Organic Anion Transporter 2 mediated Hepatic Uptake Contribute to the Clearance of High Permeability–Low Molecular Weight Acid and Zwitterion Drugs: Evaluation using 25 drugs.

Emi Kimoto, Sumathy Mathialagan, Laurie Tylaska, Mark Niosi, Jian Lin, Anthony A. Carlo, David A. Tess, Manthena V. S. Varma

Running title: OAT2-mediated hepatic uptake clearance

Corresponding Author: Manthena V. Varma, ADME Sciences, Medicine Design, Worldwide Research and Development, MS 8220-2451, Pfizer Inc., Groton, CT 06340; Phone:+1-860-715-0257. Fax: +1-860-441-6402. E-mail: manthena.v.varma@pfizer.com

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ABBREVIATIONS: AFE, average sold error; ECCS, extended clearance classification system; CL_{int}, intrinsic clearance; CL_{int,met}, intrinsic metabolic clearance; CL_{int,h}, intrinsic hepatic clearance; CL_{plasma,h}, plasma hepatic clearance; CYP, cytochrome-P-450; IVIV, in vitro-in vivo; HHEP, human hepatocytes; HLM, human liver microsomes; MW, molecular weight; NSAIDs, non-steroidal anti-inflammatory drugs; NTCP, Na+-taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; PBPK, physiologically-based pharmacokinetic; PHH, plated human hepatocytes; PS_{active}, active uptake clearance; PS_{passive}, passive clearance; REF, relative expression factor; SLC, solute carrier; UGT, Uridine 5'-diphospho-glucuronosyltransferase.
ABSTRACT

High permeability–low molecular weight acids/zwitterions (i.e., extended clearance classification system class 1A, ECCS 1A drugs) are considered to be cleared by metabolism with minimal role of membrane transporters in their hepatic clearance. However, marked disconnect in the in vitro–in vivo (IVIV) translation of hepatic clearance is often noted for these drugs; wherein, metabolic rates measured using human liver microsomes and primary hepatocytes tend to underpredict. Here, we evaluated the role of organic anion transporter 2 (OAT2)-mediated hepatic uptake in the clearance of ECCS 1A drugs. For a set of 25 ECCS 1A drugs, in vitro transport activity was assessed using transporter-transfected cells and primary human hepatocytes. All but 2 drugs showed substrate affinity to OAT2, while 4 (bromfenac, entacapone, fluorescein and nateglinide) also showed OATP1B1 activity in transfected cells. The majority of these drugs (21 of 25) showed active uptake by plated human hepatocytes, with rifamycin SV (pan-transporter inhibitor) reducing the uptake by about 25-95%. Metabolic turnover was estimated for 19 drugs, after a few showed no measurable substrate depletion, in liver microsomal incubations. IVIV extrapolation using in vitro data was evaluated to project human hepatic clearance of OAT2-alone substrates considering (i) uptake transport only (ii) metabolism only, and (iii) transporter-enzyme interplay (extended clearance model). The transporter-enzyme interplay approach achieved improved prediction accuracy (average fold error =1.9 and bias =0.93) compared to other 2 approaches. In conclusion, this study provides functional evidence for the role of OAT2-mediated hepatic uptake in determining the pharmacokinetics of several clinically important ECCS 1A drugs.
INTRODUCTION

We recently proposed a framework called extended clearance classification system (ECCS) to predict rate-determining clearance mechanism of drugs or new molecular entities using simple molecular properties including ionization state and molecular weight (MW), and in vitro membrane permeability (Varma et al., 2015; El-Kattan and Varma, 2018). According to this validated system, ECCS class 1A drugs (i.e. high permeability–low MW <400 Da acids/zwitterions) are thought to be primarily cleared by metabolism as the rate-determining step. However, despite predominance of CYP- and/or UGT-mediated metabolic pathways, many studies indicated a marked disconnect in the in vitro–in vivo (IVIV) translation for this class of drugs. That is, metabolic rates measured by substrate-depletion and/or metabolite-formation in the incubations of human liver microsomes and human hepatocytes considerably underpredict human hepatic clearance of ECCS 1A drugs (Obach, 1999; Brown et al., 2007; Bowman and Benet, 2016; Wood et al., 2017). Several previous studies discussed plausible causes for IVIV disconnect in hepatic clearance measured using hepatocytes and liver microsomes – which include, permeation rate limitation, diffusion through unstirred water layer, suboptimal substrate concentration, reagent handling and assay methodologies (Hallifax and Houston, 2012; Poulin et al., 2012; Bowman and Benet, 2016; Wood et al., 2017). While the causes are thought to be multifactorial, empirical correction of prediction bias was suggested as a pragmatic approach for pharmacokinetic predictions in drug discovery (Hallifax and Houston, 2012; Poulin et al., 2012; Wood et al., 2017).

Membrane transporters play a key role in the absorption, distribution, clearance and elimination (ADCE) of drugs (Shitara et al., 2006; Giacomini et al., 2010; El-Kattan and Varma, 2018). Particular emphasis has been given to hepatic uptake clearance mediated by organic anion
transporting polypeptides (OATPs), where many high MW acids/zwitterions (ECCS class 1B/3B) are shown to be substrates (Giacomini et al., 2010; Shitara et al., 2013; Varma et al., 2015; Varma et al., 2017b). Organic anion transporter 2 (OAT2), a member of solute carrier 22A (SLC22A7), is expressed in liver and kidney; and is known to transport several xenobiotics and endogenous compounds (eg. creatinine and cGMP) (Lepist et al., 2014; Shen et al., 2016). Although protein abundance of OAT2 in human liver is relatively similar to other major uptake transporters such as OATP1B, NTCP and OCT1 (Nakamura et al., 2016; Vildhede et al., 2018), little is known about its role in the clinical pharmacokinetics of drugs (Shen et al., 2016). We recently identified the role of OAT2-mediated hepatic uptake in the clearance of tolbutamide and R- and S-warfarin, which were previously assumed to be cleared by metabolism involving cytochrome P-450 (CYP)2C9/19 (Bi et al., 2018a; Bi et al., 2018b). Mechanistic in vitro studies and physiologically-based pharmacokinetic (PBPK) modeling and simulations demonstrated significance of OAT2-CYP2C interplay in their clinical pharmacokinetics (Bi et al., 2018a; Bi et al., 2018b). Given these drugs are high permeability–low MW acids, we hypothesized that OAT2-mediated hepatic uptake contribute to the clearance of ECCS class 1A drugs.

The main objective of this investigation was to evaluate the role of transporter-mediated hepatic uptake in the pharmacokinetics of high permeability–low MW acids and zwitterions. To this end, for a set of about 25 ECCS class 1A drugs (Varma et al., 2015), in vitro transport activity was characterized using transporter-transfected cells and primary human hepatocytes, and metabolic clearance was measured in human liver microsomes (HLM) incubations supplemented with cofactors for oxidation and glucuronidation pathways. Moreover, IVIV extrapolation was evaluated to quantitatively predict human hepatic clearance considering the uptake and metabolic clearances separately and assuming transporter-enzyme interplay (extended clearance model).
MATERIALS AND METHODS

Chemicals and Reagents

Rosuvastatin was purchased from Carbosynth (Compton, UK). R- and S-warfarin, and rifamycin SV were purchased from Sigma-Aldrich (St. Louis, MO). \(^{3}\)H-cGMP was purchased from PerkinElmer Life Sciences (Boston, MA). All other test compounds were obtained from the Pfizer chemical inventory system. InvitroGro-HT and CP hepatocyte media were purchased from BioreclamationIVT (Baltimore, MD). Collagen I coated 24-well plates were obtained from Corning (Kennebunk, ME). Dulbecco’s modified Eagle's medium (DMEM), fetal bovine serum, nonessential amino acids, GlutaMAX-1, sodium pyruvate, penicillin and streptomycin solution were obtained from Invitrogen. Cryopreserved human plateable hepatocytes lot Hu8246 (female, Caucasian, 37 year old) was obtained from Thermo Fisher Scientific (Carlsbad, CA). BCA protein assay kit was purchased from PierceBiotechnology (Rockford, IL). NP-40 protein lysis buffer was purchased from Thermo-Fisher (Franklin, MA). Human embryonic kidney (HEK)293 cells expressing human OATP1B1 were obtained from Absorption Systems (Exton, PA). HEK293 cells stably transfected with human OAT2(tv-1) were obtained from the laboratory of Ryan Pelis (Dalhousie University, Canada).

*In vitro* Transport Studies using Transporter-transfected Cells

HEK293 cells singly transfected with OATP1B1 or OAT2 and wild-type cells were seeded at a density of 0.5 to 1.2×10^5 cells/well on BioCoat™ 48 or 96-well poly-D-lysine coated plates (Corning Inc., Corning NY), grown in DMEM containing 10% fetal bovine serum, 1% sodium pyruvate, 1% non-essential aminoacids and 1% GlutaMAX for 48 hours at 37°C, 90% relative
humidity, and 5% CO2. In addition, OAT2-HEK cells were supplemented with 1% gentamycin and hygromycin B (50 μg/mL) and OATP1B1-HEK cells were supplemented with 1% HEPES.

Methods adopted for uptake studies are similar to that previously reported by our group (Bi et al., 2017; Mathialagan et al., 2017b; Bi et al., 2018a). Stock solutions of all compounds were made in DMSO. Cells were rinsed three times with warm uptake buffer (HBSS with 20mM HEPES, pH 7.4) followed by incubating test compounds at 1µM (or 10µM in some cases with analytical sensitivity issues) with a final concentration of 1% DMSO. At the end of incubation (2min) the cellular uptake was terminated by washing the cells four times with ice-cold transport buffer and then the cells were lysed with 0.2 mL of 1% NP-40 in water (radiolabelled compounds) or methanol containing internal standard (non-labelled compounds). Transporter functionality was validated using in vitro probe substrates: 0.5 μM [3H]-cGMP (OAT2), or 0.5 μM rosuvastatin (OATP1B1) as described previously (Bi et al., 2017; Mathialagan et al., 2017a). Intracellular accumulation was determined either by mixing the cell lysate with scintillation fluid followed by liquid scintillation analysis (PerkinElmer Life Sciences, Boston, MA) for radiolabelled compounds or by LC-MS/MS analysis for non-labelled compounds. The total cellular protein content was determined using a Pierce BCA Protein Assay kit according to the manufacturer’s specifications. The uptake ratio was calculated as a ratio of accumulation in the transfected cells to the accumulation in wild-type cells.

**Determination of Uptake Clearance using Short-Term Cultured Plated Human Hepatocytes (PHH)**

The hepatic uptake assay was performed using short-term cultured cryopreserved human hepatocytes as described previously (Bi et al., 2017; Bi et al., 2018a). Briefly, plateable
cryopreserved human hepatocytes (Hu8246 lot, Thermo Fisher Scientific, Carlsbad, CA) were thawed in a water bath at 37°C and placed on ice. The cells were then poured into In VitroGro-HT medium at 37°C at a ratio of one vial/50 mL in a conical tube. The cells were centrifuged at 50×g for 3 min and resuspended at 0.75×10^6 cells/mL in In VitroGro-CP medium. Cell viability was determined by trypan blue exclusion and exceeded 85%. Hepatocyte suspensions were plated in collagen-coated 24-well plates at a density of 0.375×10^6 cells/well in a volume of 0.5 mL/well and incubated overnight (~18 hours). Cells were first rinsed twice with HBSS or ice-cold HBSS and preincubated with HBSS in the presence or absence of 1 mM rifamycin SV or ice-cold HBSS for 10 min at 37°C or 4°C. After aspirating the preincubation buffer, 0.5 mL of HBSS or ice-cold HBSS containing a substrate (1 or 10 µM) was added in the presence or absence of 1 mM rifamycin SV, a pan-SLC inhibitor (Bi et al., 2017; Mathialagan et al., 2017a; Bi et al., 2018a). The uptake was terminated at designated times (0.25, 0.5 and 1 or 0.5, 1 and 2 min) by adding 0.5 mL of ice-cold standard HBSS after removal of the incubation buffer. Cells were then washed three times with 0.5 mL of ice-cold HBSS. Hepatocytes were lysed with methanol containing the internal standard for LC−MS/MS quantification.

A two-compartment mathematical model (compartments representing the media and cell) was developed to estimate the intrinsic passive clearance ($P_{S_{passive}}$) and total intrinsic active uptake clearance ($P_{S_{active}}$) by simultaneously fitting the cell accumulation ($A_{cell}$) data in PHH with and without rifamycin SV (1 mM), a pan-inhibitor of active uptake transporters. This model is analogous to the method described previously to analyze transport data in other cell systems (Poirier et al., 2008). Equations 1–6 are used in this modeling process:
\[ V_{ew} \cdot \frac{dC_{ew}}{dt} = -(PS_{\text{passive}} + PS_{\text{active}}) \cdot C_{ew} \cdot f_{u,ew} + PS_{\text{passive}} \cdot C_{iw} \cdot f_{u,iw} \]

\[ V_{iw} \cdot \frac{dC_{iw}}{dt} = (PS_{\text{passive}} + PS_{\text{active}}) \cdot C_{ew} \cdot f_{u,ew} - PS_{\text{passive}} \cdot C_{iw} \cdot f_{u,iw} \]

\[ V_{iw} = PR \cdot CpPR \cdot VpC \]

\[ A_{cell} = \left( A_{iw} + A_{ew} \cdot (1 - f_{u,ew}) \right) \cdot PR \]

\[ C_{ew} = \frac{A_{ew}}{V_{ew}} \]

\[ C_{iw} = \frac{A_{iw}}{V_{iw}} \]

\[ Eq. 1 - 6 \]

Where, \( C_{ew}, C_{iw}, A_{ew}, A_{iw}, V_{ew} (0.5 \text{ mL}), V_{iw}, f_{u,iw}, \) and \( f_{u,ew} \) represent concentration (C), amount (A), volume (V), and unbound fraction (\( f_u \)) of the extracellular (ew) and intracellular (iw) compartments. \( PR \) is the measured protein concentration per well, \( CpPR \) is the number of cells per measured protein (1.5 million cells/mg (Sohlenius-Sternbeck, 2006)), and \( VpC \) is cell volume (2.6 \( \mu \text{L} \)/million cells, internal data) measured assuming a spherical structure (17.2 \( \mu \text{m} \) diameter, internal data).

**In Vitro Metabolism in Human Liver Microsomes (HLM) Incubations**

Human pooled liver microsomes (HLM-103 lot prepared from a mixed gender pool of 50 donors, Sekisui XenoTech, LLC, Kansas City, KS), (final protein concentration, 1 mg/mL) were diluted in 0.1 M potassium phosphate buffer (pH 7.4), containing 5 mM magnesium chloride. The microsomes were then activated by adding alamethicin at a final concentration at 10 \( \mu \text{g/mL} \) and allowed to remain on ice for 15 min (Walsky et al., 2012). The stock solutions were first
prepared in DMSO (10 mM), then subsequent sub-stock 100×solutions (0.1 mM) were prepared in 50% acetonitrile:water. The final concentration of acetonitrile in the incubation was 0.5% (v/v). The total incubation volume in the experiment was 0.5 mL. The microsome/buffer mixture were then pre-warmed at 37°C for 5 min on a heat block prior to the addition of both cofactors NADPH (1.3 mM) and UDPGA (5 mM). The reaction was initiated immediately, following the addition of cofactors, with compound solutions at a final concentration of 1 µM (Kilford et al., 2009). The incubations were terminated by removing (50 µL) aliquots of the reaction mixture at 0, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min time points (n=2 at each time point) and were added to acetonitrile (200 µL) containing a cocktail of internal standards. The samples were then vortex for 1 min and centrifuged (Allegra X-12R, Beckman Coulter, Fullerton, CA) at 3000 rpm for 5 min. The supernatant (150 µL) was transferred to a 96-deepwell injection block containing (150 µL) of HPLC water containing 0.2% formic acid and mixed before LC-MS/MS analysis for the disappearance of compound. The apparent metabolic intrinsic clearance ($CL_{int,met,app}$) was determined based on the substrate-depletion half-life estimated from the ratio of the peak area response of each compound to that of the internal standard, as described earlier (Di et al., 2012).

**In Vitro Substrate-depletion Assay using Human Hepatocytes**

The high throughput human hepatocyte substrate-depletion assay was performed in a 384-well formatted as described previously (Di et al., 2012). Briefly, The cryopreserved human hepatocytes (DCM lot, 10 donor pool, BioreclamationIVT, Westbury, NY) were thawed, and re-suspended in Williams E medium supplemented with HEPES and Na$_2$CO$_3$. The cells were counted using the Trypan Blue exclusion method. Test compounds were added to suspension
human hepatocytes in WEM buffer and incubated at 37°C in a humidified CO₂ incubator (75% relative humidity, 5% CO₂/air) for 4 hours. The final incubation contained 0.5 million cells/mL and 1 µM test compound in 15 µL total volume with 0.1% DMSO. At various time points (0, 3, 10, 30, 60, 120, 240 min), an aliquot of the sample was taken and quenched with cold acetonitrile containing internal standard (CP-628374). The samples were centrifuged (Eppendorf, Hauppauge, NY) at 3000 rpm for 10 min at 4°C, and the supernatants were transferred to new plates, which were sealed prior to LC-MS/MS analysis. The apparent metabolic intrinsic clearance ($CL_{\text{int,met,app}}$) was determined based on the substrate-depletion half-life estimated from the ratio of the peak area response of each compound to that of the internal standard, as described earlier (Di et al., 2012).

**LC-MS/MS Analysis**

Sample analysis was conducted using LC-MS/MS system comprised of a AB Sciex 5500 or 6500 triple quadrupole mass spectrometer equipped with an electrospray source (AB Sciex, Framingham, MA), Agilent Technologies Infinity 1290 HPLC (Santa Clara, CA), and CTC Leap autosampler (Pflugerville, TX) was programmed to inject 10 µL of sample on to a ACE 1.7µm Excel C18-PFP 2.1 × 30 mm (Advanced Chromatography Technologies Ltd, Scotland), Halo C18 2.7µm, 100 Å 2.1 × 30 mm (Mac Mod, Chadds Ford, PA), or a Kinetex C18 2.6µm, 100 Å, 3 × 30 mm (Phenomenex, Torrence, CA) analytical column. A binary gradient was employed consisting of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) and monitored using the multiple reaction monitoring (MRM) mode for the m/z transitions, as described in Supplemental Table 1.
Data analysis

In vivo hepatic intrinsic clearance ($\text{CL}_{\text{int},h}$) was calculated from human intravenous clearance data assuming the well-stirred liver model (Pang and Rowland, 1977).

$$\text{CL}_{\text{int},h} = \frac{\text{CL}_{\text{blood},h}}{f_{u,b} \cdot \left(1 - \frac{\text{CL}_{\text{blood},h}}{Q_h}\right)} \quad \text{Eq. 7}$$

where $\text{CL}_{\text{blood},h} = (\text{CL}_{\text{plasma}} - \text{CL}_{\text{renal}})/R_b$ is the hepatic blood clearance obtained from intravenous total plasma clearance corrected for renal clearance and blood-to-plasma ratio ($R_b$). $f_{u,b}$ is fraction unbound in blood (fraction unbound in plasma ($f_{u,p}$)/$R_b$), and $Q_h$ is hepatic blood flow (20.7 mL/min/kg (Kato et al., 2003)).

Predicted $\text{CL}_{\text{int},h}$ were calculated under 3 different scenarios: (i) considering total hepatic uptake alone (Eq. 8) measured using plated human hepatocytes, (ii) considering metabolic clearance alone (Eq. 9) measured by substrate-depletion in HLM, and (iii) extended clearance model (Eq. 10) accounting for transporter-enzyme interplay using both uptake transport (from plated human hepatocytes) and metabolic (HLM) data (Sirianni and Pang, 1997; Liu and Pang, 2005; Shitara and Sugiyama, 2006; Camenisch and Umehara, 2012; Varma et al., 2014; Patilea - Vrana and Unadkat, 2016; Kimoto et al., 2017). A schematic of the step-wise approach employed in IVIV extrapolation is given in Supplemental Figure 1.

$$\text{CL}_{\text{int},h} = \text{PS}_{\text{active}} \cdot \text{REF} + \text{PS}_{\text{passive}} \quad \text{Eq. 8}$$

$$\text{CL}_{\text{int},h} = \text{CL}_{\text{int,met}} \quad \text{Eq. 9}$$

$$\text{CL}_{\text{int},h} = \text{PS}_{\text{active}} \cdot \text{REF} + \text{PS}_{\text{passive}} \left(\frac{\text{CL}_{\text{int,met}}}{\text{PS}_{\text{passive}} + \text{CL}_{\text{int,met}}}\right) \quad \text{Eq. 10}$$

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PS$_{active}$ and PS$_{passive}$ are sinusoidal intrinsic active uptake clearance and intrinsic passive uptake clearance, respectively. CL$_{int,met}$ represents the intrinsic metabolic clearance measured using HLM coinoculated with both NADPH and UDPGA. CL$_{int,met}$ is equal to measured apparent intrinsic clearance (CL$_{int,met,app}$) divided by microsomal binding (f$_{u,mic}$) or hepatocyte binding (f$_{u,hep}$). Active basolateral and canalicular efflux was assumed to be negligible (Sirianni and Pang, 1997; Shitara et al., 2013; Varma et al., 2014). The in vitro intrinsic values were scaled assuming the following: $1.5 \times 10^6$ hepatocytes/mg-measured protein, $118 \times 10^6$ hepatocytes/g-liver, 39.8 mg microsomal protein/g-liver, 24.5 g liver/kg-body weight (Varma et al., 2013). Relative expression factor (REF) – correction for the expression difference in the hepatocytes and human liver – of 1.8 and 2.0 were applied for OAT2 and OATP1B1 substrates, respectively, based on our previous published quantitative proteomics data (Kimoto et al., 2012; Vildhede et al., 2018). The hepatocyte lot (Hu8246) used for uptake studies showed expression levels of OAT2 similar to the median expression levels of 30 single donor cryopreserved hepatocyte lots (Vildhede et al., 2018).

Hepatic blood and plasma clearances are calculated assuming the well-stirred liver model (Eq. 11), where $\text{CL}_{\text{blood},h} = \text{CL}_{\text{plasma},h}/R_b$

$$\text{CL}_{\text{blood},h} = \frac{Q_n \cdot f_{u,b} \cdot \text{CL}_{\text{int},h}}{Q_h + f_{u,b} \cdot \text{CL}_{\text{int},h}}$$

Eq. 11

Predictive precision and accuracy were assessed with average fold error (AFE) Eq. 12 and bias Eq. 13.
\[
\text{AFE} = 10 \frac{\sum \log_{10} \text{Predicted}}{\sum \log_{10} \text{Observed}}
\]

Eq. 12

\[
\text{Bias} = 10 \frac{\sum \log_{10} \text{Predicted}}{\sum \log_{10} \text{Observed}}
\]

Eq. 13

N is the number of observations.
RESULTS

Compound Selection

In a previously published dataset of 368 compounds spanning across six classes of ECCS – classified based on their ionization, MW and transcellular permeability – about 10% (n=36) compounds are binned in class 1A (Varma et al., 2015; El-Kattan et al., 2016). Human intravenous clearance and renal clearance data are available for all these drugs. Of these 36 high permeability (>5x10^6 cm/s) – low MW (<400Da) acids/zwitterions, 3 compounds (milrinone, pralidoxime and clinafloxacin) show predominantly renal clearance, while others involve hepatic clearance mechanisms. All the compounds were considered for the current study with a few exceptions: control substances (hexobarbital and thiopental) and lack of compound availability (acenocoumarol, perindopril and sinitrodil). Dataset of 25 compounds with in vitro hepatic uptake measurements is available after a few compounds were dropped (acetylsalicylic acid, amniosalicylic acid, dichloracetic acid, diflunisal, milrinone and valproic acid) due to analytical challenges and/or low cellular accumulation in uptake studies. Of the 25, 6 compounds returned no measurable metabolic turnover in the human liver microsomal incubations under the experimental conditions employed. Therefore, the final set of 19 compounds was considered for IVIV extrapolation of transporter-enzyme interplay via extended clearance model (Supplemental Figure 1).

In vitro transport in OAT2 and OATP1B1 transfected cells

ECCS 1A compounds were evaluated for their substrate affinity to OAT2 and OATP1B1 using transfected HEK293 cells. All the compounds studied showed uptake ratio (i.e. ratio of accumulation in transfected cells to wild-type cells) of more than 1.5 in OAT2-HEK cells
suggesting they are actively transported by OAT2, with the exception of clinafloxacin and nateglinide (Figure 1). While 85% of compounds showed uptake ratio of >2, compounds such as ibuprofen, meloxicam, R-warfarin and sulfamethoxazole yielded uptake ratios of more than 5.

In contrary, only 4 of 25 compounds (bromfenac, entacapone, fluorescein, and nateglinide) showed uptake ratio of >1.5 in OATP1B1-HEK cells, implying majority of the compounds are not transported by OATP1B1; and these four compounds are OAT2/OATP1B1 dual substrates with the exception of nateglinide (OATP1B1-alone). Positive controls of OAT2 (cGMP) and of OATP1B1 (rosuvastatin) showed robust signal supporting the performance of the cell lines employed. Diclofenac, tolbutamide and R/S-warfarin were previously reported to be OAT2 substrates using transfected cells (Zhang et al., 2016; Bi et al., 2018a; Bi et al., 2018b). On the other hand, fluorescein and nateglinide were previously shown to be OATP1B1 substrates (De Bruyn et al., 2011; Takanohashi et al., 2012; Izumi et al., 2016). For all the other compounds, the noted transporter substrate affinity was previously unknown to our best knowledge.

**In vitro uptake in human hepatocytes**

Figure 2 shows the time-dependent uptake of ECCS class 1A drugs by human hepatocytes plated in short-term culture. Rifamycin SV (1mM), a pan-SCL inhibitor (including OAT2 and OATP1B1 (Bi et al., 2017; Mathialagan et al., 2017a; Bi et al., 2018a)), significantly reduced uptake of all compounds – with a few exceptions. Clinafloxacin, naproxen, thalidomide and tiagabine uptake was not altered by rifamycin SV under the experimental conditions employed. In case of diclofenac, pioglitazone, rosiglitazone and tolcapone, presence of rifamycin SV lead to increased hepatocyte accumulation (data not shown). While little is known on the stimulation of OAT2 activity, several previous studies suggested plausible allosteric interaction for the
substrate-dependent stimulation of the in vitro OATPs activity in the presence of an inhibitor (Roth et al., 2011). Nonetheless, for these 4 drugs, hepatic uptake was significantly reduced when incubated on ice (4°C). Uptake time-course data was fitted to a 2-compartment mathematical model to estimate intrinsic active uptake clearance (PS_{active}) and intrinsic passive clearance (PS_{passive}), assuming 1mM rifamycin SV (or 4°C) completely inhibited active uptake (Table 1). Estimated PS_{active} ranged from 0 (clinafloxacin) to 300 µL/min/mg (pioglitazone), while PS_{passive} ranged from 0.2 (thalidomide) to 138 µL/min/mg (rosiglitazone). Finally, compounds in the dataset were categorized in 3 bins based on their transport characteristics observed with transfected cells and plated human hepatocytes: (1) OAT2 substrates with significant active uptake in human hepatocytes (referred to as OAT2-alone substrates, n=17), (2) OAT2/OATP1B1 substrates with significant active hepatic uptake (OAT2/OATP1B1 dual substrates, n=4), and (3) compounds with no measurable active transport in transfected and/or plated hepatocytes (i.e., no active hepatic uptake, n=4). Nateglinide was recognized by only OATP1B1, however, it was included in dual substrates category for further data analysis. Generally, PS_{active} is higher than PS_{passive} for the OAT2-alone substrates in this set (Figure 3A). Interestingly, uptake clearance was lower for OAT2/OATP1B1 dual substrates compared to substrates for OAT2 alone.

In vitro metabolic clearance in human liver microsomes (HLM) and primary human hepatocytes (HHEP)

Intrinsic clearance of all compounds was measured by substrate-depletion method using pooled HLM with both NADPH and UDPGA cofactors in the incubations (Table 1). The measured intrinsic clearance (CL_{int,met,app}) (not corrected for the microsomal binding (f_{u,mic})) spanned a broad range from 1.9 (meloxicam, tolbutamide) to 140 µL/min/mg-protein (diclofenac). A few
compounds (gliclazide, isoxicam, piroxicam, sulfamethoxazole and R/S-warfarin) did not show measurable substrate depletion during the 180 min incubations. From our previous study, apparent metabolic clearance in hepatocytes corrected for cell-to-media concentration (Kpuu) was available for R/S-warfarin (Bi et al., 2018a). Microsomal binding was determined by equilibrium dialysis and the values ranged from no binding to approximately 50% bound. It is noteworthy that the $P_{S_{\text{passive}}}$ measured in plated hepatocytes is equal or higher than microsomal metabolic clearance for 10 of 13 (~76%) OAT2-alone substrates (Figure 3B).

Additionally, we measured apparent intrinsic clearance following substrate-depletion in suspension human hepatocyte incubations. Values ranged from 1.6 (gliclazide) to 66.5 $\mu$L/min/million-cells (diclofenac) (Table 1). For OAT2-alone substrates, where the substrate-depletion was measurable in both systems (n=15), hepatocellular $CL_{\text{int,met}}$ values were on average 2.6-fold higher than the microsomal $CL_{\text{int,met}}$ (Supplemental Figure 2).

**In vitro-in vivo (IVIV) extrapolation to human hepatic clearance**

A summary of human hepatic clearance predictions from the in vitro data is presented in Table 2; and the predicted hepatic clearance values ($CL_{\text{int,h}}$ and $CL_{\text{plasma,h}}$) are plotted verses observed clearance values in Figure 4. IVIV extrapolation was evaluated assuming well-stirred considerations under three variations: (i) uptake-determined clearance, where total hepatic uptake ($P_{S_{\text{active,REF}}} + P_{S_{\text{passive}}}$) was assumed to be the rate-determining step (Eq. 8), (ii) metabolism-determined clearance, where liver microsomal clearance was scaled alone (Eq. 9), and (iii) transporter-enzyme interplay, where hepatic transport and microsomal metabolic clearance were integrated using extended clearance model (Eq. 10) (Supplemental Figure 1).
For OAT2-alone substrates, AFE for $\text{CL}_{\text{int,h}}$ predictions based on uptake-determined clearance is \(\sim 8.2\), where majority of the compounds are overpredicted (bias \(\sim 6.4\)) (Table 3). Metabolism-determined clearance alone underpredicted (AFE \(\sim 5.2\) and bias \(\sim 0.20\)) in vivo $\text{CL}_{\text{int,h}}$ for all compounds with a few exceptions (pioglitazone, and pralidoxime). Only 5 of 13 (38\%) predictions are within 3-fold of observed values. However, prediction accuracy and bias markedly improved when employing extended clearance model (AFE\~1.9 and bias \(\sim 0.90\)). About 8 (62\%) and 11 (85\%) of the 13 predictions of OAT2-alone substrates are within 2-fold and 3-fold error of the observed values, respectively. Similar predictive performance could be seen for the plasma hepatic clearance predictions (Table 3).

OAT2/OATP1B1 dual substrates were considerably underpredicted in all scenarios. A scaling factor of 10.6 for active hepatic uptake (instead of OATP1B1 REF of 2.0) in extended clearance model (Eq. 10), which was previously reported by our group (Varma et al., 2014; Kimoto et al., 2017), improved clearance predictions for dual substrates to some extent (Supplemental Figure 3). Due to limited microsomal stability data, no particular conclusions could be dawn for compounds with no-active uptake in plated human hepatocytes.

Finally, direct scaling of intrinsic clearance measured by substrate-depletion in suspension human hepatocytes reasonably predicted hepatic clearance (Figure 5, Table 3). AFE and bias are \(\sim 2.3\) and 0.63, respectively, for the OAT2-alone substrates, with \(\sim 47\%\) and 73\% predictions within 2-fold and 3-fold error, respectively. For OAT2/OATP1B1 dual substrates, this method underpredicted in vivo $\text{CL}_{\text{int,h}}$ on an average of about \(\sim 10\)-fold.
Discussion

This study, using a diverse set of 25 drugs, provided evidence for the functional role of OAT2-mediated hepatic uptake in the pharmacokinetics of high permeability–low MW acid and zwitterion drugs (i.e., ECCS 1A drugs). These drugs generally undergo extensive hepatic metabolism by CYP2C, UGTs and other enzymes, and are primarily eliminated from body as metabolites (Varma et al., 2015; El-Kattan et al., 2016). Here, a majority of ECCS 1A drugs demonstrated substrate affinity to OAT2 and active hepatic uptake. Clearly, hepatic clearance of OAT2-alone substrates was well predicted when employing extended clearance model – assuming transporter-enzyme interplay. However, scaling human liver microsomal clearance alone, assuming metabolism-determined clearance, showed the tendency of systemic underprediction (~5-fold). To our knowledge, this is the first study suggesting OAT2 role in the hepatic uptake clearance and thus the pharmacokinetics for a majority of high permeability–low MW acid and zwitterion drugs. These findings are of clinical importance as many such drugs are widely prescribed in a variety of therapeutic areas including inflammation, diabetes, Parkinson disease and thrombosis.

Based on the in vitro mechanistic studies and PBPK analyses, we recently demonstrated that OAT2 plays a key role in the overall hepatic clearance of tolbutamide and R/S-warfarin, which are ECCS class 1A drugs (Bi et al., 2018a; Bi et al., 2018b). We therefore set out to investigate the role of OAT2 in the hepatic clearance for an unbiased set of 25 drugs in this class. With the exception of clinafloxacin and nateglinide, all drugs showed OAT2-mediated transport in transfected cells. These drugs also showed significant active uptake in primary human hepatocytes plated in short-term cultures with a few exceptions under the experimental conditions employed (clinafloxacin, naproxen, thalidomide and tiagabine). Collectively, these in
vitro studies provided functional evidence for active hepatic uptake driven by OAT2 for a majority of the high permeability-low MW acids/zwitterions. Further work is needed to understand the role of OAT2 in the clearance of other classes of drugs.

We employed pooled liver microsomes to estimate in vitro metabolic intrinsic clearance by following substrate-depletion time course. Previous studies have shown that CL$_{\text{int,met}}$ obtained from substrate-depletion and metabolite-formation approaches are often comparable (Jones and Houston, 2004; Di et al., 2013). The presence of both cofactors, NADPH and UDPGA, in the incubations of alamethicin-activated microsomes allowed the simultaneous assessment of oxidation and glucuronidation pathways (Fisher et al., 2000; Kilford et al., 2009). Moreover, values obtained here are generally consistent with the reported CL$_{\text{int}}$ measured using liver microsome and hepatocytes (Obach, 1999; Brown et al., 2007). However, the microsomal CL$_{\text{int,met}}$ underpredicted in vivo CL$_{\text{int,h}}$ by an average of ~5-fold (bias ~0.20). This observation is also consistent with several previous reports highlighting that the in vitro CL$_{\text{int,met}}$ systematically underpredict clearance of acid drugs that undergo extensive metabolism (Obach et al., 1997; Obach, 1999; Brown et al., 2007; Wood et al., 2017). There does not appear to be any trend between the predictive accuracy and the known primary enzyme involved (CYP2C versus UGT). The current study suggests lack of consideration to OAT2-mediated hepatic uptake as a major source for IVIV disconnect noted previously in this physicochemical space.

Our recent mass spectrometry-based targeted proteomics studies indicated about 1.8-fold higher abundance of OAT2 in human liver tissue samples (n=52) compared to the expression in single-donor cryopreserved hepatocytes (n=30) (Vildhede et al., 2018). Similarly, in our previous studies ~2-fold higher OATP1B1 expression was observed in liver tissues relative to the levels in cryopreserved hepatocytes (Kimoto et al., 2012). Therefore, REF of 1.8 and 2.0 were applied
when scaling in vitro active uptake to predict hepatic clearance for OAT2-alone substrates and OAT2/OATP1B1 substrates, respectively. We examined the sensitivity of OAT2 REF (1.8 versus 1.0 – later assuming no correction for transporter abundance) and noted only a marginal effect on the overall predictive performance of extended clearance model (AFE 1.9 versus 2.0; bias 0.9 versus 0.6). However, consistent to many earlier reports (Jones et al., 2012; Ménochet et al., 2012; Varma et al., 2012b; Li et al., 2014; Varma et al., 2014), a scaling factor much larger than the OATP1B1 expression differences was needed to recover hepatic clearance of OATP1B1 substrates. To this end, a previously derived empirical scaling factor for the active uptake clearance (SF_{active} ~10.6 (Varma et al., 2014)) provided reasonable predictions for the 4 OAT2/OATP1B1 substrates in the current dataset (Supplemental Figure 3). Collectively, direct IVIV extrapolation of OAT2-alone substrates suggests that OAT2 functional activity, unlike OATP1B1, is well preserved in cryopreserved human hepatocytes.

Depletion of parent was also assessed in pooled human hepatocytes and the predictive performance of this system is better than that from liver microsomes (Table 3, Figure 5). Interestingly, hepatocytes on an average yielded ~2.6-fold higher substrate-depletion than was measured in microsomal incubations (Supplemental Figure 3). This is likely due to higher free cell-to-media concentrations (Kpuu) driven by OAT2-mediated uptake in the hepatocytes. In our previous studies we observed a Kpuu of ~4-5 for R/S-warfarin in the suspension human hepatocytes (Bi et al., 2018a). Although further studies are needed to understand the reasons leading onto differences in the measurements between the two systems, this study suggests that suspension human hepatocytes can be employed as a pragmatic screening tool to predict hepatic clearance in this physico-chemical space.
“Rapid-equilibrium” between blood and liver compartments is often presumed for high permeability drugs, resulting in a theory that metabolism is the rate-determining process in their hepatic clearance. Transcellular permeability of the OAT2-alone substrates in our dataset, measured across monolayers of MDCK-low efflux cells, ranged from about 8 (sulfamethoxazole) to 35×10^{-6} cm/s (fenoprofen). In the same experimental conditions, permeability of ECCS class 1B drugs (high permeability–high MW acids/zwitterions) span a similar range, ~5.5 (atorvastatin, pitavastatin) to 16×10^{-6} cm/s (repaglinide) (Varma et al., 2015; El-Kattan et al., 2016). ECCS class 1B drugs are well proven to involve OATP-mediated hepatic uptake in their systemic clearance (Shitara et al., 2013; Varma et al., 2017a). Indeed, many evidences are presented for “uptake-determined” clearance for high permeable and metabolically labile drugs such as atorvastatin, bosentan and glyburide (Zheng et al., 2009; Watanabe et al., 2010; Maeda et al., 2011; Yoshikado et al., 2017). Under the similar principles, the current study demonstrate that OAT2-mediated active uptake contribute to the systemic clearance of high permeability–low MW acids/zwitterions. Interestingly, no correlation was apparent between the transcellular permeability across MDCK cell monolayers and passive uptake clearance measured in human hepatocytes (in the presence of rifamycin SV or 4°C) for the compounds in the current dataset (Supplemental Figure 4). Drugs in our dataset have a median acid pKa of ~4.4 (range 2.2 to 7.9), implying that they exists in >99.9% ionized form (at physiological pH 7.4) – a form that is expected to have negligible passive diffusion across the cell membrane (Avdeef, 2001). This is consistent with the low passive uptake clearance, and implies dependence on the active transport mechanism to achieve high total hepatic uptake noted in the hepatocytes. While cell membrane composition difference across the systems may contribute to the observed poor correlation (Simons and Ikonen, 1997), role of uptake transporters cannot be ruled out in the transcellular
permeability model (Dobson and Kell, 2008; Ahlin et al., 2009; Kell et al., 2011). Overall, potential role of uptake transporters in hepatic clearance should be duly evaluated for the acids and zwitterions irrespective of the membrane permeability across cell culture models (eg. MDCK or Caco-2).

We observed a minimal overlap in the substrate specificity for OAT2 versus OATP1B1, which is in general agreement that OATPs preferentially accept high MW (>400 Da) acids/zwitterions (class 1B) as substrates (Varma et al., 2012a). Of the 4 OATP1B1 substrates identified in the ECCS 1A space, nateglinide and fluorescein were previously reported to be OATPs substrates (De Bruyn et al., 2011; Takanohashi et al., 2012; Izumi et al., 2016), while this is the first report implying bromfenac, a NSAID for ocular inflammation, and entacapone, an catechol-O-methyltransferase inhibitor used in Parkinson’s disease, involve OATP1B1-mediated hepatic uptake clearance. Clinical drug-drug interaction studies with OATP inhibitors, rifampicin and cyclosporine, may be helpful to further define uptake-determined clearance in these cases. MW of these 4 OATP1B1 substrates range from 305 to 334 Da. While the occurrences are low, this study suggests that low MW acids/zwitterions may involve OATP1B1-mediated clearance.

Although a plethora of drug-drug interaction and pharmacogenetic studies have shown the relevance of CYP2C and UGT mechanisms, our findings will open up the field to consider OAT2-mediated hepatic uptake as a source of variability in the pharmacokinetics and pharmacodynamics of drugs in ECCS 1A class. According to extended clearance concept (Shitara et al., 2013; Patilea-Vrana and Unadkat, 2016), extended clearance model (Eq. 10) is reduced such that the hepatic clearance can be approximated by $PS_{uptake} \times CL_{met}/PS_{passive}$, when $PS_{passive} > CL_{int,met}$ – which is often the case for the compounds in the current dataset (Figure 3B). Therefore, functional variability in OAT2 and enzymatic metabolism can lead to variability in
pharmacokinetics for these drugs. For instance, genetic polymorphism in CYP2C8 and CYP2C9 was shown to be associated with systemic clearance of many ECCS 1A drugs including ibuprofen, piroxicam, tolbutamide and S-warfarin (Rettie et al., 1994; Kirchheiner et al., 2002; García-Martín et al., 2004; Perini et al., 2005). For the latter two drugs, we previously demonstrated that a PBPK model with permeability-limited hepatic disposition (transporter-enzyme interplay) quantitatively describe the pharmacogenetic effects when considering genotype, phenotype and fraction metabolism by CYP2C9 (Bi et al., 2018a; Bi et al., 2018b). However, our preliminary literature search revealed limited knowledge on the clinically relevant OAT2 inhibitors and functional polymorphic variants of SLC22A7 (gene encoding OAT2) (Bi et al., 2018a). Further studies are needed in these areas to assess OAT2 role in clinical settings.

This is important as changes in uptake or metabolic clearance will have a proportional impact on hepatic clearance, and a simultaneous change in both mechanisms in same direction can result in a marked change in clinical pharmacokinetics. Moreover, OAT2-mediated uptake can lead to high free liver-to-plasma concentrations (Kpuu), which may contribute to the liver-specific pharmacological and/or toxicological activities. It will be of interest to understand the association between liver Kpuu and drug-induced hepatotoxicity noted for several of the OAT2-alone substrates in our dataset (eg. diclofenac, ibuprofen, pioglitazone, piroxicam, rosiglitazone, tolcopone, etc. (Boelsterli, 2003; Morgan et al., 2010; Chen et al., 2011)).

In conclusion, our systemic evaluation provided robust evidence for the role of a previously unrecognized OAT2-mediated hepatic uptake in the clearance of several high permeability–low MW acid and zwitterion drugs. For this class of drugs or new chemical entities (ECCS class 1A), uptake transport characterization and considerations to transporter-enzyme interplay is important
for predicting clinical pharmacokinetics and assessing variability due to drug-drug interactions and other intrinsic and extrinsic factors.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Kimoto, Mathialagan, Tylaska, Niosi, Lin, Tess, and Varma.

Conducted experiments: Kimoto, Mathialagan, Tylaska, Niosi, Lin, and Carlo.

Performed data analysis: Kimoto, Mathialagan, Tylaska, Niosi, Lin, Tess, and Varma.

Wrote or contributed to the writing of the manuscript: Kimoto, Mathialagan, Tylaska, Niosi, Lin, Carlo, Tess, and Varma.
CONFLICT OF INTEREST

All authors are full-time employees of Pfizer Inc. The authors have no conflicts of interest that are directly relevant to this study.
References


Bowman CM and Benet LZ (2016) Hepatic Clearance Predictions from In Vitro–In Vivo Extrapolation and the Biopharmaceutics Drug Disposition Classification System. Drug Metabolism and Disposition 44:1731-1735.


Legends for Figures

Figure 1. OAT2- and OATP1B1-mediated transport of ECCS 1A drugs in the overexpressing cells. Uptake was measured in HEK293 cells transfected with OAT2 (A) and OATP1B1 (B), and presented as ratio to uptake in wild-type HEK293 cells. Bars and error bars represent mean and s.d. (n=3). Dashed and dotted vertical lines represent uptake ratio of 1 and 1.5, respectively. Uptake ratio >1.5 was used as a criteria for transport activity.

Figure 2. Time-course of cellular uptake of ECCS 1A drugs in the plated human hepatocytes. Drugs were binned in three categories: OAT2-alone substrates (A), OAT2/OATP1B1 dual substrates (B) and no active hepatic uptake (C). Data points and curves represent uptake in control condition (black), in the presence of 1mM rifamycin SV (red) and at 4°C (blue). Curves are data fit to the 2-compartment mathematical model. Uptake rates of tolbutamide and R/S-warfarin were obtained from our previous studies (Bi et al., 2018a; Bi et al., 2018b). Incubation concentration is 10μM for fenoprofen, ibuprofen, pralidoxime, naproxen and thalidomide, and 1μM for all others.

Figure 3. Comparison of passive hepatic uptake with the active uptake clearance measured using plated human hepatocytes (A). Comparison of passive hepatic uptake measured using plated human hepatocytes with the intrinsic metabolic clearance measured in microsomal incubations (B). Circles represent OAT2-alone substrates, triangles represent OAT2/OATP1B1 dual...
substrates, and squares represent drugs with no measurable active uptake in human hepatocytes. Solid and dashed diagonal blue lines indicate unity and 3-fold range, respectively.

Figure 4. In vitro-in vivo extrapolation to predict human hepatic clearance of ECCS 1A drugs. Observed versus predict hepatic clearance assuming (A) total uptake clearance alone measured using plated human hepatocytes, (B) metabolic clearance alone measured using human liver microsomes, and (C) extended clearance model considering active and passive uptake transport and microsomal metabolic clearance. Left and right panels represent hepatic intrinsic clearance and hepatic plasma clearance, respectively. Circles represent OAT2-alone substrates, triangles represent OAT2/OATP1B1 dual substrates, and squares represent drugs with no measurable active uptake in human hepatocytes. Solid, dotted and dashed diagonal blue lines indicate unity, 2-fold, and 3-fold range, respectively. AFE and bias (red diagonal lines) of OAT2-alone substrates are shown.

Figure 5. Comparison of hepatic clearance predicted from intrinsic clearance measured using substrate-depletion in human hepatocytes with the observed in vivo hepatic clearance. Left and right plots represent hepatic intrinsic clearance and hepatic plasma clearance, respectively. Circles represent OAT2-alone substrates, triangles represent OAT2/OATP1B1 dual substrates, and squares represent drugs with no measurable active uptake in human hepatocytes. Solid, dotted and dashed diagonal blue lines indicate unity, 2-fold, and 3-fold range, respectively. AFE and bias (red diagonal lines) of OAT2-alone substrates are shown.
Table 1. Summary of drug properties and in vitro transport and metabolism measurements of the 25 high permeability, low molecular weight acid and zwitterion drugs evaluated.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Therapeutic Class</th>
<th>Permeability (10^-6 cm/s m²)</th>
<th>Molecular weight</th>
<th>Acidity</th>
<th>Ionization</th>
<th>Uptake Transporters</th>
<th>Major Elimination Pathway</th>
<th>Main Enzyme(s)</th>
<th>Plasma free fraction (f_{un})</th>
<th>Blood-to-plasma ratio</th>
<th>Micromolar free fraction (f_{mic})</th>
<th>Hepatocyte free fraction (f_{hep})</th>
<th>PHH P_{app} Uptake (µL/min/mg)</th>
<th>PHH P_{app} Uptake (µL/min/mg)</th>
<th>PHH P_{app} Uptake (µL/min/mg)</th>
<th>HLM CL_{app} (µL/min/M-cells)</th>
<th>HHEP CL_{app} (µL/min/M-cells)</th>
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<td>Bromfenac</td>
<td>NSAID</td>
<td>17.8</td>
<td>334</td>
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<td>OATP1B1, OAT2</td>
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<td>CYP2C9</td>
<td>0.002</td>
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<td>9.3</td>
<td>25.1</td>
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<td>253</td>
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<td>OAT2</td>
<td>Met</td>
<td>CYP2C9</td>
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<td>31.7</td>
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aMeasured in vitro across MDCK-low efflux cells (Di et al., 2011).

bObtained from (Varma et al., 2015).

cObtained from (El-Kattan et al., 2016) and other literature.

dMeasured by equilibrium dialysis method as described by (Di et al., 2012).

eExtracted from published literature. When data were not available, blood-to-plasma ratio of 0.55 was used.

fMeasured by equilibrium dialysis method as described by (Di et al., 2012).

gCalculated from $f_{u,\text{mic}}$ using equation (Cory Kalvass and Maurer, 2002): $f_{u,\text{HHEP}} = 1 / (1 + (1/f_{u,\text{mic}} - 1) * 0.5/0.8)$. Where, $f_{u,\text{HHEP}}$ was assumed equivalent to $f_{u,\text{mic}}$ under similar assay conditions (i.e., microsomes at 1 mg/mL is similar to hepatocytes at 1 million cells/mL) (Austin et al., 2005). Values, 0.5 million-cells/mL represent the cell density of the hepatocytes substrate-depletion assay, and 0.8 mg/mL represent protein concentration of the $f_{u,\text{mic}}$ assay.

hTaken from (Bi et al., 2018a).

A – Acid; Z – zwitterion; Met – metabolism.
n.d. – no measureable substrate depletion under the experimental conditions.

<table>
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<th>$f_{u,\text{HHEP}}$</th>
<th>$f_{u,\text{mic}}$</th>
<th>$f_{u,\text{HHEP}}$</th>
<th>$f_{u,\text{mic}}$</th>
<th>$f_{u,\text{HHEP}}$</th>
<th>$f_{u,\text{mic}}$</th>
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<th>$f_{u,\text{mic}}$</th>
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</table>

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Table 2. In vitro-in vivo extrapolation of transport and metabolism data assuming hepatic clearance is determined by total uptake, metabolism-alone and extended clearance model. Clearance predictions from pooled hepatocytes substrate-depletion studies were also summarized.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intrinsic hepatic clearance (mL/min/kg)</th>
<th>Plasma hepatic clearance (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observeda</td>
<td>Predicted</td>
</tr>
<tr>
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<td>Hepatocyte total uptake</td>
<td>Liver microsomes metabolism</td>
</tr>
<tr>
<td><strong>OATP1B1/OAT2 substrates</strong></td>
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<td></td>
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<td>Bromfenac</td>
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<td><strong>OAT2-alone substrates</strong></td>
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<td></td>
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</table>

*aObserved plasma hepatic clearance was obtained from (Davis et al., 2000; Delrat et al., 2002; Scordo et al., 2002; Obach et al., 2008; Varma et al., 2009; El-Kattan et al., 2016). Observed intrinsic hepatic clearance was calculated from plasma hepatic clearance using Eq. 7.*
Table 3. Summary of predictive performance of various in vitro-in vivo extrapolation approaches employed.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intrinsic hepatic clearance</th>
<th></th>
<th></th>
<th></th>
<th>Plasma hepatic clearance</th>
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<tr>
<td></td>
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<td>Liver microsomes</td>
<td>Extended</td>
<td>Hepatocyte</td>
<td>Hepatocyte total uptake</td>
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<td>Within 3-fold error (%)↑</td>
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</table>

N-number of compounds; AFE-average fold error (Eq. 12). Bias was calculated for only OAT2-alone drugs (Eq. 13). $\uparrow$-number of predictions within 2- and 3-fold of the observed values.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

(A) Total uptake clearance (uptake-only)

(B) Microsomal metabolic clearance (metabolism-only)

(C) Extended clearance model (Transporter-enzyme interplay)
Hepatocyte substrate-depletion clearance

Figure 5.