), which was shown to completely block the function of OATP transporters, was selected as an inhibitor for assessing the initial rates of active uptake. The optimized SCHH model was applied in a retrospective investigation of compounds with known clinically significant OATP-mediated uptake and was further applied to explore drug-drug interactions (DDIs). Greater than 50% inhibition of active uptake by Rif SV was found to be associated with clinically significant OATP-mediated DDIs. We propose that the in vitro active uptake value could therefore serve as a cutoff for class 3 and 4 compounds of biopharmaceutics drug disposition classification system (BDDCS), which could be integrated into the International Transporter Consortium (ITC) decision tree recommendations (Giacomini et al., 2010) to trigger clinical evaluations for potential DDI risks. Furthermore, kinetics of in vitro hepatobiliary transport obtained from SCHH, along with protein expression scaling factors, offer an opportunity to predict complex in vivo processes using mathematical models, such as physiologically-based pharmacokinetics models (PBPK).
Introduction

Hepatobiliary elimination are primary elimination routes for many endogenous and exogenous xenobiotics. Hepatic uptake and efflux transporters respectively located on sinusoidal or canalicular membranes, contribute to the vectorial transport of drugs and their metabolites from systemic circulation to bile (Meier et al., 1997; Kullak-Ublick et al., 2000). Two classes of hepatic uptake transporters, sodium-dependent and sodium-independent transporters, co-exist on the sinusoidal membrane of hepatocytes with overlapping substrate specificities. For example, the sodium-dependent transporter, sodium-taurocholate co-transporting polypeptide (NTCP), is shown to transport the organic anion transporting polypeptide (OATP) substrates atorvastatin and rosuvastatin (Ho et al., 2006; Choi et al., 2011). OATP1B1/1B3, organic anion transporter 2 (OAT2) and organic cation transporter 1 (OCT1) are specifically expressed in the liver and transport structurally diverse substrates in a sodium-independent manner. In the last decade, significant advances toward the prediction of \textit{in vivo} NME clearance using \textit{in vitro} models have been well-documented. Indeed, \textit{in vitro} human liver microsomes and isolated hepatocytes were shown to be important tools in drug discovery and development to confidently predict \textit{in vivo} human drug metabolism for compounds.
predominately eliminated by cytochrome P-450 (Obach, 1999; McGinnity et al., 2004).

However, human PK prediction remains very challenging for compounds where drug
transporters are involved in the clearance mechanism. In 2010, the International
Transporter Consortium (ITC) recommended decision trees using in vitro systems to
assess the risk of in vivo transporter mediated drug-drug interactions (DDIs) (Giacomini
et al., 2010). For OATP transporters, the investigation cascade is initiated by the criteria
of active hepatic uptake if hepatic clearance is an important route of elimination, e.g. >0.3
of total clearance (Giacomini et al., 2010). However, the models and the extent of active
hepatic uptake that would trigger the investigation cascade remain undetermined and
suitable in vitro tools to assess the active/passive hepatic uptake need to be further
validated. We hypothesized that clinically relevant OATP mediated DDI is associated
with significant active uptake in vitro.

Recently, a physiologically-based pharmacokinetic (PBPK) model, in which
physiological compartments representing organs/tissues are connected with blood flow,
was developed to predict in vivo clearance and time profiles of drug elimination using in
vitro models such as suspension hepatocytes and canalicular membrane vesicles
(Watanabe et al., 2009). The approach is generally accepted as a useful tool to predict
tissue concentration, drug-drug interaction, and the effects of enzyme/transporter genetic polymorphisms on drug exposure. Practically, parameters for distinct clearance processes including passive diffusion, transporter-mediated hepatic uptake (sinusoidal), metabolism, and transporter-mediated efflux (canalicular) into the bile could be determined \textit{in vitro} and used to predict \textit{in vivo} human.

The sandwich cultured hepatocyte model (SCH) built by culturing hepatocytes between two layers of gelled matrix in a sandwich configuration, forms a bile canalicular network and provides the three-dimensional orientation and proper localization of hepatobiliary transporters that mimic \textit{in vivo} conditions and allow the vectorial transport of xenobiotics (Bi et al., 2006). In 2006, Turncliff \textit{et al.} evaluated the hepatobiliary disposition of metabolites of terfenadine generated in sandwich cultured rat hepatocytes (SCRH) (Turncliff et al., 2006). The advancement of \textit{in vitro} models for the clearance prediction in human has further reflected an increase in the mechanistic understanding of the hepatic vectorial elimination process (Kotani et al., 2011). For example, by using LC-MS/MS quantification methods, the expression of biliary efflux transporters in sandwich cultured rat hepatocytes (SCRH) were determined and used to improve the extrapolation from \textit{in vitro} to \textit{in vivo} for the compounds excreted from bile (Li et al., 2009; Li et al., 2010). Meanwhile, a decrease in functional uptake in SCRH was observed raising
concerns for its suitability as a model for active hepatic uptake evaluations (Li et al., 2010; Kotani et al., 2011). However, unlike SCRH, based on our in house unpublished results and the recently published data (Kotani et al., 2011), active uptake functions in sandwich cultured human hepatocytes (SCHH) are well-maintained and the system appears promising as a single in vitro model for evaluation of candidate drug uptake and biliary excretion. The objectives of this research are three folds; first, confirm the maintenance of hepatic-specific uptake transporter function and expression in SCHH; second, optimize the experimental conditions needed to define the active hepatic uptake and biliary excretion to obtain in vitro parameters that could be used as inputs for mathematical PBPK model for in vivo prediction (Jones et al., 2012); and third, obtain an in vitro cutoff values for active uptake in SCHH that would be appropriate to trigger the clinical investigations recommended by the ITC.

Materials and Methods

Reagent and hepatocytes

HPLC grade acetonitrile, water and methanol were purchased from Burdick & Jackson (Muskegon, MI) and EMD Chemicals, Inc (Gibbstown, NJ), respectively. Hanks
balanced salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA).

Rosuvastatin, atorvastatin, pitavastatin valsartan, fluvastatin, pravastatin, cerivastatin,
buprenorphine, and midazolam were obtained from Sequoia Research Products Ltd.
(Oxford, UK). Rifamycin SV (Rif SV) was purchased from Sigma-Aldrich (St. Louis, MO). Ammonium acetate was obtained from Mallinckrodt (Phillipsburg, NJ).

[^3H]Taurocholate (TC, 4.6 Ci/mmol) was purchased from PerkinElmer Life Sciences
(Boston, MA). The BCA kit was purchased from Pierce Biotechnology (Rockford, IL).

The ProteoExtract® native membrane protein extraction kit was purchased from
Calbiochem (Temecula, CA). Trypsin was purchased from Promega (Madison, WI).
Matrigel™ (phenol red free) and collagen coated 24 well plates were obtained from BD
Biosciences (Franklin Lakes, NJ). In Vitro GRO-HT, In Vitro GRO-CP and In Vitro
GRO-HI hepatocytes media were purchased from Celsis In Vitro Technologies
Inc. (Baltimore, MD). Cryopreserved human hepatocyte (lot Hu4165) was purchased from
CellzDirect (Pittsboro, NC).

**Sandwich cultured human hepatocytes (SCHH)**

Cryopreserved hepatocytes were thawed and plated as described previously (Bi et
al., 2006). In brief, hepatocytes were thawed in a water bath at 37°C, and then
immediately poured into 50 mL in pre-warmed In Vitro GRO-HT medium in a conical
tube. The cells were then centrifuged at \(50 \times g\) for 3 minutes and resuspended \(0.85 \times 10^6\) cells/mL in In Vitro GRO-CP medium. Cell viability was determined by trypan blue exclusion. On day 1, hepatocyte suspensions were seeded in BioCoat 24-well plates in a volume of \(0.5\) mL/well. After incubation overnight at \(37^\circ C\), the hepatocytes were overlaid with \(0.5\) mL IN VITRO GRO-HI medium with Matrigel\textsuperscript{TM} (0.25 mg/mL). The IN VITRO GRO-HI media were refreshed every 24 hr.

**Extraction and digestion of membrane protein**

At day 5 post culture, SCHH were detached from cell culture plates and washed with HBSS. Along with suspension hepatocytes, the membrane protein fraction of hepatocytes was extracted as described previously (Li et al., 2008) using the ProteoExtract\textsuperscript{®} native membrane protein extraction kit (Calbiochem). Briefly, hepatocyte pellets were homogenized in extraction buffer I of the kit containing a protease inhibitor cocktail followed by incubation at \(4^\circ C\) for 10 minutes with gently rocking. The homogenate was centrifuged at \(16,000 \times g\) for 15 minutes at \(4^\circ C\). The supernatant containing cytosolic proteins was discarded and the pellets were re-suspended in extraction buffer II of the kit containing a protease inhibitor cocktail. After 60 minutes of incubation at \(4^\circ C\) with gentle rocking, the suspension was centrifuged at \(16,000 \times g\) for
15 minutes at 4 °C. The protein concentration in the membrane fractions was determined using the BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL).

**LC-MS/MS quantitative measurement of OATP1B1, 1B3 and 2B1 transporters**

Proteotypic peptides and stable isotope label (SIL) peptides of OATP1B1, 1B3, and 2B1 were selected (Table 1) and synthesized as surrogate analytes for the corresponding protein quantification by LC-MS/MS (Li et al., 2008). LC-MS quantification for human OATP proteins and two of six peptides identified (NVTGFFQSFK and SSPAVEQQLLVSGPGK) were also reported by Ji et al (Ji et al., 2012). The digestion condition were optimized by our previous report (Balogh et al., 2012). Briefly, 80 μg of membrane fraction protein was reduced with 6 mM dithiothreitol and alkylated with iodoacetamide in 25 mM ammonium bicarbonate digestion buffer containing 10% sodium deoxycholate monohydrate (DOC), and then digested by trypsin (the final concentration of DOC during digestion was 1%). At the end of digestion, samples were acidified with an equal amount of 0.2% formic acid in water with SIL internal standards, and centrifuged at 14,000 x g for 5 minutes. The supernatants were transferred to a new plate and dried down. The samples were reconstituted with 0.1% formic acid in water and analyzed by LC-MS/MS.
The calibration curve was prepared using the synthetic proteotypic peptide with a fixed concentration of each SIL peptide as the internal standard. Sample quantification was conducted by coupling a triple quadrupole mass spectrometer (TQ-MS, API4000, Applied Biosystem, Foster City, CA) to a Shimadzu High-performance liquid chromatography (LC) system (SLC-10A, WoolDale, IL) and HTS PAL Leap autosampler (Carrboro, NC). A Phenomenex Kinetex 2.6 μm C18 100A column (3.0 × 100 mm) was used for peptide chromatography. A linear gradient elution program was conducted to achieve chromatographic separation with mobile phase A (0.1% formic acid in HPLC Grade Water), and mobile phase B (0.1% formic acid in acetonitrile). A sample volume of 10 μl was injected onto the LC column at a flow rate of 0.5 mL/min. The parent-to-product transitions for the proteotypic peptide generally represent the doubly charged parent ion to the single charged product y ions for each peptide (Table 1). Data were processed by integrating the appropriate peak areas for the analyte peptides and the SIL internal standard peptides in Analyst 1.4.2 (Applied Biosystems, Foster City, CA).

**Determination of hepatic uptake in SCHH**

SCHH were rinsed twice with 0.5 ml of 37°C regular HBSS buffer or Ca$^{2+}$/Mg$^{2+}$-free HBSS containing 1 mM EGTA, and then replaced with fresh regular HBSS buffer or Ca$^{2+}$/Mg$^{2+}$-free HBSS containing 1 mM EGTA. The disruption of the bile canalicular
network was achieved by pre-incubation with Ca\(^{2+}/\)Mg\(^{2+}\)-free HBSS containing 1 mM EGTA buffer for 10 minutes. For determination of the effects of various inhibitors on rosvavastatin uptake in SCHH, parallel incubations were conducted in the presence of the uptake transporter inhibitors, Rif SV (100 μM), Cyclosporin A (CsA, 10 μM), and gemfibrozil (30 μM). SCHH uptake was initiated by the addition of 500 μL containing substrate at a concentration indicated in the figure legends, with or without inhibitors. Reactions were terminated at designated time points by quickly washing the hepatocytes three times with ice-cold HBSS buffer. The cells were either lysed with 0.5% Triton-100 (radiolabeled compounds) or 100% methanol containing internal standard (non-radiolabeled compounds). Samples were analyzed either by LC-MS/MS, respectively.

**LC-MS/MS Analysis of Probe Substrates**

LC-MS/MS analysis of probe substrates was conducted with an API 4000 triple quadruple mass spectrometer (PE Sciex, Ontario, ON, Canada) coupled with a turbo ion spray interface in positive ion mode, and connected with a Shimadzu LC (SLC-10A) system (WoolDale, IL) and Gilson 215 autosampler. The mass spectrometer was controlled by Analyst 1.4.2 software (Applied Biosystems). The Gilson autosampler was independently controlled by Gilson 735 software and synchronized to Analyst via contact closure. The HPLC method consisted of a step gradient with 25 uL samples loaded onto
a 1.5 x 5 mm Showadenko (Tokyo, Japan) ODP 13 µm particle size column using 95%
2mM ammonium acetate/5% 50/50 methanol/acetonitrile. Incubation samples and
standard curve samples were eluted with 10% 2mM ammonium acetate in 50/50 of
methanol/acetonitrile. Peak area counts of analyte compound and internal standard (IS)
were integrated using DiscoveryQuant Analyze as an add-on to Analyst 1.4.2. The
obtained values underwent data analysis by averaging the analyte area divided by the
internal standard analyte for each concentration.

**Data Analysis**

The apparent *in vitro* biliary clearance (CL\textsubscript{bile}) (Liu et al., 1999) and percent active
uptake were determined by equations 1 and 2, respectively. The hepatic uptake of test
compounds was estimated from the initial uptake phase (0.5 to 1 min) while biliary
excretion was assessed at 10 minutes. The data represent the results from a single study
run in triplicate or duplicate and a minimum of two experiments were performed. The
standard deviations or coefficient variation (CV%) was listed in the legends of figure or
table.

\[ CL_{\text{bile}} = \frac{Accumulation_{(\text{medium, HBSS})} - Accumulation_{(\text{Ca}^{2+} + \text{Mg}^{2+}, \text{Free})}}{Incubation\_time*concentration_{(\text{medium})}} \] (Eq 1)

\[ \% \text{ active uptake} = 100 - (\text{slope}_{+\text{Rif SV}})/(\text{slope}_{-\text{Rif SV}}) \] (Eq 2)
Results

Protein expression of OATP1B1, 1B3, and 2B1 in suspension hepatocytes and SCHH

As functional active uptake has been shown previously in SCHH both in house and in the literature (Kotani et al., 2011), in the present study, the protein expression of OATP1B1, 1B3, and 2B1 in SCHH was measured by LC-MS/MS and compared with that in suspension hepatocytes. Peptides proteolytically released from the target OATP proteins were quantified using external synthetic peptide calibration curves. The peptide fragments were monitored using multiple reactions monitoring (MRM) (Table 1). Sample preparation, digestion, and detection limits of OATP protein quantification were previously evaluated (Balogh et al., 2012). As shown in Table 2, while OATP1B3 and 2B1 expression levels were reduced to approximately half of that in suspension hepatocytes, OATP1B1 expression in SCHH was slightly higher (1.5 fold) than that found in suspension hepatocytes. The results provide support for OATP-mediated active hepatic uptake in SCHH, which indicates that SCHH could be a suitable tool for the assessment of active OATP uptake. In addition, the protein expression obtained by LC-MS/MS measurement could be integrated into a mathematical model as a component of scaling factors for in vivo extrapolation from in vitro.
Inhibition of active uptake in SCHH

The active component of hepatic uptake can be determined by the total hepatic uptake (PS\textsubscript{uptake}) minus the passive diffusion (PS\textsubscript{passive}) in an \textit{in vitro} hepatocyte model. Generally, passive diffusion in hepatocytes can be obtained by either co-incubation with an OATP inhibitor or by conducting the uptake experiment at low temperature, e.g. at 4ºC. We previously reported that the lack of uptake might be an artificial effect of a rigid cell membrane at 4ºC and the uptake in the hepatocyte model determined at 37ºC and 4ºC might not truly reflect the functional active uptake (Kimoto et al., 2011). To select a suitable inhibitor blocking carrier-mediated active hepatic uptake in SCHH, time-dependent accumulation of rosuvastatin, a known substrate of OATP proteins, was investigated in the presence or absence of known OATP inhibitors, rifamycin SV (Rif SV, 100 \textmu M), cyclosporin A (CsA, 10 \textmu M) and gemfibrozil (30 \textmu M). As depicted in Figure 1, rosuvastatin was actively transported into SCHH and the uptake was linear up to 1.5 minutes. It is worthwhile to note that a positive Y-intercept was obtained by extending the trendline of rosuvastatin uptake. Non-specific binding on hepatocyte surface has been proposed to contribute the intercept and was not included here for the calculation of initial uptake rate. The initial uptake rates of rosuvastatin estimated from 0.5 to 1 minute were inhibited by all three OATP inhibitors, Rif SV, CsA and gemfibrozil, by 95, 80, and
78%, respectively. The active uptake of rosuvastatin was almost completely inhibited in the presence of Rif SV at 100 μM.

**Hepatic uptake and biliary excretion for compounds that are the substrates of phase I/II metabolizing enzymes or hepatic uptake transporters in SCHH**

As mentioned above, rosuvastatin uptake in SCHH was nearly abolished by co-incubation with 100 μM Rif SV. The results agree with previous reports that Rif SV (100 μM) totally blocks OATP functions in OATP transfected cell lines (Vavricka et al., 2002).

To determine if Rif SV also affects metabolizing enzymes, biliary excretion, and active hepatic uptake by transporters other than OATPs, initial uptake and biliary excretion of various compounds were further tested in SCHH. Buprenorphine and midazolam, which are metabolized by UGT1A1 and CYP3A4, respectively, were used as compounds that penetrate the hepatocytes via passive diffusion. TC were selected as probe substrates of NTCP transporters. Rosuvastatin as a known OATP substrate was again tested in SCHH for characterizing the initial uptake and biliary excretion kinetics. In addition, rosvuvastatin and taurocholate (TC) are excreted from bile through biliary efflux transporters such as multidrug resistant protein 2 (MRP2), breast cancer resistant protein (BCRP) or bile salt exporting pump (BSEP) (Ito et al., 2010). The uptake (PS\textsubscript{uptake} and PS\textsubscript{passive}) and biliary excretion (CL\textsubscript{bile}) in SCHH of the compounds are shown in Figure 2
and Table 3. As expected, Rif SV (100 μM) inhibited approximately 93% and 83% of the uptake rate of rosuvastatin and TC in SCHH, respectively. Following a decrease of uptake into SCHH caused by the inhibition of Rif SV, biliary excretion of rosuvastatin and TC were also substantially decreased (Table 3). Although significant increases in the intracellular concentration of buprenorphine or midazolam in SCHH were detected in the presence of Rif SV (Figure 2), the initial uptake rates of the compounds were not significantly altered. The intracellular accumulation of buprenorphine or midazolam by Rif SV might be due to the inhibition of CYP3A4 and UGT1A1 activities in SCHH. Since no biliary excretion was detected for buprenorphine and midazolam (Table 3) both in the absence and presence of Rif SV, the results revealed that Rif SV did not change the elimination profile by switching elimination pathways between metabolizing enzyme-mediated clearance and transporter-mediated biliary excretion.

**Hepatic uptake in SCHH for the compounds that undergo clinically significant DDIs with OATP inhibitors**

As noted above, SCHH has been characterized as a suitable model to assess in vitro hepatic uptake (active and passive) and biliary excretion. To evaluate the risk of in vivo OATP-mediated DDIs from the in vitro SCHH model, a literature review was
conducted to compile the OATP substrates that undergo significant DDIs in the clinic (>2 fold increase in AUC) when co-administered with OATP inhibitors. The substrates are listed in Table 4 and we conducted uptake and biliary excretion assays in SCHH to determine the in vitro active uptake (PS\textsubscript{active}) and CL\textsubscript{bile}. To avoid potential saturation of active uptake transporters, the substrate concentrations selected either were below the OATP \textit{Km} known from literature reports or lack of saturation was confirmed by our preliminary experiments. As indicated in Table 4, initial uptake rates into SCHH for the compounds ranged from 2 to 45 uL/min/mg protein. Rif SV inhibitable active hepatic uptake ranged from 55% to 84% in SCHH for the compounds reported to undergo OATP-mediated DDIs. Although the Rif SV inhibitable active uptake (%) did not correlate with the AUC changes that are observed in clinic (Figure 3A), these compounds were actively uptaken into hepatocyte to the extent of greater than 50% (Figure 3A). On the other hand, an increase of passive diffusion into hepatocytes tended to diminish the fold increase in AUC change caused by OATP inhibitors (Figure 3B), suggesting OATP mediated DDI risk could be low for high permeable compounds.

**Discussion**

Adverse clinically significant DDIs represent major challenges in drug discovery and development. In the last two decades, preclinical in vitro in vivo models were used to...
effectively predict human pharmacokinetics. However, the predictions were successful for the compounds that are mainly eliminated renally or via cytochrome P-450 metabolizing enzymes (Obach, 2009). Predicting hepatic transporter mediated clearance continues to be challenging due to large interspecies differences in hepatic transporter homology or expression, and lack of validated in vitro tools (Lai, 2009). Hepatic drug elimination is generally composed of a series of processes that include: entrance into hepatocyte via passive diffusion and active uptake mediated by hepatic transporters; metabolic elimination through phase I and/or phase II enzymes; and excretion to bile and/or back to systemic circulation by the efflux transporters. To assess processes involved in hepatic uptake and biliary excretion for in vivo prediction, the development of an in vitro model that mimics the complexity of the hepatic transport system has become imperative.

Human hepatocyte in vitro models are widely accepted as a valuable tool for investigating drug metabolic liability and gene induction and toxicity, as well as serving as effective screening tools for NMEs. The cellular polarity that allows vectorial transport in vivo is disrupted rapidly when cells are isolated from the intact organ. Therefore, the restoration of the bile canalicular network in the cultured hepatocyte model is desired for investigating the vectorial transport of drug candidates. SCHH has been generally
accepted as a good model to aid in predicting biliary clearance in humans (Bi et al., 2006).

Following the previous efforts to understand the expression of hepatobiliary efflux transporters in SCHH (Li et al., 2009), in the present study, we report, for the first time, the maintenance in SCHH of uptake hepatic transporters, OATP1B1, 1B3 and 2B1 as measured by LC-MS/MS. This provides molecular evidence to support SCHH as a model for obtaining functional hepatic uptake parameters for \textit{in vivo} prediction.

Moreover, to better define the uptake clearance in SCHH, ideally an inhibitor that can block all known hepatic uptake transporters with minimum effect on passive diffusion and biliary excretion is needed. To meet these criteria, we measured rosvastatin uptake in SCHH co-incubated with several known OATP transporter inhibitors. Under the concentrations applied, these inhibitors can completely block OATP1B1 or 1B3 activity in OATP gene overexpressing systems (Vavricka et al., 2002; Yamazaki et al., 2005; Letschert et al., 2006). As a result, Rif SV (100 μM) was shown to completely knock down rosvastatin uptake in SCHH. The inhibitor selected provided the ability in assessing the sum of active hepatic uptakes contributed by OATP transporters expressed in hepatocytes. In contrast, Rif SV (100 μM) had no effect on the passive diffusion of midazolam and buprenorphine into hepatocytes. Rif SV also increased the intracellular accumulation of buprenorphine and midazolam through the inhibition of metabolizing...
enzymes, such as UGT1A1 and CYP3A4. These inhibitory effects were minimized through optimization of experimental conditions by calculating the initial uptake rate to avoid the artificial effect on hepatic uptake caused by the inhibitory effects of Rif SV on metabolizing enzymes. Moreover, the reduced biliary excretion is observed followed the decreases of hepatic uptake. Since the intracellular accumulation of the compounds was not observed (Table 3, 4 and Figure 2) with or without Ca\textsuperscript{2+}, we speculate that the reduced biliary excretion was caused by the inhibitory effects on hepatic uptake. However, direct inhibitory effects on hepatobiliary efflux transporters still remain to be further investigated as Rif SV appears as an inhibitor of efflux transporters including bile salt export pump (BSEP) (Wang et al., 2003).

Three isoforms (OATP1B1, OATP1B3 and OATP2B1) are considered to play a pivotal role in the hepatic uptake of xenobiotics and endogenous compounds on the sinusoidal membrane of hepatocytes (Giacomini et al., 2010). The OATPs transport drugs from a wide range of therapeutic classes including the 3-hydroxymethylglutaryl-CoA reductase inhibitors (statins), angiotensin II receptor antagonists (e.g., olmesartan and valsartan), angiotensin-converting enzyme inhibitors (enalapril and temocaprilat), the H\textsubscript{1}-receptor antagonist fexofenadine, and the endothelin receptor antagonist, bosentan (Giacomini et al., 2010). As the OATP proteins are poorly conserved evolutionarily as
evidenced by the lack of human orthologues in rodents, extrapolation of \textit{in vivo} human from rodent models remains limited. The ITC recommends that the clinical investigation cascade should be initiated when active hepatic transport is involved in the liver clearance pathway and outlines decision trees for \textit{in vitro} evaluation of hepatic uptake using a hepatocyte model to predict the potential risk of clinical DDIs. The investigation cascades are considered to be similar to the \textit{in vitro} studies of drug metabolism and interaction (Giacomini et al., 2010). As a result, developing \textit{in vitro} models that can predict OATP mediated DDIs is an efficient and inexpensive approach that could reduce or eliminate the need for further clinical investigation. However, the extent of \textit{in vitro} active hepatic uptake necessary to trigger the clinical assessment of DDI was not provided (Giacomini et al., 2010). In addition, the performance of the \textit{in vitro} hepatocyte models should be evaluated to increase confidence in obtaining outcomes through retrospective analysis. With this in mind, a literature review was conducted and the compounds with reported OATP-mediated clinical DDI were tested in our optimized SCHH model. As expected, active hepatic uptake of the compounds with clinically significant OATP DDIs was observed. The contribution of the active portion to overall hepatic uptake was high, being 55 to 84\% of the total uptake rate. As hepatocyte lot differences in transporter expression exist, it is important that we confirmed the OATP
expression in SCHH, and compared the active hepatic uptake in multiple hepatocyte lots (Supplemental Table 1). These results suggest that 50% active hepatic uptake in SCHH could be the in vitro cutoff to trigger the clinical risk assessment of hepatic transporter-mediated DDIs.

The relationship among solubility, passive permeability, and the effects of drug transporter and metabolizing enzymes on drug disposition has been well addressed by the biopharmaceutics drug disposition classification system (BDDCS) (Wu and Benet, 2005). As depicted in Figure 3B, the fold increase of AUC tended to decrease as the PS\text{passive} in SCHH increased. The results suggest that low permeability drugs that rely primarily on transporter uptake for entry are prone to clinical DDIs mediated by OATP transporters. When applying the classification system, transporter effects are predicted to be minimal for high permeability/high solubility Class 1 compounds (Wu and Benet, 2005). In the gastrointestinal space, good solubility is essential for saturation of efflux transporters to obtain good absorption. Once the compounds are absorbed (into the systemic space), high permeability/low solubility Class 2 compounds could behave similarly to Class 1 compounds, in that the high permeability can overcome the transporter effects, and therefore reduce the risk of transporter mediated DDIs by competing drug entering into hepatocytes. This simple categorization under BDDCS suggests that the high vs low
permeability designation reflects the differences in freedom of access to phase I and/or phase II metabolizing enzymes within hepatocytes. In addition to the factors described here, hepatic blood flow needs to be considered as under flow limited conditions the impacts of inhibition of uptake transport could be reduced.

It is interesting to note that atorvastatin is categorized as class 2 in the previous report (Wu and Benet, 2005). However, atorvastatin was shown to be have limited diffusion into SCHH in present study, and could be categorized as a class 4 compound (9.3 uL/min/mg of atorvastatin vs. 91 ul/min/mg of propranolol, data not shown). In this regard, additional investigations are proposed to better understand the passive diffusion cutoff using cellular uptake model, e.g SCHH. Some exceptions were also found in the relationship between passive permeability and AUC increase observed in clinic. While cerivastatin was a high permeable compound in SCHH (15 μL/min/mg), a significant AUC increase (4.8 and 5.6 fold) was reported when cerivastatin was co-administered with the OATP inhibitors, CsA and gemfibrozil, respectively (Figure 3B, circle marked). Cerivastatin interacted with OATP proteins and is also extensively metabolized by CYP2C8 and CYP3A4 in liver via demethylation of the benzylic methyl ether moiety (Muck, 2000). CsA and Gemfibrozil are potent inhibitors of OATP transporters, as well as inhibiting CYP metabolizing enzymes. These clinical observations could be explained
by the inhibition of multi-clearance pathways resulting in a sum of effects on overall hepatic clearance. Due to lack of exposure data, it is also worthwhile to note that outliers were anticipated with co-administration of CsA, as the inhibition of OATP transporters may have been insufficient in vivo (Figure 3 below the dotline). Collectively, caution should be raised in that the interplay between transporters and metabolizing enzymes could generate a multi-level complexity in predicting clinical DDI from an in vitro system.

In conclusion, SCHH maintains hepatic transporter expression and functional activity, and appears to be a well-characterized in vitro model for prediction of in vivo human PK. Rif SV inhibited the active uptake of OATP transporters, demonstrating it was an OATP inhibitor useful to estimate the extents of active and passive uptake in SCHH. The optimization of the experimental design minimized the impacts of inhibitory effects on metabolizing enzymes. Retrospective analysis for the compounds that undergo clinically significant hepatic transporter-mediated DDIs suggested that 50% or greater active hepatic uptake in SCHH with low passive permeability would have a potential risk of clinical DDIs and this value could serve as a cutoff to trigger the clinical investigation cascade for DDI risk assessment.
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Contributed new reagents or analytic tools: Y.B., E.K.


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Reference:


Footnotes:

The authors were all employees of Pfizer during this research and declare no conflicts of interest. YB and EK are equal contributors.
Figure Legends

Figure 1. Inhibitory effect of CsA, gemfibrozil and Rif SV on rosuvastatin uptake in SCHH. The uptake of rosuvastatin (1µM) was measured at 37 °C in the presence and absence of CsA (10 µM), gemfibrozil (30 µM) and rif SV (100 µM). Data are presented as mean ± SD.

Figure 2. Hepatic uptake and biliary excretion of several compounds in SCHH. The hepatic uptake was investigated at 37 °C in the presence and absence of Rif SV (100 µM) or in the buffer with/without Ca^{2+}/Mg^{2+}. A: midazolam (1µM), B: buprenorphine (0.2µM), C: TC (1µM) and D: rosuvastatin (1µM). Data are presented from single studies run in duplicate or triplicate. A minimum of two experiments were performed on different day to verify coefficient of variation (CV%) <15%.

Figure 3. The correlation between AUC fold-changes with OATP inhibitors observed in the clinic and the active uptake (A) or passive diffusion (PS_{passive}) (B) obtained from SCHH with Rif SV (100 µM) inhibition. Circle: AUC changes of cerivastatin with CsA or gemfibrozil. Dot line: 2-fold AUC changes for clinical relevance.
### TABLES

Table 1. Target peptides and MRM transitions monitored for human OATP proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Q1</th>
<th>Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>NVTGFFQSFK</td>
<td>587.8</td>
<td>961.4, 860.4, 656.3</td>
</tr>
<tr>
<td></td>
<td>NVTGFFQSFK*</td>
<td>591.8</td>
<td>969.4, 868.4, 664.3</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>NVTGFFQSLK</td>
<td>570.8</td>
<td>927.4, 826.4, 622.3</td>
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<tr>
<td></td>
<td>NVTGFFQSL*K</td>
<td>574.3</td>
<td>934.4, 833.5, 629.3</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>SSPAVEQQLLVSGPGK</td>
<td>798.9</td>
<td>711.9, 445.2, 1155.6</td>
</tr>
<tr>
<td></td>
<td>SSPAVEQQLLVSGPGK*</td>
<td>802.9</td>
<td>715.9, 453.2, 1163.6</td>
</tr>
</tbody>
</table>

m/z: mass to charge ratio of the ion. *, stable isotope labeled amino acid.
Table 2. Quantification of OATP1B1, 1B3 and 2B1 in suspension hepatocytes and SCHH of lot Hu4165. The protein expression of OATP1B1, 1B3, and 2B1 in SCHH was measured by LC-MS/MS and compared with that in suspension hepatocytes. * P<0.05, as compared to suspension.

<table>
<thead>
<tr>
<th></th>
<th>Suspension</th>
<th>SCHH at day 5</th>
<th>% change of suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(fmol/μg protein)</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>3.42±0.11</td>
<td>5.28±0.22</td>
<td>154*</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>1.50±0.15</td>
<td>0.88±0.11</td>
<td>59*</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>1.84±0.15</td>
<td>1.23±0.12</td>
<td>67*</td>
</tr>
</tbody>
</table>
Table 3. Comparison of initial rate of hepatocyte uptake in absence or presence of Rif SV.

Substrates were incubated in the presence and absence of the inhibitor Rif SV (100 µM) for passive and active uptake, or in the buffer with/without Ca²⁺ for in vitro biliary clearance (CLbile). Data are presented from single studies run in duplicate or triplicate. A minimum of two experiments were performed on different day to verify coefficient of variation (CV%) <15%.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Transporters/enzymes involved</th>
<th>Control</th>
<th>100 µM rifamycin SV</th>
<th>Active hepatic uptake (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PSuptake</td>
<td>CLb</td>
<td>PSpa ssive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(µL/min/mg protein)</td>
<td>(µL/min/mg protein)</td>
<td></td>
</tr>
<tr>
<td>Midazolam</td>
<td>CYP3A4</td>
<td>28</td>
<td>0.0</td>
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<tr>
<td>Buprenorphine</td>
<td>UGT</td>
<td>39</td>
<td>0.0</td>
<td>43</td>
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<tr>
<td>TC</td>
<td>NTCP</td>
<td>42</td>
<td>16.3</td>
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<tr>
<td>Rosuvastatin</td>
<td>OATP1B1, OATP1B3</td>
<td>9.3</td>
<td>2.5</td>
<td>1.2</td>
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</table>
Table 4. OATP related drug-drug interactions and hepatic active uptake in SCHH.
Clinical substrates were incubated in the presence and absence of the inhibitor Rif SV (100 μM) for passive and active uptake, or in the buffer with/without Ca^{2+} for in vitro biliary clearance (CLbile). Data are presented as the mean of duplicates or as the mean (SD) of 3~5 experiments.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Substrates</th>
<th>Control</th>
<th>Rif SV (100 μM)</th>
<th>Active uptake (%)</th>
<th>AUC changes (Fold)</th>
<th>Cmax changes (Fold)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Substrate concentration (μM)</td>
<td>P\text{Suptake}</td>
<td>CL\text{bile}</td>
<td>P\text{Passive}</td>
<td>CL\text{bile}</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ul/min/mg protein</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CsA</td>
<td>Atorvastatin</td>
<td>1</td>
<td>22(5.9)</td>
<td>1.4</td>
<td>9.3(1.3)</td>
<td>~0</td>
<td>58 (6)</td>
</tr>
<tr>
<td></td>
<td>Atorvastatin</td>
<td>1</td>
<td>2.4 (0.91)</td>
<td>0.4</td>
<td>0.5 (0.35)</td>
<td>0.1</td>
<td>79.1(18)</td>
</tr>
<tr>
<td></td>
<td>Pravastatin</td>
<td>1</td>
<td>8.3 (1.5)</td>
<td>2.6</td>
<td>1.5(1.0)</td>
<td>0.5</td>
<td>84(14)</td>
</tr>
<tr>
<td></td>
<td>Repaglinide</td>
<td>0.2</td>
<td>61(38)</td>
<td>0.2</td>
<td>24(6.4)</td>
<td>0.4</td>
<td>55(17.7)</td>
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<tr>
<td></td>
<td>Erythromycin</td>
<td>1</td>
<td>36.5(4.2)</td>
<td>0.4</td>
<td>11.9(0.7)</td>
<td>67(5.6)</td>
<td>3.6</td>
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<tr>
<td>Gemfibrozil</td>
<td>Cervastatin</td>
<td>1</td>
<td>31.8(5.0)</td>
<td>0.2</td>
<td>15(1.7)</td>
<td>~0</td>
<td>55 (3)</td>
</tr>
<tr>
<td></td>
<td>Pravastatin</td>
<td>1</td>
<td>2.4 (0.91)</td>
<td>0.4</td>
<td>0.5 (0.35)</td>
<td>0.1</td>
<td>79.1(18)</td>
</tr>
<tr>
<td></td>
<td>Rosuvastatin</td>
<td>1</td>
<td>8.3 (1.5)</td>
<td>2.6</td>
<td>1.5(1.0)</td>
<td>0.5</td>
<td>84(14)</td>
</tr>
<tr>
<td>Rifaxin</td>
<td>Atorvastatin</td>
<td>1</td>
<td>22(5.9)</td>
<td>1.4</td>
<td>9.3(1.3)</td>
<td>~0</td>
<td>58 (6)</td>
</tr>
<tr>
<td></td>
<td>Pravastatin</td>
<td>1</td>
<td>2.4 (0.91)</td>
<td>0.4</td>
<td>0.5 (0.35)</td>
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<tr>
<td></td>
<td>Atorvastatin</td>
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<td>22(5.9)</td>
<td>1.4</td>
<td>9.3(1.3)</td>
<td>~0</td>
<td>58 (6)</td>
</tr>
<tr>
<td>Tipranavir</td>
<td>Rosuvastatin</td>
<td>1</td>
<td>8.3 (1.5)</td>
<td>2.6</td>
<td>1.5(1.0)</td>
<td>0.5</td>
<td>84(14)</td>
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<tr>
<td>Atazanavir</td>
<td>Rosuvastatin</td>
<td>1</td>
<td>8.3 (1.5)</td>
<td>2.6</td>
<td>1.5(1.0)</td>
<td>0.5</td>
<td>84(14)</td>
</tr>
</tbody>
</table>
Figure 2

(A) Uptake (pmol/mg protein) over time (min) for different conditions: W/\(\text{Ca}^2+\), W/O \(\text{Ca}^2+\), W/\(\text{Ca}^{2+}\)+Rif SV, and W/O \(\text{Ca}^{2+}\)+Rif SV.

(B) Uptake (pmol/mg protein) over time (min) for similar conditions as in (A).

(C) Uptake (pmol/mg protein) over time (min) for additional conditions.

(D) Uptake (pmol/mg protein) over time (min) for comparison with (A) and (B).
Figure 3

**Graph A**

- **Y-axis**: AUC changes (fold)
- **X-axis**: Active uptake (%)
- **Legend**:
  - Gemfibrozil
  - Ilopinavir and ritonavir
  - CsA
  - Rifampicin
  - Tipranavir and ritonavir
  - Atazanavir and ritonavir

**Graph B**

- **Y-axis**: AUC change (fold)
- **X-axis**: PS, passive (µl/min/mg protein)
- **Legend**:
  - Gemfibrozil
  - Ilopinavir and ritonavir
  - CsA
  - Rifampicin
  - Tipranavir and ritonavir
  - Atazanavir and ritonavir

- **Equation**: $R^2 = 0.488$
Supplementary Material

Journal: 
Drug Metabolism and Disposition

Title of Article: 

In vitro evaluation of hepatic transporter-mediated clinical drug-drug interactions: hepatocyte model optimization and retrospective investigation

Author’s names

Yi-an Bi, Emi Kimoto, Samantha Sevidal, Hannah M. Jones, Hugh A. Barton, Sarah Kempshall, Kevin M. Whalen, Hui Zhang, Chengjie Ji, Katherine S Fenner, Ayman F. El-Kattan, and Yurong Lai

Supplemental table 1: Rosuvastatin uptake in different hepatocytes lots of SCHH.

<table>
<thead>
<tr>
<th>hepatocyte lot</th>
<th>Rosuvastatin (1 μM)</th>
<th>Passive hepatic uptake (incubation with Rif SV 100 uM)</th>
<th>active uptake (% protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD109</td>
<td>12.8±3</td>
<td>1.7±1.2</td>
<td>87±5</td>
</tr>
<tr>
<td>Hu4165</td>
<td>8.3±1.5</td>
<td>1.5±1.0</td>
<td>84±14</td>
</tr>
<tr>
<td>Hu4168</td>
<td>8.0±2.8</td>
<td>0.8±0.7</td>
<td>89±8</td>
</tr>
<tr>
<td>Hu4241</td>
<td>7.0</td>
<td>0.34</td>
<td>95%</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD